

**Nationwide Surveillance on enteric fever to estimate the disease
burden in Bangladesh**



A DISSERTATION SUBMITTED TO BRAC UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE IN
BIOTECHNOLOGY

**SUBMITTED BY
AMENA MAHMOOD
EXAMINATION ROLL NO-13276004
REGISTRATION NO-13276004
SESSION: FALL 2013**

**DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES
MASTER OF SCIENCE IN BIOTECHNOLOGY
BANGLADESH
www.bracu.ac.bd
JULY, 2016**

DECLARATION

I hereby solemnly declare that the work reported in the thesis entitled '**Nationwide surveillance on enteric fever to estimate the disease burden in Bangladesh**' submitted to the Department of Mathematics and Natural Sciences, BRAC University in partial fulfillment of the degree of Master of Science in Biotechnology has been carried out by me under joint supervision of Professor Dr. Naiyyum Choudhury, Former Coordinator, Biotechnology and Microbiology Programmes, BRAC University and Professor Dr. Firdausi Qadri, Head of Mucosal Immunology Immunology & Vaccinology Laboratory, Enteric and respiratory infections, Infectious Disease Division, International Center for Diarrheal Disease Research, Bangladesh (icddr,b) in the icddr,b laboratories and that it has not been submitted to any other university or any academic institution for a degree or a diploma.

(Amena Mahmood)

ID: 13276004

Session: Fall 2013

Certified:

Professor Dr. Firdausi Qadri

Supervisor

Head, Mucosal Immunology and
Vaccinology Laboratory,
Enteric and respiratory infections,
Infectious Disease Divisions,
International Center for Diarrhoeal
Disease Research, Bangladesh (icddr,b)

Professor Dr. Naiyyum Choudhury

Supervisor

Coordinator, Biotechnology and
Microbiology Program,
Department of Mathematics and Natural
Sciences
BRAC University
Dhaka-1212, Bangladesh

Acknowledgement

At the very beginning, I express in the most humble way from the core of my heart, the gratitude to almighty Allah for blessings, guidance, protection, help and wisdom in all aspects of my life. I thank Almighty Allah (The Most Gracious, The Most Merciful) to enable me to work on my thesis to the best of my abilities and to keep me in good health throughout.

I convey my gratitude to Professor Dr. A.A.Ziauddin Ahmed, Professor and Chairperson, Department of Mathematics and Natural Sciences for kindly allowing me to undertake my postgraduate studies in the department and for his valuable suggestions and continuous encouragement all throughout my studies.

I am greatly indebted to my respected supervisor Professor Dr. Naiyyum Choudhury, Department of Mathematics and Natural Sciences, BRAC University, for his sensible and creative advice, unique assistance, scholastic guidance and excellent academic counseling.

I am overwhelmed to express my respect, sincere gratitude and heartfelt thanks to my supervisor Professor Dr. Firdausi Qadri, Head of Mucosal Immunology Immunology & Vaccinology Laboratory, Enteric and respiratory infections, Infectious Disease Division, International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b) for her pearls of wisdom, affectionate guidance, cordial supervision, endless inspiration, constructive criticism, and specially for encouraging me to think independently in the fascinating field of Immunology. Without her continuous help this part of research work was indeed unachievable.

I offer my profound reverence to my honorable teacher Dr. Aparna Islam, Dr. Mahboob Hossain and Dr. Mohammad Sorowar Hossain, Department of Mathematics and Natural Sciences for their wise advice, affectionate guidance, inspiration and incessant help over my days in the department.

I am most grateful to all my teachers of the Department of Mathematics and Natural Sciences, BRAC University, for their suggestions and encouragement.

I would particularly like to express my deepest thanks to Dr. Farhana Khanam, Deputy Project Co-ordinator, Mucosal Immunology Immunology & Vaccinology Laboratory, icddr,b for her hearty, dateless, incessant cooperation and encouragement throughout the study. I gratefully acknowledge her for her advice, supervision, and crucial contribution throughout my research and thesis writing periods.

I would also like to give heartily thanks to Dr. Ashraful islam, Abu Sayeed, Motaher Hossain, Naoshin Nishat, Nazia Rahman for their constant encouragement, sound advice, good company, great cooperation and lots of good ideas. It is great pleasure for me to receive ancillary help from Dr. Taufiqur Rahman Bhuiyan, Dr. Yasmin Ara Begum, Muhammad Ikhtear Uddin, Mr.Rasheduzzaman Rashu and other members of the immunology Laboratory who have contributed in various ways during this work.

I would like to express deep indebtedness to all my friends for their enthusiastic inspiration and company during my thesis work.

I would like to thank everybody who was important to the successful realization of this thesis, as well as expressing my apology that I could not mention all of you personally one by one.

Finally I like to express my outmost gratitude to my parents, my husbands and my family for their endless moral support and kind prayers during my thesis work.

The Author

Department of Mathematics and Natural Sciences

BRAC University

July, 2016.

**DEDICATED
TO
MY BELOVED PARENTS**

Abstract

Enteric fever is a life threatening febrile illness caused by *Salmonella* Typhi and Paratyphi found highly prevalent in developing countries like Bangladesh. The government and non-government organization is working together for minimizing the burden of disease through active surveillance, ongoing collection of data, analysis and interpretation of data. The aim of the current study was to estimate the prevalence in different sentinel sites using data of laboratory confirming diagnosis, identification of high risk population, risk factors for acquiring the disease, and antimicrobial susceptibility of *S. Typhi* and *S. Paratyphi A*. During the study period May, 2014 to December, 2015 specimens were collected from ten different hospital facilities divided into seven divisions. The TPTest and blood culture methods were used for understanding the disease burden as well as serotyping of enteric fever. Total 2036 and 2068 specimens were tested by TPTest and blood culture respectively. A total of 530 (26.03%) specimens were positive by the TPTest; whereas 59 (2.85%) cases were positive by blood culture. Among blood culture positive specimens 46 (78%) cases were *S. Typhi* and 13 (22%) were *S. Paratyphi A*. The highest number of enteric fever positive cases was found in Dhaka (TPTest 57.17%; blood culture 59.32%) division followed by Barisal and Chittagong. During the two years of surveillance two peak seasons have been observed. The first peak was observed in month of August and second peak was November-December of 2014 and 2015. Enteric fever was more prevalent among adults aged ≥ 18 years (278 was positive by TPTest, 16 was positive by blood culture); whereas younger children aged 6-17 years (176 was positive by TPTest, 22 was positive by culture) were also found susceptible. Males (36 cases was culture positive) were more positive than females (10 cases was culture positive). A higher number of the febrile patients had taken antibiotics (33.6%) before enrolment rationalized the lower incidence rate of blood culture. Among the isolated *S. Typhi* and *S. Paratyphi A* strains, 100% had shown reduced susceptibility to ciprofloxacin. This study demonstrates that enteric fever is highly prevalent in Bangladesh. The findings of the study also imparts the importance of proper vaccination program with an effective and immunogenic vaccine.

Index	Page Number
Contents	I-IV
List of Tables	V
List of figures	VI-VII
Abbreviations	VIII

Chapter one: Introduction

1.1	Enteric Fever, a common febrile illness	1
1.2	Epidemiology	
1.2.1	Global Perspective	1-2
1.2.2	Epidemiology of Bangladesh	2-3
1.2.3	Surveillance of Enteric Fever in Bangladesh	3-4
1.3	General characteristics of Salmonella Typhi and Paratyphi	
1.3.1	The Organism	4
1.3.2	Classification	4-5
1.4	Antigenic structure of Salmonella enterica serover Typhi	6-7
1.5	Genomic Variation of Salmonella enterica serover Typhi	7-8
		8
1.6	Pathogenesis	

1.7	Transmission and Risk factor	10-11
1.8	Clinical Manifestation	12
1.9	Diagnosis of Enteric Fever	12
1.9.1	Blood Culture	12
1.9.2	Widal Test	12
1.9.3	Typhidot Test	13
1.9.4	TPTest	13
1.10	Treatment	13
1.11	Prevention	14
1.12	Vaccination of Enteric Fever	14
1.13	Rationale of the study	14
1.14	Hypothesis of the study	14
1.15	Objectives	14

Chapter two:

Methods and Materials

2.1	Overview of the study	15
2.2	Study Site	15-16
2.3	Sample Collection	18

	18-19
2.4 Study Design	19
2.5 Ethical Issues	20
2.6 Laboratory Based Method Used in the study	21
2.7 Blood Culture	
2.8 Biochemical Test	21-22
2.8.1 Triple Sugar Iron (TSI) Agar Medium	22
2.8.2 Motility Urea Indole (MIU) Medium	22
2.8.3 Citrate Agar	
2.9 Serological detection of <i>Salmonella</i> and serovers	24
2.9.1 Step: 1 Identification of <i>Salmonella</i> species	25
2.9.2 Step: 2 Identification of <i>Salmonella</i> Typhi	25
2.9.3 Step: 3 Identification of <i>Salmonella</i> Paratyphi A	25
2.9.4 Step: 3 Identification of <i>Salmonella</i> Paratyphi B	25
2.9.5 Step: 5 Test for Negative Control	
2.10 Typhoid and Paratyphoid Test (TPTest)	27
2.10.1 Separation of Peripheral blood Mononuclear Cells by density gradient centrifugation	27-28
2.10.2 Collection of Antibody in Lymphocyte Secretion (ALS) specimen	28-31
2.10.3 Determination of membrane	

Chapter three: Results

3.1 Study subjects	32
3.2 Prevalence of enteric fever in Bangladesh	32
3.2.1 Number of cases detected by TPTest method in Bangladesh	34
3.2.2 Distribution of enteric fever cases in Bangladesh by Blood culture method	35-36
3.2.3 Overall prevalence of enteric fever (TPTest and blood culture) in Bangladesh	36-37
3.3 Serotype of Identified Organism	37-38
3.3.1 Distribution of both serover (Typhoid and Paratyphoid fever) in Bangladesh	38
3.3.1 Isolation of other organism	39-40
3.4 Seasonality of enteric fever in Bangladesh	41-42
3.5 Demographic and clinical characteristics of patients in Bangladesh	42-43
3.5.1 Age and sex distribution of enteric fever	43
3.5.2 Source of water	44-45
3.6 Antibiotic Resistance Pattern of the Salmonella Typhi and Paratyphi A	45-46
3.7 Private health care and disease surveillance	

Chapter four: Discussion	47-50
Chapter five: References	51-53

Appendices	
-------------------	--

LIST OF TABLES

Table no.	Table name	Page no.
1	Overview of the biochemical test	25
2	Baseline demographic data of the enteric fever patients (Total number of patients=2113)	42
3	Important clinical features of the enteric fever patients (Total number of patients=2113)	43
4	Characteristics of enteric fever positive patient's enrolled in the study sites from May, 2014 to Dec, 2015	44
5	Antibiotic susceptibility pattern of isolated S. Typhi strains (n=46)	45
6	Antibiotic susceptibility pattern of isolated S. Paratyphi A strains (n=13)	46

Figure no	Figure name	Page no.
------------------	--------------------	-----------------

1.1	The global scenario of enteric fever.	2
1.2	<i>Salmonella</i> Typhi (http://salmonellatyphi.org)	3
1.3	The classification of the genus <i>Salmonella</i>	4
1.4	Antigenic structure of <i>Salmonella</i> (https://www.studyblue.com)	7
1.5	Circular representation of the <i>S. typhi</i> genome. http://www.nature.com/	8
1.6	Schematic representation of the <i>Salmonella</i> pathogenicity. http://www.nature.com/	9
1.7	Schematic representation of host–pathogen interactions during pathogenesis of <i>Salmonella</i> infections. Pathogenesis (Adapted from Hansen-Wester and Hensel, 2001)	10
1.8	Month-wise variation of typhoid and paratyphoid incidence in Kolkata.	11
2.1	The ten study site is divided in seven divisions	17
2.2	The ten sentinel site of enteric fever surveillance in Bangladesh.	18
2.3	BD BACTEC® blood culture bottle.	19
2.4	Overview of the study design	20
2.5	The flow chart of the laboratory procedure	21
2.6	The three different medium used for the biochemical detection of organism	24

2.7	(A) TSI medium before incubation (B) TSI medium after incubation	25
2.8	Serological identification test	28
2.9	Separation of PBMC from blood sample.	30
2.10	The mechanism of MP specific kinetic ELISA from ALS sample.	32
3.1	The mechanism of MP specific kinetic ELISA from ALS sample.	35
3.2	Incidence rate of enteric fever detected by TPTest method in study sites.	35
3.3	Incidence rate of enteric fever detected by TPTest method in study sites.	36
3.4	Prevalence of enteric fever from May 2014 to December2015	37
3.5	Cases of Enteric patients detected by TPTest and blood culture method.	38
3.6	Distribution of isolated organism in percentage	39
3.7	Isolation of <i>Salmonella</i> Typhi and Paratyphi A in Different divisions of Bangladesh	40
3.8	The other organism isolates during the study period.	41
3.9	Monthly distribution of enteric fever in Bangladesh	42
3.10	Number of TPTest positive infection cases From May to December of 2014 and 2015.	42

3.11	Source of water distribution among the participants of study	45
3.12	Distribution of total sample in private and Government health facilities.	48

1.1 Enteric Fever, a common febrile illness

Salmonella enterica serover Typhi and Paratyphi A cause an infectious disease called enteric fever. This is a serious public health sector concern in many developing countries. In 2010, data has shown the incidence rate of typhoid and paratyphoid fever was 3.9/1000 persons per year. [1] Lack of safe drinking water and food, unhygienic sanitation is the considerable reason of the enteric fever to become endemic in some region. Although the incidence of enteric fever is quite high but the actual data on the diagnosis of febrile patient and epidemiology is not sufficient. [2] Difficulty to distinguish typhoid and paratyphoid fever from other febrile illness makes it more sensitive topic to be deal with. Despite the high incidence, typhoid fever surveillance is also not strong in South Asian regions, and vaccination efforts have been limited. Hence, enhancing laboratory based surveillance, increasing preventive measures, and improving infrastructure would support the immunization program for the enteric fever.

1.2 Epidemiology

1.2.1 Global Perspective

Enteric fever is a life threatening febrile illness, making it a globally concerned topic. In 2000, the estimated number of illness due to typhoid fever was 21.7 million, resulting in 217,000 deaths. About 5.4 million illness is estimated as paratyphoid fever. [3] Developing countries are suffering from typhoid and paratyphoid fever because of the resource poor infrastructure and the lack of monitoring over the health related issues. While developed countries have overcome this limitation by improving the sewage system, supplying pure water and the advance level of antimicrobial drug. But still the developed countries are facing enteric fever affected patient due to their travelling across the endemic country. A report shows that about 80% of people traveling from the USA to India, Philippines, Pakistan, El Salvador and Haiti are affected by typhoid fever. The most risk zone has been found while traveling to the Indian subcontinent, and the number of people increased from 23.4 to 81.2 per 100 000.[4]

Another surveillance in Egypt reveals the incidence rate of typhoid fever is 59/100,000 persons annually and the number of young typhoid patients was significantly higher compared to other febrile patients enrolled for surveillance. [5]

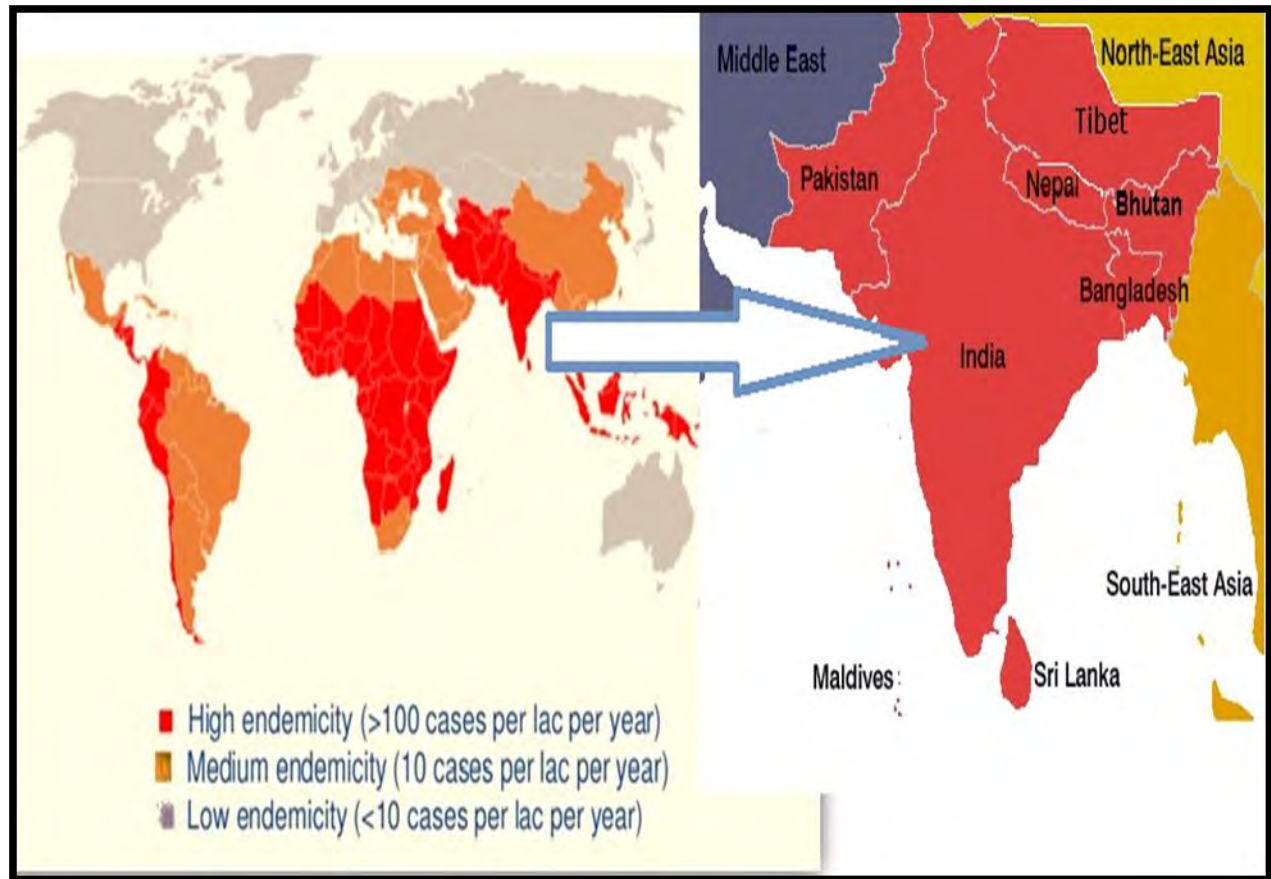


Figure 1.1: The global scenario of enteric fever.

In South Asian region, the enteric fever incidence has been encountered in the province Punjab in India. The total number of patients was 340 where *S. Typhi* and Paratyphi A was 334 and 6 respectively. Again the surveillance study was conducted over the densely populated area of Kolkata and it showed the lower number of paratyphoid fever in Kolkata compared to typhoid fever although the patients were older[6]. Whereas a significant number of blood culture positive *S. Typhi* and *S. Paratyphi A* has been isolated in two urban settlement of Karachi in Pakistan respectively 67% and 36% out of 4027 blood sample collected from people[7]

From the above mentioned figure (figure 1.1), the prevalence of typhoid fever is showing different pattern depending on the region and comparatively younger people are more exposed to this organisms, So we can conclude that, the geographical pattern of typhoid fever is remaining consistent and the higher incidence found in India, Indonesia and Pakistan, the lower incidences in the site of Viet Nam and China.[8]

World Health Organization (WHO) emphasizes to identify the outbreak and conduct the surveillance to estimate the low and high risk population. During 2009-2013, WHO recommended South Asia Region (SEAR) and Western Pacific Region (WPR) for vaccination in 48 countries. But 23 out of 48 countries have collected surveillance data on typhoid cases. So it has been recommended to identify the high risk zone for control, proper disease estimation and calculation of incidence.[9]

1.2.2 Epidemiology of Bangladesh:

Enteric fever in Bangladesh is very common while the country is suffering from the unsafe drinking water and food, inadequate sanitation, unhealthy street food and poor hand wash practice during eating. In 2013, a study conducted in Bangladesh has shown that *S.Typhi* is the most frequent pathogen causing the invasive bacterial disease. Total number of isolated pathogen was 143, where 93 cases (65%) was *S.Typhi* and 15 cases (10%) was *S.Paratyphi A* [10]. The children under 5 years of age living in urban slum constitute 85% of total Enteric fever incidence in Bangladesh. The older persons are 8.9 times less threatened than children.[11] Though the incidence of enteric fever is higher but the estimation of enrolled patient's number is not updated. The expanded immunization programmed of typhoid vaccine in this region could be considered, but there is no national level laboratory based surveillance data regarding typhoid and paratyphoid fever. The monitoring of disease outbreak and the profiling of enrolled patient is essential for the understanding of health issue within a country.

1.2.3 Surveillance of Enteric Fever in Bangladesh

The globalization of trade and commerce not only influences the sharing of commodities but also food safety is becoming major concern. Enteric illness is a food borne and water borne disease, a predominant public health concern in Bangladesh but due to absence of trustworthy data it has not been addressed adequately. Even there is no national level surveillance data to find out the determination of actual burden. The present study has been conducted for the appropriate collection of data from the nine districts which covered the seven division of Bangladesh. With the culture confirmation results, an enteric fever detection technique TPTest [12] is also being used for the incorporation of laboratory and

research based systematic and collaborative data management which is increasing the development of policy level decision making for updating resources.

1.3 General Characteristics of *Salmonella* Typhi and Paratyphi A

1.3.1 The Organism

Salmonella enterica subspecies enterica serovar Typhi is gram negative rod-shaped, facultative, intracellular, and flagellated organism. They are motile and cannot produce lactose. Theobald Smith (1885) first discovered the organism and Daniel Elmer (1850-1914) described the organism. The organism is human restricted organism and habituated in intestinal tract of human host.



Figure 2.3 : *Salmonella* Typhi (<http://salmonellatyphi.org>)

1.3.2 Classification

The beginning of the *salmonella* nomenclature was quite confused. The Kauffmann and White scheme is used for the classification of Genus *Salmonella*, which has two species named *Salmonella enterica* and *Salmonella bulgori*. *Salmonella enterica* is divided into 6 subspecies and subdivided into more than 2000 serovers.[13]

The scientific classification is given *as follows*:

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Salmonella
Species	<i>Salmonella enterica</i>

The classification is shown below:

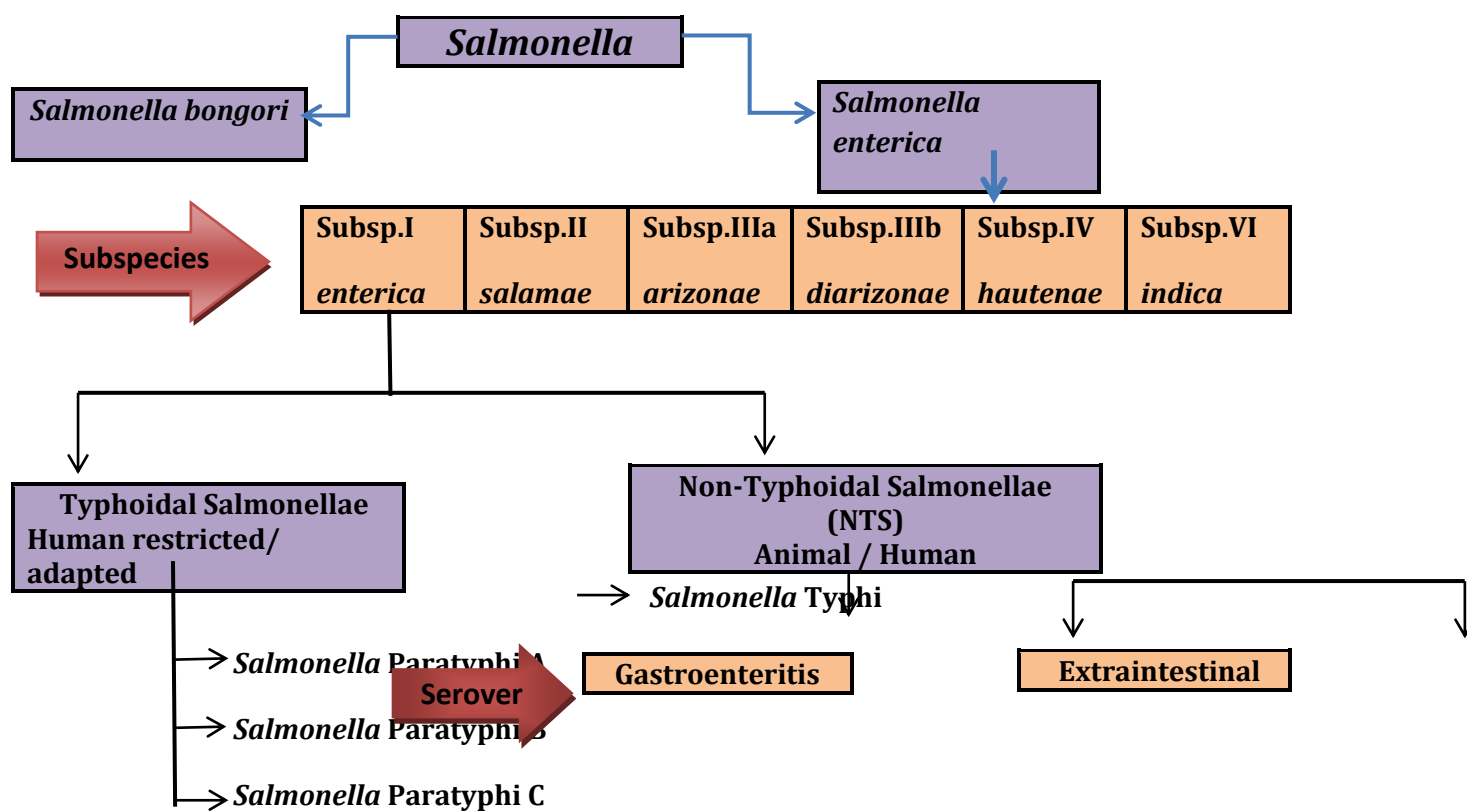


Figure 1.4: The classification of the genus *Salmonella*

1.4 Antigenic Structure of *Salmonella enterica* serovar Typhi

The knowledge of antigenic structure of outer membrane protein and virulent factor may enhance the development of vaccine against different antigen. *Salmonella enterica* composed of the bilayered outer membrane that surrounded the inner membrane, periplasmic space and the peptidoglycan layer. [14]

The Kauffmann-White classification scheme described three major antigen found on the surface of *S.Typhi* , O-antigen, Vi-capsule and H-flagella.

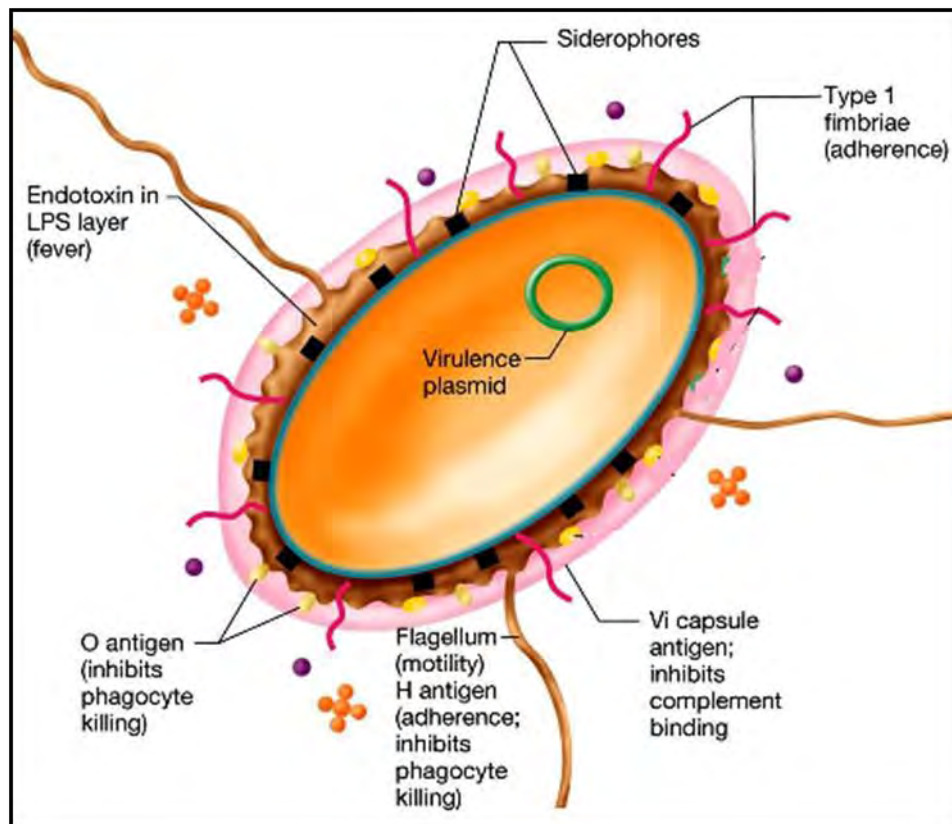


Figure 1.5: Antigenic structure of *Salmonella* (<https://www.studyblue.com>)

The O-antigens are heat stable and alcohol resistant. O-polysaccharide chains contain repeating sugar which is the responsible of O-antigen specificity.

The extracellular polysaccharide layer that shields the *S.Typhi* is produced by Vi capsule. The role of Vi-antigen is very important for the survival from host innate immune system. It helps the bacteria to get protection from phagocytosis and complement mediated killing. [15] The prevention of Toll like receptor-4 (TLR-4) recognition and the limit of the deposition of C3 results the low clearance of bacteria.

The H-antigen possess two phase named phase 1 (fliC gene encoded the protein) and phase 2 (fliB gene encoded the protein). Bacteria is able to transform between these two phase. They are heat labile. Bacterial H-antigen becomes a useful tool for the determination of source and mode of spread.

1.5 Genomic Variation of *Salmonella enterica* serover Typhi

The advancement in the field of genetic studies has revealed the whole genomic sequence of *S.typhi*. About 90% genes are shared with *S.typhimurium* and many genes shared with *E.coli*.

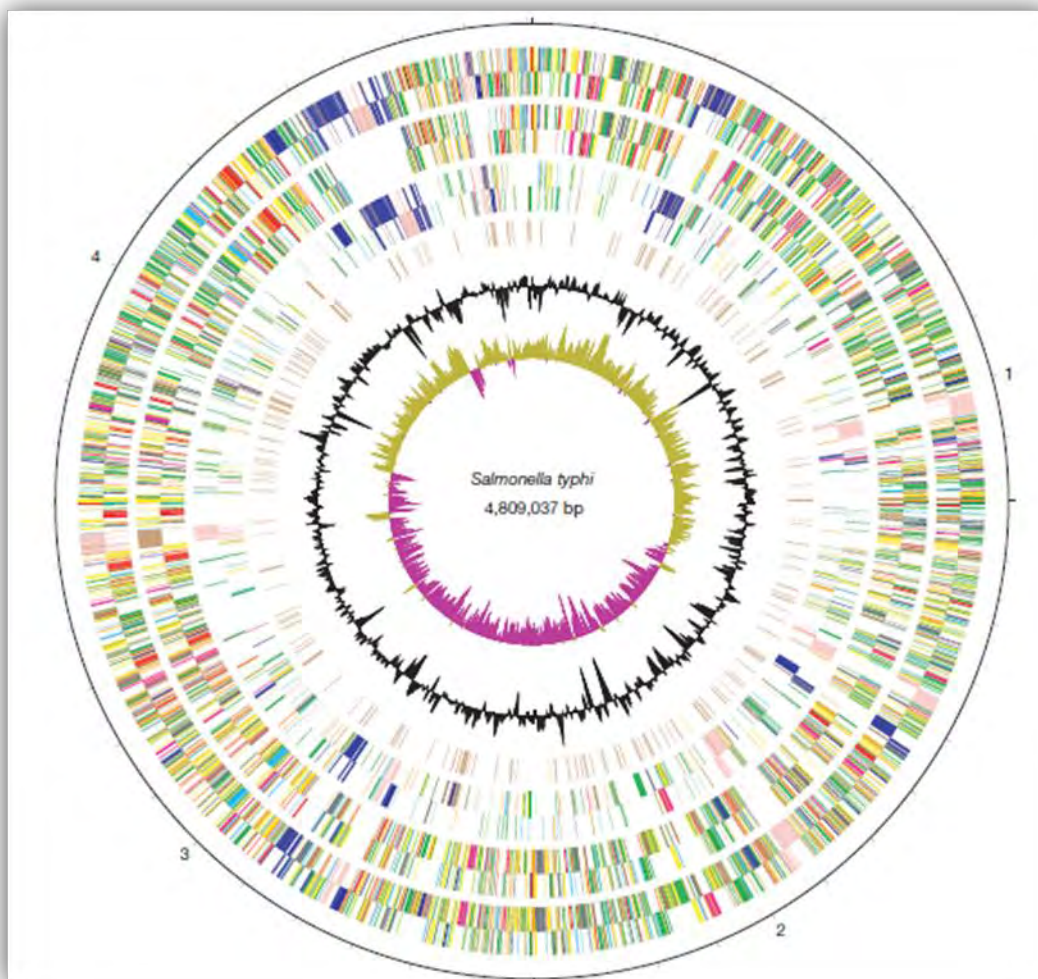


Figure 1.6: Circular representation of the *S. typhi* genome. <http://www.nature.com/>

The whole genome of *salmonella enteric* serovar Typhi has been isolated and the emerging of multi drug resistance *S.Typhi* strain becomes alarming for the developing countries. There are two strain have been isolated Ty2 and CT18. The vaccine study of typhoid fever is associated with Ty2 strain which contain no plasmid. Other strain CT18 contain two plasmid named pHCM1, is conjugative and the first line of microbial drug resistance encoded by it.[16] The other plasmid pHCM2 contain the sequence most common with the virulence plasmid of *Yersinia pestis*. The extensive number of pseudo gene was found in the chromosome but 124 out of 204 pseudo genes are inactivated by the stop codon. This huge number of pseudo gene may be the regulator of *S.Typhi*'s host restriction in case of compare to other strain and would play an important role during microbial drug resistance.

1.6 Pathogenesis

Healthy individuals have the ability to contain the organism in the intestine through the local immune defense mechanism whereas *S.Typhi* is able to spread over the deeper tissue of human as well as the liver, spleen and bone marrow. The presence of a specific gene called pathogenicity islands (**PIs**) has distinguished the virulence factor of pathogenic bacteria from non- pathogenic species. In case of Enteric fever, the virulent factor clustered in certain area of *Salmonella* pathogenicity island (SPI), till today 15 SPI have been discovered whereas two of them are SPI-1 and SPI-2 .The invasion and intracellular proliferation has been controlled by genes in SPI-1, the intracellular pathogenesis is controlled by genes in SPI-2. T3SS (Type 3 Secretion System) is a complex protein crucial for the invasion of virulent factor into the host cell. The structure of T3SS form a base interact the target cell and a needle like structure form the conduit within the cytoplasm of pathogen and the cell membrane of target cell. The combination of SPI-1 and T3SS allows the require protein to be transferred from the bacterial cytoplasm to the host cell.[17]

Whenever the salmonella internalized into host cell, a vacuole is formed around the organism called Salmonella-containing vacuole (SCV). The mature SCV enter into macrophages within peyer's patches in the sub mucosal space. The role of SCV is crucial for the survival of bacteria inside the host. The SPI-2 and T3SS combined and suppress the dendritic cell's activity and help salmonella for persistence inside the host cell.

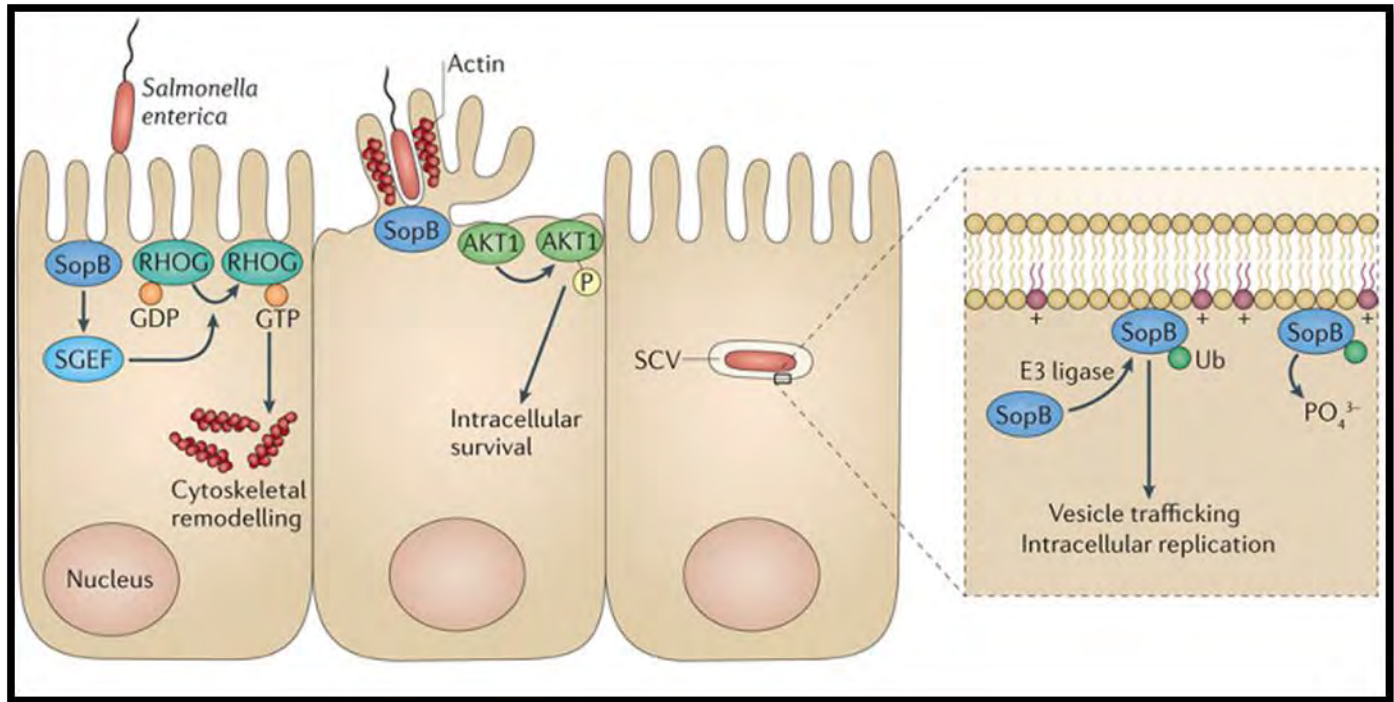


Figure 1.7: Schematic representation of the *Salmonella* pathogenicity.
<http://www.nature.com/>

As a result of the bacterial presents in the intestinal lymphoid tissue as well as the secretion into mesenteric nodes, the thoracic duct and the circulatory system of blood helps to persist the bacteria move across the reticuloendothelial system within 24 hour of their ingestion. During the incubation period the bacteria survive and replicate in cells of monocytic lineage.

Salmonella Pathogenicity Island (SPI1) function is required for the initial stages of salmonellosis, i.e. the entry of *Salmonella* into non- phagocytic cells by triggering invasion and the penetration of the gastrointestinal epithelium. Furthermore, SPI1 function is required for the onset of diarrheal symptoms during localized gastrointestinal infections.

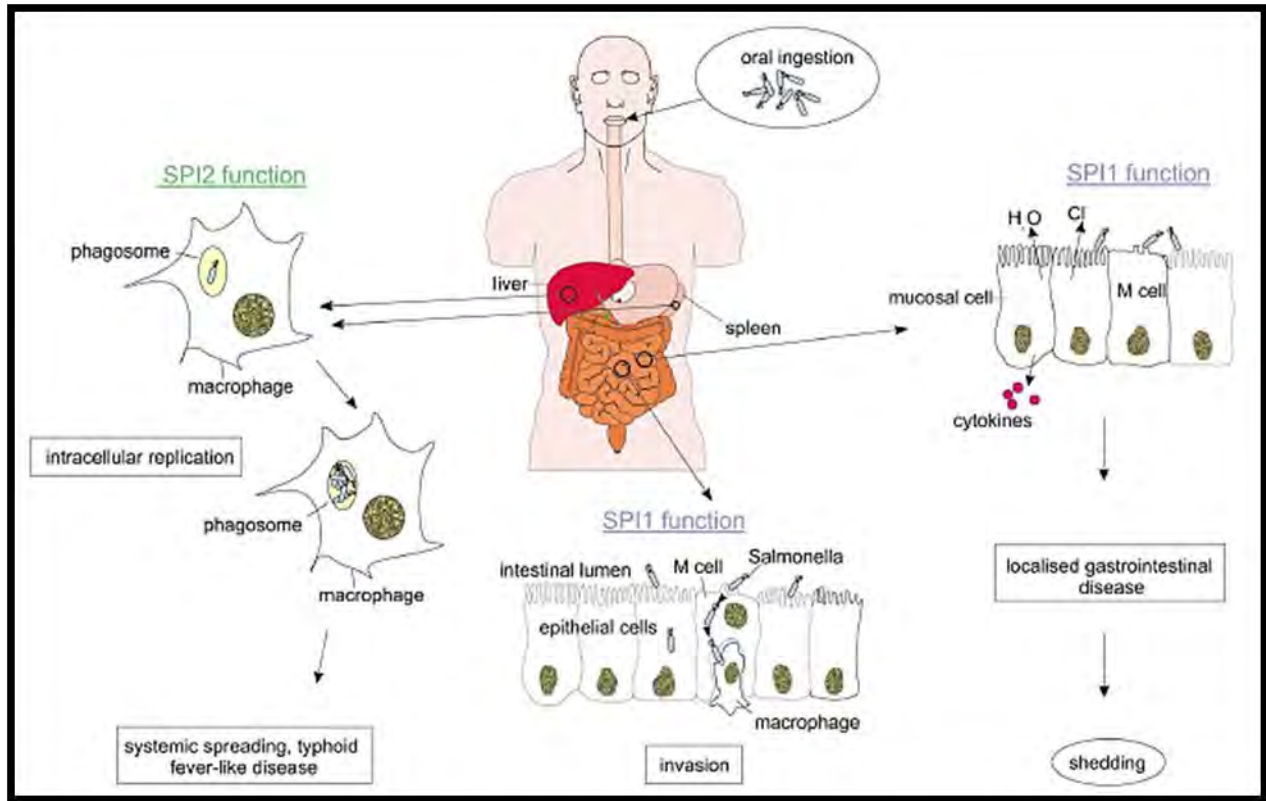


Figure 1.8: Schematic representation of host–pathogen interactions during pathogenesis of *Salmonella* infections. Pathogenesis (Adapted from Hansen-Wester and Hensel, 2001)

The function of SPI2 is required for later stages of the infection, i.e. systemic spread and the colonization of host organs. The role of SPI1 for survival and replication in host phagocytes appears to be essential for this phase of [18]

1.7 Transmission and Risk factor

The pathogen causes disease is transmitted via the fecal-oral route and the severity of the disease is depending on the virulence capacity of the bacteria and the infective dose. The infectious dose of *S.Typhi* in volunteers could be (1000-1) million organisms. The bacteria spread in the environment through stool of the infected patient or recently recovered patients from the illness as he excretes the organism via stool for a long time. The contamination of food and water takes place due to the poor sanitation facility; the food washed by contaminated water, unhygienic food handling practice. On the other hand *S.Typhi* can withstand the optimum temperature so the raw meat and the half cooked food may onset the illness. A recent study from urban slums in Bangladesh has shown that eating raw papaya is associated with the disease. Papaya has a neutral pH and its cut

surface can support the growth of various microorganisms.[19] Eating lettuce salad and *cig kofte*, a traditional food of Turkey became source of typhoid fever. [20] Apart from the biological factor of enteric fever transmission, there are also some socio-economic factors which differ from one country to another. The prevalence of enteric fever was significant among the children of parents having medium level of education and the poor economic condition of family. [21] [22] Environmental factor impact the cases of enteric fever, A data is shown below describing that typhoid and paratyphoid fever tends to be more common during the hot dry season because the water level decreased and the concentration of bacteria become high in pond and river water.

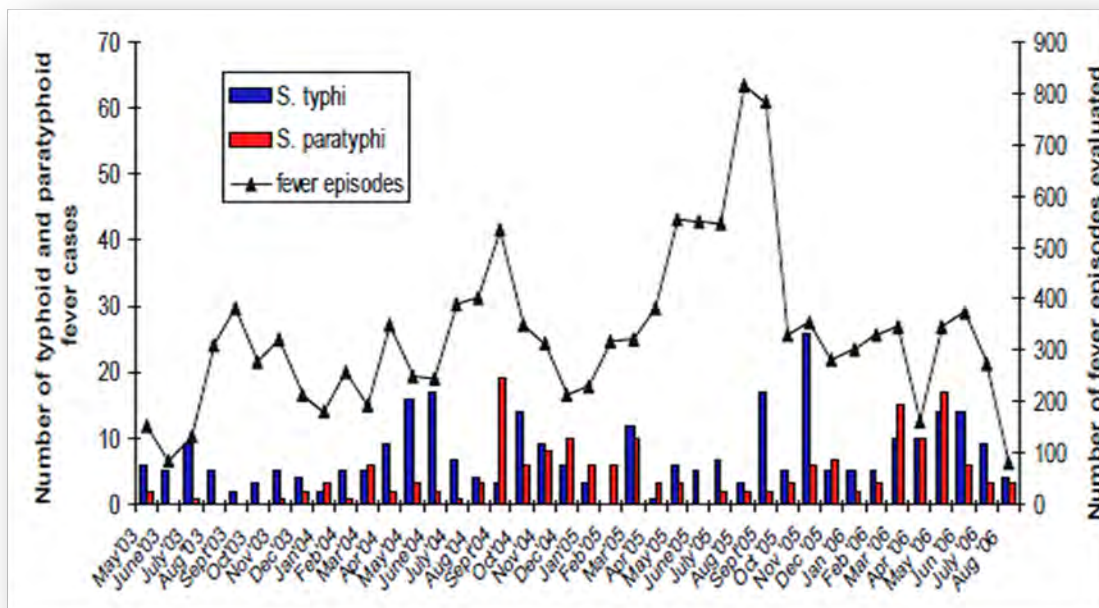


Figure 1.9: Month-wise variation of typhoid and paratyphoid incidence in Kolkata.

1.8 Clinical Manifestation

Typhoid and Paratyphoid fevers are very sensitive systemic disease. In most developing countries the typhoid patients do not take as serious as it is. The reason behind is non-specific feature like consistent low grade fever, diarrhea and vomiting. The symptoms most often overlap with other febrile illness like Malaria, Dengue, Kalajar. The onset of the fever lasts for 3-4 weeks depending on the virulence of the organism and infectious dose. [1]The temperature rises gradually with dull frontal headache, myalgia, a dry bronchitic cough, anorexia, nausea, malaise and chills. Acute case of typhoid fever has an incubation period of 10-14 days however it can progress into multiple organ failure resulting intravascular coagulation and central nervous system involvement may result in early death. Intestinal perforation occurs in 1-3% of cases in hospital. Higher fatal case observed intermittent confusion, insomnia, and dizziness in 3-10% cases[23]. The bleeding of intestinal vessel occurs due to erosion of the peyer's patch in 10-20% cases. Other gastrointestinal symptoms include:

- Diarrhea presents especially in immunocompromised cases and infants
- Constipation in adults
- Abdominal tenderness
- Mild hepatosplenomegaly has been observed after first week.
- Bradycardia may be observed relative to the condition of fever.
- Epistaxis in early stage of illness.
- Rose spots featured small, pale red, blanching, slightly raised maculae seen on the chest during first week.

The clinical manifestation of Paratyphoid fever is similar with typhoid fever except the less severity along with shorter incubation period of *S.Paratyphi* A and B. But jaundice and thrombosis often manifest with paratyphoid fever

1.9 Diagnosis of Enteric Fever

Instant diagnosis and the following microbial drug treatment reduced the complication of enteric fever and prevent the spreading of bacteria.[24] The aim of all diagnostic procedure is to distinguish the typhoid and paratyphoid fever from other febrile illness.

1.9.1 Blood culture

Blood culture of *S. Typhi* and *S. Paratyphi A* is regarded as the standard diagnostic technique worldwide. About 50% enteric patients found positive in this method.[25] The sensitivity is higher during the first week of infection. The volume of blood, the use of antimicrobial drug and the timing of blood collection is an important factor for the determination of bacteria.[26, 27]

1.9.2 Widal test

Widal test is used to determine the agglutinating antibody against O and H antigens of *S. Typhi*. Minimum requirement of blood is 1 ml and the acute serum is used to perform the test. The sensitivity and the specificity of the test are moderate. Because the cut-off value of the agglutination test varies depends on the prevalence area and the incident time of disease. But the standard antibody titer for O-antigen is 1:80 and H-antigen cut-off value is 1:160 indicate recent typhoid infection. [28]

1.9.3 Typhidot test

Typhidot is rapid immunoassay test. It can detect the specific IgM and IgG antibodies against the outer-membrane protein ST50 of *S. T.* The test is simple and economic but the specificity is 75% and the sensitivity is 95%. It is able to carry out within three hour. Due to the Persistence of IgG in blood for more than two years after typhoid infection, typhidot test is going through some evaluation.[29]

1.9.4 TPTest

TPTest can detect *Salmonella Typhi* specific IgA responses based on the secretion of antibodies from peripheral blood lymphocytes. The test needs a small volume of blood and the early diagnosis is feasible. The sensitivity of the test is 100% and the specificity ranges between 78-97%.[12]

1.10 Treatment

The infected patient is clinically diagnosed and the conformed typhoid fever or other Salmonella infection was treated with ampicillin or trimethoprim-sulfamethoxazole.[30] Ciprofloxacin and ceftriaxone were reserved for MDR infections. But the recent studies on antibiotics resistance pattern in Bangladesh has shown ciprofloxacin resistance with high MIC (Minimum Inhibitory Concentration) found in 2006.[31]

1.11 Prevention

Typhoid and Paratyphoid fever is foodborne and waterborne disease. The preventive measure is crucial step for the reducing enteric fever rate in Bangladesh. The infrastructure as well as the social awareness could be helpful for eradicating infected patients. The rapid urbanization and changing food habits may lower the prevalence of typhoid and paratyphoid fever.[32]

The following preventive measure should be taken as follows:

- Safe drinking water should be made available to the population through a piped system or from tanker trucks.
- Wells must be checked for pathogens and treated if necessary.
- A particular attention must be paid to the disinfection and the storage of the water however safe its source.
- Washing hands with soap before preparing or eating food; avoiding raw food, shellfish, ice; eating only cooked and still hot food or re-heating it.

1.12 Vaccination of Enteric fever

WHO recommended the vaccination among the high risk population for the control of enteric fever. There are three vaccine has been developed named inactivated whole-cell typhoid vaccine, Vi-polysaccharide and the live attenuated Ty21a vaccine. The emerging of multidrug resistance *S.Typhi* is emphasizing the new vaccine formulation and the demand for the development of vaccine especially against paratyphoid fever is urgently needed.[32]

1.13 Rationale of the Study

The objective of the study is to estimate the prevalence of the enteric fever in different regions of Bangladesh. We would be able to compare the results of one region with that of the other. The possible outbreak of enteric fever in the urban and rural area as well as the management of risk factor responsible for the disease outbreak is monitored.

1.14 Hypothesis of the study

The surveillance system will be useful to estimate the prevalence of enteric (typhoid and paratyphoid) fever in Bangladesh.

1.15 Objective

The objective of the study was to estimate the prevalence of enteric fever caused by *S. Typhi* and *Paratyphi* among the individual who came to seek treatment from the study site across Bangladesh. The Principal objectives of the present study were as follows:

- a) Diagnosis of Typhoid and Paratyphoid fever through blood culture and TPTest method by using the blood sample of infected patient.
- b) The possible outbreak of enteric fever in the urban and rural area as well as the management of risk factor which is responsible for the disease outbreak.
- c) Identification of the high risk population and the respective endemic area for the typhoid and paratyphoid fever and comparison of high risk population between the seven divisions of Bangladesh.

Chapter: 2 Methods and Materials

2.1 Overview of the Study

The enteric fever is causing the febrile illness towards the huge number of population living in the city and rural area of Bangladesh. This study is designed for the understanding of different risk factor, isolation and identification of pathogen by the collection of blood sample. The whole study was supported by IEDCR (Institute of Epidemiology, Disease Control and Research), A Government organization collaborate with Mucosal Immunology and Vaccinology Laboratory, icddr. The major concern of this collaboration was systemic and ongoing collection of data and the confirmation through laboratory analysis of clinical sample for the proper utilization of resources and advancement of public health sector of Bangladesh.

2.2 Study Site

The study was held in the Mucosal Immunology and Vaccinology laboratory at the icddr, in Dhaka, Bangladesh. Blood specimen is collected from ten sentinel site across the seven division of Bangladesh. The study site has been organized in such a way that overall incidence rate of enteric fever in Bangladesh would estimate. The demographic, Socioeconomic, clinical data has been collected from every patient and required volume of blood sample is collected for identify the risk factor, prevalence area, and the development of the prevention as well as the control measurement against typhoid and paratyphoid fever.

Dhaka	• 100 Bedded district hospitals, Narsindgi • Uttara Adhunik Medical College Hospital • Dhaka Medical College Hospital, Dhaka
Chittagong	• 250 bedded district Sadar Hospital, Cox's bazar • Bangladesh Institute of Tropical and Infectious Disease, (BITID) Chittagong
Rajshahi	• Adhunik Sadar Hospital, Naogaon
Khulna	• District Sadar Hospital, Shatkhira
Barisal	• 250 bedded district Hospital Patuakhali
Rangpur	• District Sadar Hospital, Thakurgaon
Sylhet	• Adhunik District Sadar Hospital, Habigonj

Figure 2.1: The ten study site is divided in seven divisions.

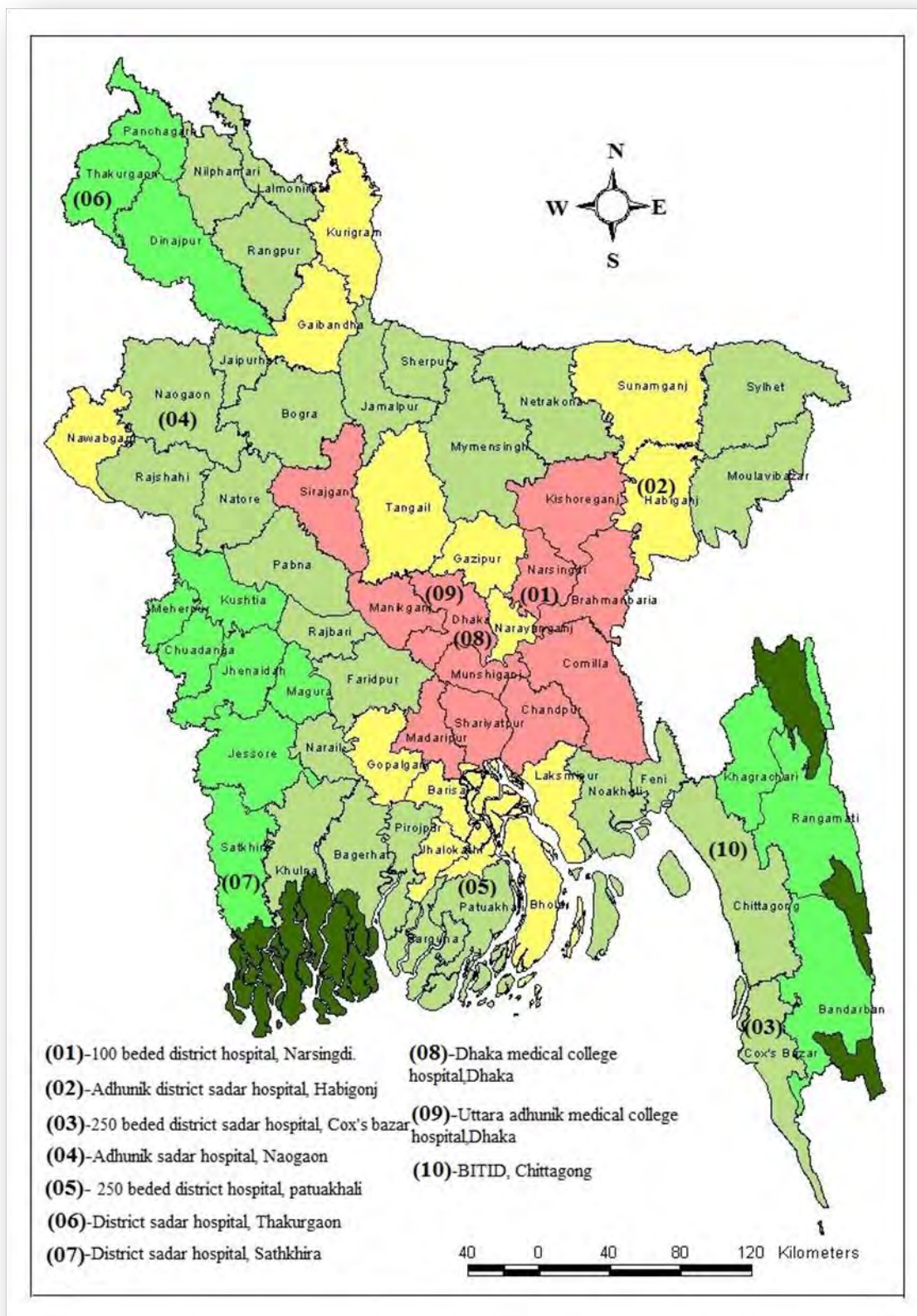


Figure 2.2: The ten sentinel site of enteric fever surveillance in Bangladesh.

2.3 Sample Collection

The study was conducted from May 2014 to December 2015. The blood sample was collected from 2113 patients attending the hospitals. The inclusion criteria set for the patient was sustained fever more than three consecutive days and the temperature above 38°C / 100.4°F. During the sample collection, one portion of blood is taken into blood culture bottle and rest of the blood is taken into heparinized tube. The patient ID is written in the culture bottle and the heparinized tube for the identification. Then the sample has been sent to icddr laboratory via IEDCR.



Figure 2.3: BD BACTEC® blood culture bottle.

2.4 Study Design

About 8 mL and 5 mL venous blood was collected from the adult age ≥ 18 years and child age 0-17 years respectively. The collected blood specimen was used to diagnose typhoid and paratyphoid fever using blood culture and TPTest (Typhoid and Paratyphoid Test) technique.[12]

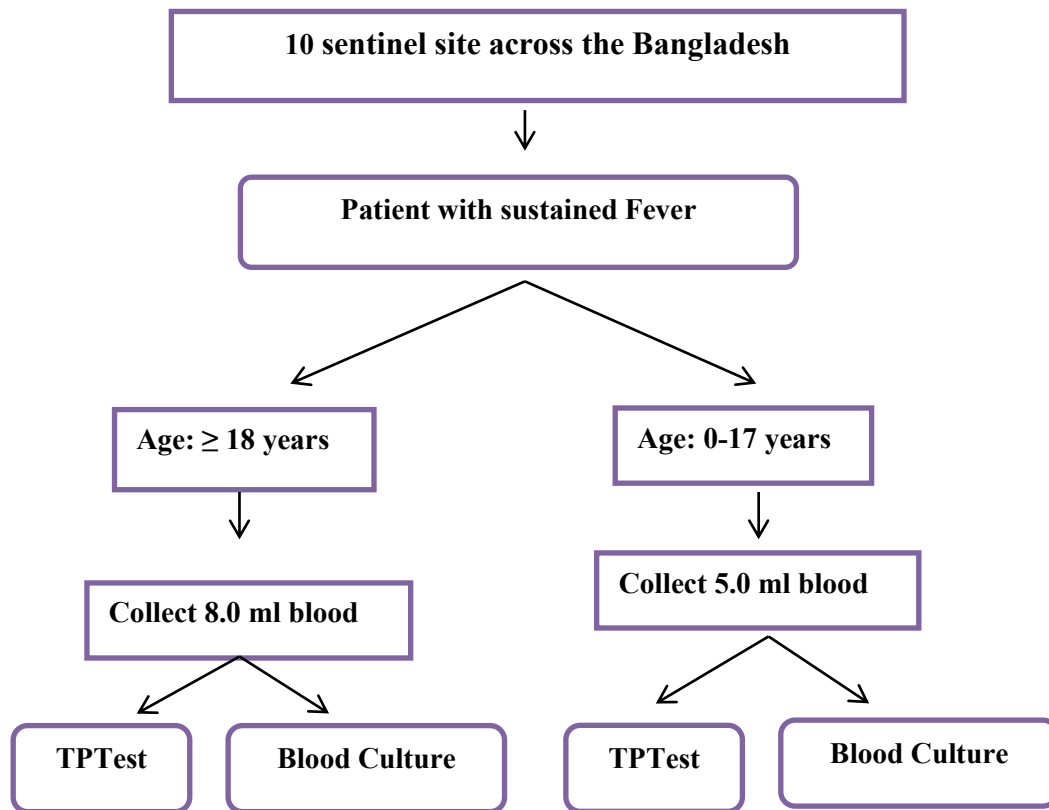


Figure 2.4: Overview of the study design

2.5 Ethical Issues

This surveillance study was approved by the Research Review Committee (RRC) and the Ethical Review Committee (ERC) of icddr, b. The written consent was taken from the participants during the enrollment in the study. In case of children, the consent was taken

from the legal guardian. The purpose, risk and benefits was written and explained to the participants

2.6 Laboratory Based Method Used in the Study

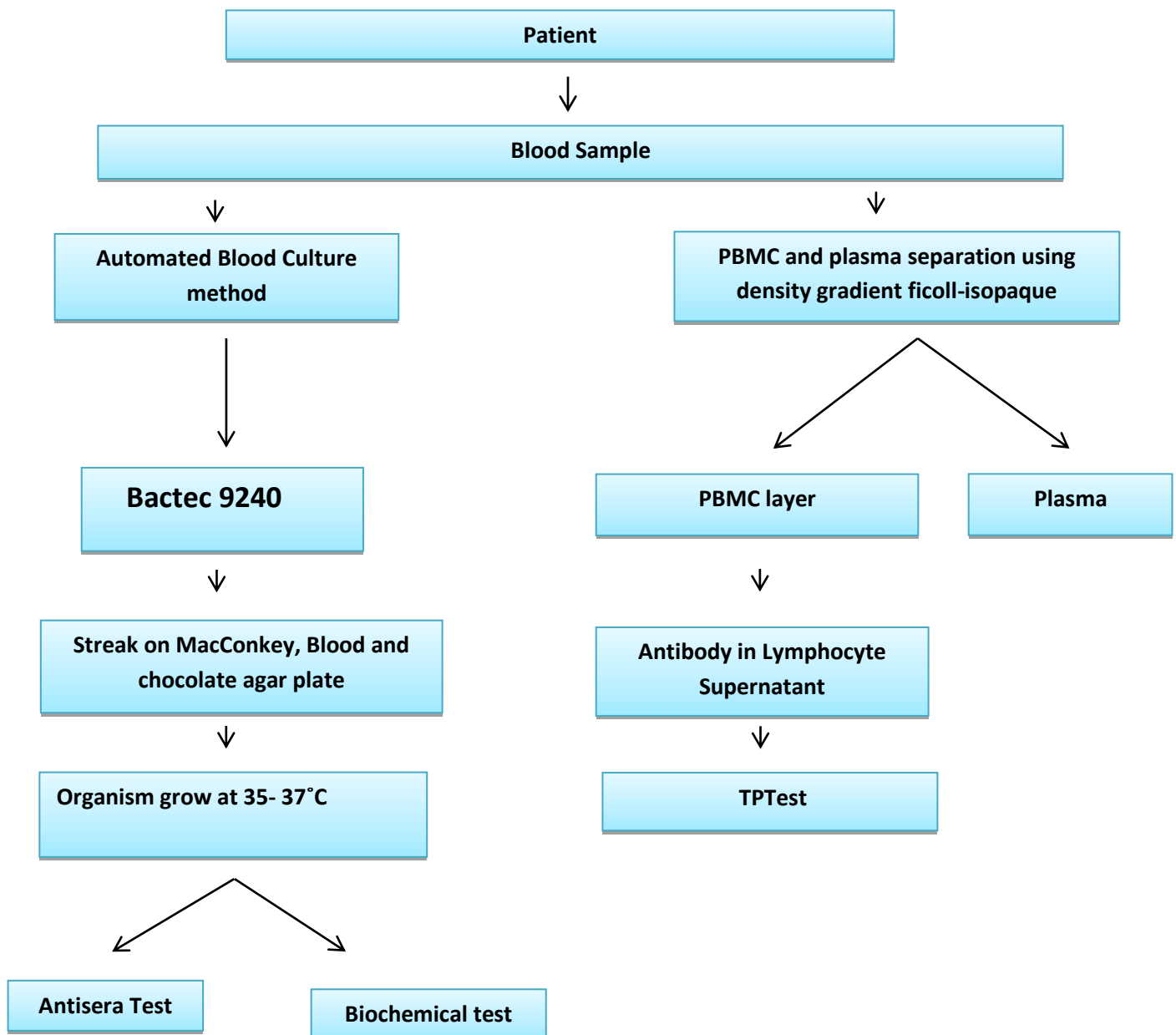


Figure 2.5: The flow chart of the laboratory procedure

2.7 Blood Culture

The blood specimen collected in yellow cap culture bottle and the procedure is performed by Bectec 9240 automated system. [33] [34] The brief description is given below:

1. Minimum 5 mL of blood was inoculated in the culture bottle and loaded into Bectec 9240 machine for a maximum period of 5 days.
2. Standard Bectec 9240 software was used for recording the results.
3. Bottles flagged as positive by the Bectec 9240 instrument and were sub cultured on MacConkey, Blood, and Chocolate agar, Then incubated overnight at 37°C.
4. After incubation plates were examined for non-lactose fermenting colonies.

2.8 Biochemical Test:

2.8.1 Triple Sugar Iron (TSI) Agar Medium

Triple Sugar Iron agar is a reliable diagnostic medium for the detection of *Salmonella* species.[27] The TSI medium is preferable over the KIA medium Because of the addition of sucrose. The reason behind is the inability of *Salmonella* and *Shigella* species to metabolize either lactose or sucrose. As a result any change that produce acidic reaction would indicate that lactose, sucrose or both is fermented and the identification would be achieved.

Major ingredients of TSI:

- Glucose – 0.1%
- Lactose – 1%
- Sucrose – 1%
- Peptone (Nitrogen source)
- Sodium thiosulphate (Sulfur source)
- Ferrous sulfate (H₂S indicator)
- pH – 7.4
- pH indicator – Phenol red (orange at neutral pH)
- Turn into pink at alkaline pH & turn into yellow at acidic pH.

These ingredients allow determining four biochemical properties of unknown organism.

- A. Lactose (+) or (-): At the surface of the slant (aerobic conditions), only fermentation of carbohydrates present in high concentration (in this case lactose) yield more

acidic products than can be oxidized to neutrality. Thus lactose (+) organisms yields a yellow slant and lactose (-) organisms yield a red slant.

- B. Glucose (+) or (-): In the largely anaerobic butt of the tube, even fermentation of the trace concentrations of glucose yields enough acid to change the P^H. Thus glucose (+) organisms also yields a yellow butt. Fermentation of lactose in the butt will obviously also change the P^H. This does not confuse the interpretation since all lactose positive organisms are also glucose-positive. If a black color from iron sulfide obscure the butt can presume it is yellow.
- C. H₂S (+) or (-): if an organism forms H₂S, the lower portion of the tube will turn black, due to formation of iron sulfide.
- D. Gas formation (+) or (-): If an organism's forms gas from glucose or lactose the agar in the butt will show bubbles or cracks.

2.8.2 Motility Urea Indole (MIU) Medium

A semisolid Motility Indole Urea medium designed for detection in Enterobacteriaceae of urease activity, motility, and indole production is used for the detection of specific organism. The MIU medium is inoculated by inserting a straight wire into tube. Then 18-24 hours incubation carried out at 37°C. A negative result showed Urea and indole remain same yellow color which indicates the presence of *Salmonella* Typhi and Paratyphi. The turbidity or growth is showed extending from the line of inoculation indicated motility of the organism. The non-motile organisms grew along the line of inoculated medium. A positive result is indicated by the formation of a red line at the interface of the reagent and the medium.

Appearance: Liquid

Color: Golden

pH: 6.8 ±0.2

Recommend Incubation: Aerobically at 37±C for 18-24 hours

Shelf Life: 182 Days.

2.8.3 Citrate Agar

Citrate agar slants contain sodium citrate (only carbon source) and ammonium ion (the sole nitrogen source). A pH indicator, Bromothymol Blue is also included. Bromothymol Blue is **green** at pH < 7.0 and **blue** at pH > 7.6. Organisms that utilize citrate for energy produce alkaline compounds as by-products. Thus, a positive result for citrate utilization is the formation of a blue color. For the conformation of *Salmonella* Typhi and Paratyphi, the color of the citrate agar would be remained green.

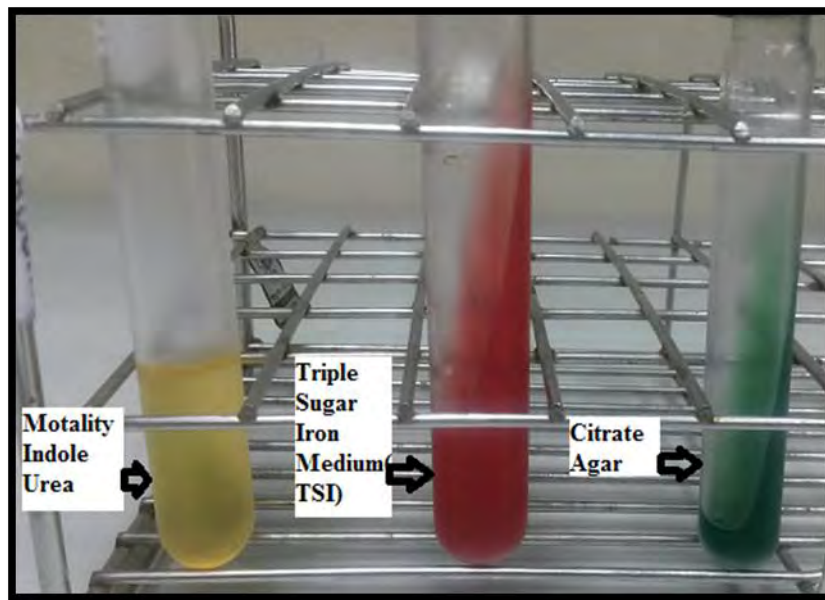


Figure 2.6: The three different medium used for the biochemical detection of organism.

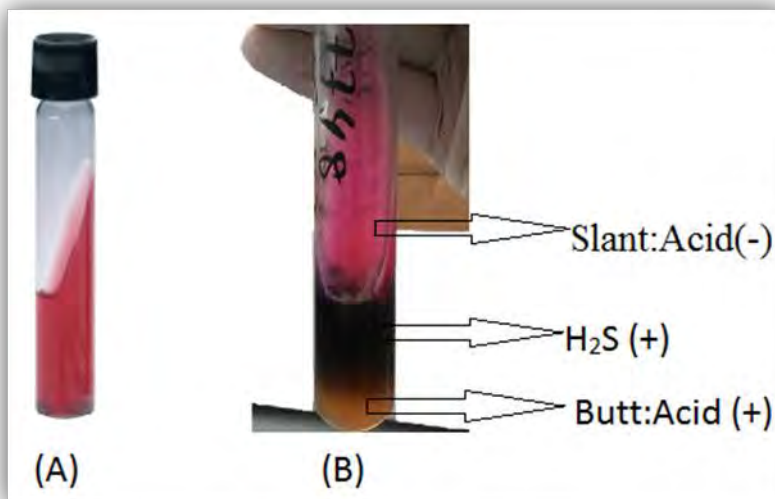


Table 1: Overview of the biochemical test

Figure 2.7: (A) TSI medium before incubation (B) TSI medium after incubation

Organism	Citrate	Motility	Indole	Urea	TSI (Triple Sugar Iron) agar			
					Slope	Butt	Black (H ₂ S)	Cracks (Gas)
<i>Salmonellae</i>								
<i>Salmonella</i> Typhi	-	+	-	-	R	Y	+ weak	-
<i>S.Paratyphi</i> A	-	+	-	-	R	Y	-	+

2.9 Serological detection of *Salmonella* and serovers

Anti-sera test has been performed for the identification of different serover of *Salmonella* containing different antigenic properties.[35] The slide agglutination test is able to isolate the *Salmonella* Typhi, Paratyphi A, Paratyphi B from the culture.

2.9.1 Step-1 Identification of *Salmonella* species

The slide has been taken and divided into three divisions with glass marker. Starting from the left, 10µL of O-Polyvalent antiserum is added in first part of dividend slide. A loop of 3 single bacterial colonies added in the reagent by inoculating tips and mixed thoroughly by tilting the slide back and forth for 1 minute. The agglutination was observed carefully with the light. When the agglutination of the mixture in the slide becomes visualized strongly while observation, it would be regarded as a *Salmonella* species.

2.9.2 Step-2 Identification of *Salmonella* Typhi

For the detection of *Salmonella* Typhi, 10 µL of Vi-Polysaccharide is added to the second part of marked slide. Again a loop of 3 single bacterial colonies is mixed with the reagents through inoculating tips. For the 1 minute, the slide is tilted back and forth until mixing is complete. Then observe the slide carefully with light. The strong agglutination would be determined the serover S.Typhi.

2.9.3 Step-3 Identification of *Salmonella* Paratyphi A

The third part of the marked slide is used for the determination of *Salmonella* Paratyphi A. 10 µL of S-Poly A antigen is added to that portion. A loop of 3 single bacterial colonies is mixed with the reagents through inoculating tips. For the 1 minute, the slide is tilted back and forth until mixing is complete. Then observe the slide carefully with light. The strong agglutination would be determined the serover S.Paratyphi A.

2.9.4 Step-4 Identification of *Salmonella* Paratyphi B

10µL of S-Poly B antigen is added to the next part of dividend slide. A loop of 3 single bacterial colonies added in the reagent by inoculating tips and mixed thoroughly by tilting the slide back and forth for 1 minute. The agglutination was observed carefully with the

light. When the agglutination of the mixture in the slide becomes visualized strongly while observation, it would be regarded as a *Salmonella* Paratyphi B.

2.9.5 Step-5 Test for Negative control

At the last part of the marked slide 10 μ L of PBS is added. Then added a loop of bacterial colony by inoculating tips into PBS and mixed it thorough the above discussed procedure for 1 minute. Then the agglutination pattern of mixture observed with light. If there were no agglutination had seen through naked eye, the procedure would be acceptable for the detection of *S.Typhi* and Paratyphi.

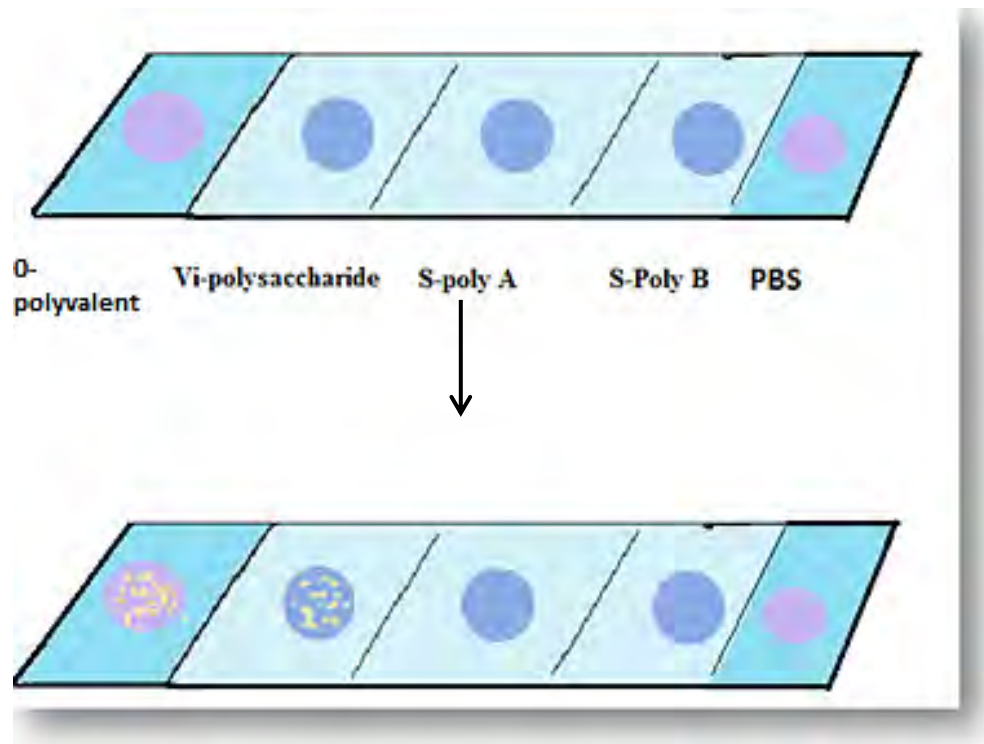


Figure 2.8: Serological identification test

3.1 Typhoid and Paratyphoid Test (TPTest)

The available diagnostic tests for the detection of typhoid and paratyphoid fever is not well-recognized. For the current study, TPTest [1] was used because of the highest specificity and sensitivity as well as the required volume of blood to be tested is feasible for the study. Detectable increase of antigen-specific antibody secreting lymphocytes in the peripheral circulation and this response can be measured in lymphocyte secretions by the TPTest as a diagnostic tool.

3.1.1 Separation of Peripheral blood Mononuclear Cells by density gradient centrifugation

- The volume of heparinized venous blood was measured and 100 μ L was withdrawn for blood grouping.
- The remaining volume of blood diluted with the Phosphate Buffer Saline (PBS) where the ratio is maintained 1:1 in the falcon tube. The identifying serial number of the patient was written in each falcon tube.
- About 3-4 mL of ficoll-Isopaque was added to falcon tube and the serial number was maintained for each tube respectively.
- The diluted blood was added to the ficoll-isopaque carefully in such a way that the ficoll layer remains in bottom of the falcon and the diluted blood do not get mixed.
- The falcon tubes were balanced and centrifuge at 772 g for 25 min at 20°C
- After centrifugation, four layers are seen in the falcon. The upper most layer is plasma, the following layer which is white and cloudy is PBMC, and the third layer in the falcon is ficoll-isopaque. The bottom layer contains pellets of RBC and granulocytes
- The plasma layer of the falcon tube was removed from the top carefully with pastaur pipette without disturbing the PBMC layer.
- Then the PBMC layer was collected in another falcon tube containing PBS.
- The volume of the PBMC containing falcon was filled upto 12 ml and was washed at 953 g (2000 rpm) for 10 minute at 20°C.
- After the first wash, the PBS is discarded and mononuclear cell pelleted is resuspended in 5 ml of PBS and 25 μ L suspended cell is withdrawn into a microcentrifuge tube for the counting of PBMC .Then 25 μ L of 0.4% trypan blue solution is added to make it 1:1 dilution and taken in haemocytometer for counting of PBMC.
- The second wash is completed at 953g (2000 rpm) for 10 minute at 20°C similar to first washed.

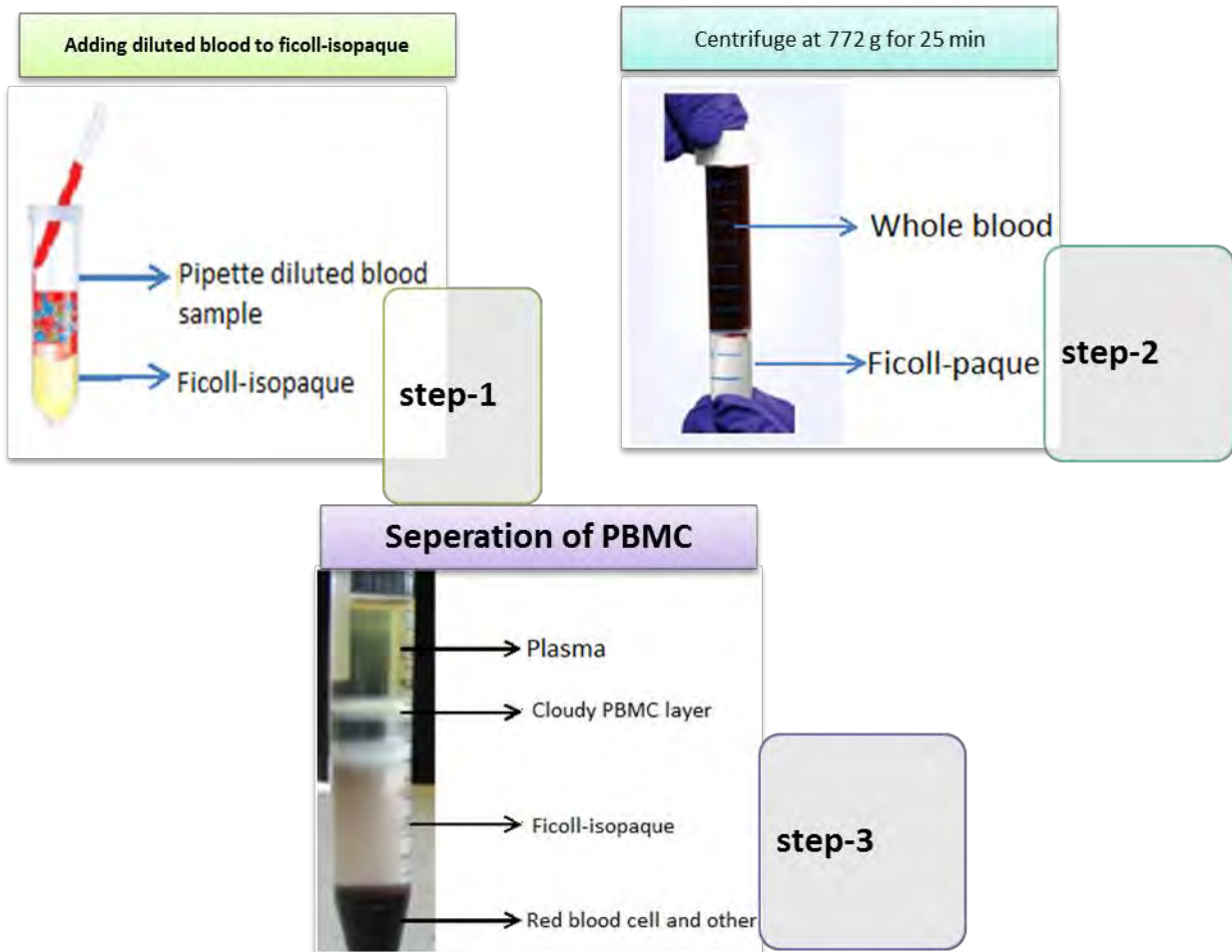


Figure 2.9: Separation of PBMC from blood sample.

3.1.2 Collection of Antibody in Lymphocyte Secretion (ALS) specimen

- The isolated PBMC is taken and resuspended in RPMI-complete medium. The required volume of RPMI is measured according to the number of PBMC. 1 mL medium is used per 1×10^7 PBMC.
- The cell culture plate is treated aseptically and cells are cultured at 37°C in 5% CO_2 incubator for 18 to 48 hours.

- For the rapid detection of enteric fever, the ELISA with the culture supernatant is carried out after 18 to 24 hours of incubation. The repetition of ELISA is done after 36 to 48 hours, if the TPTest done at 18 to 24 hours showed negative result.
- The culture supernatant is withdrawn in a micro-centrifuge tube from the culture plate after 36 to 48 hours.
- The culture supernatant in micro-centrifuge tube is centrifuged at 12000 rpm (11600xg) at 20°C for 5 minute. The supernatant is collected and protease inhibitor is added at 1% of the supernatant.
- The lymphocyte supernatant is used for TPTest and stored at -70°C if there is any remaining volume.

3.1.3 Determination of membrane preparation (MP) specific antibodies in ALS by kinetic ELISA

Nunc F plate coating:

- Membrane preparation (MP) antigen is prepared from Ty21 which is diluted with the PBS in falcon. The concentration 5.0 µg/mL is maintained for the lymphocyte culture supernatant.
- The ELISA plate is coated with 100 µL of MP-antigen in each well and is incubated at room temperature for overnight.

Blocking the plates:

- The coated plate is washed for three times with PBS.
- The plates were blocked with 200 µL/well of 1% bovine serum albumin in PBS (BSA-PBS) and is incubated for 30 minutes at 37°C.

Sample loading:

- The plates were washed three times with PBS-Tween (0.05% Tween) and once with PBS.
- ALS samples were diluted at 1:2 dilutions with 0.1% BSA-PBS-Tween.

- For positive control, pooled plasma of typhoid positive patients was taken and diluted at 1:100 dilutions with 0.1% BSA-PBS-Tween and 100µL of diluted pool solution was given in appropriate well.
- For negative control, 100 µL 0.1% BSA-PBS-Tween was given in appropriate wells.
- The plates were then incubated at room temperature for 90 minutes.

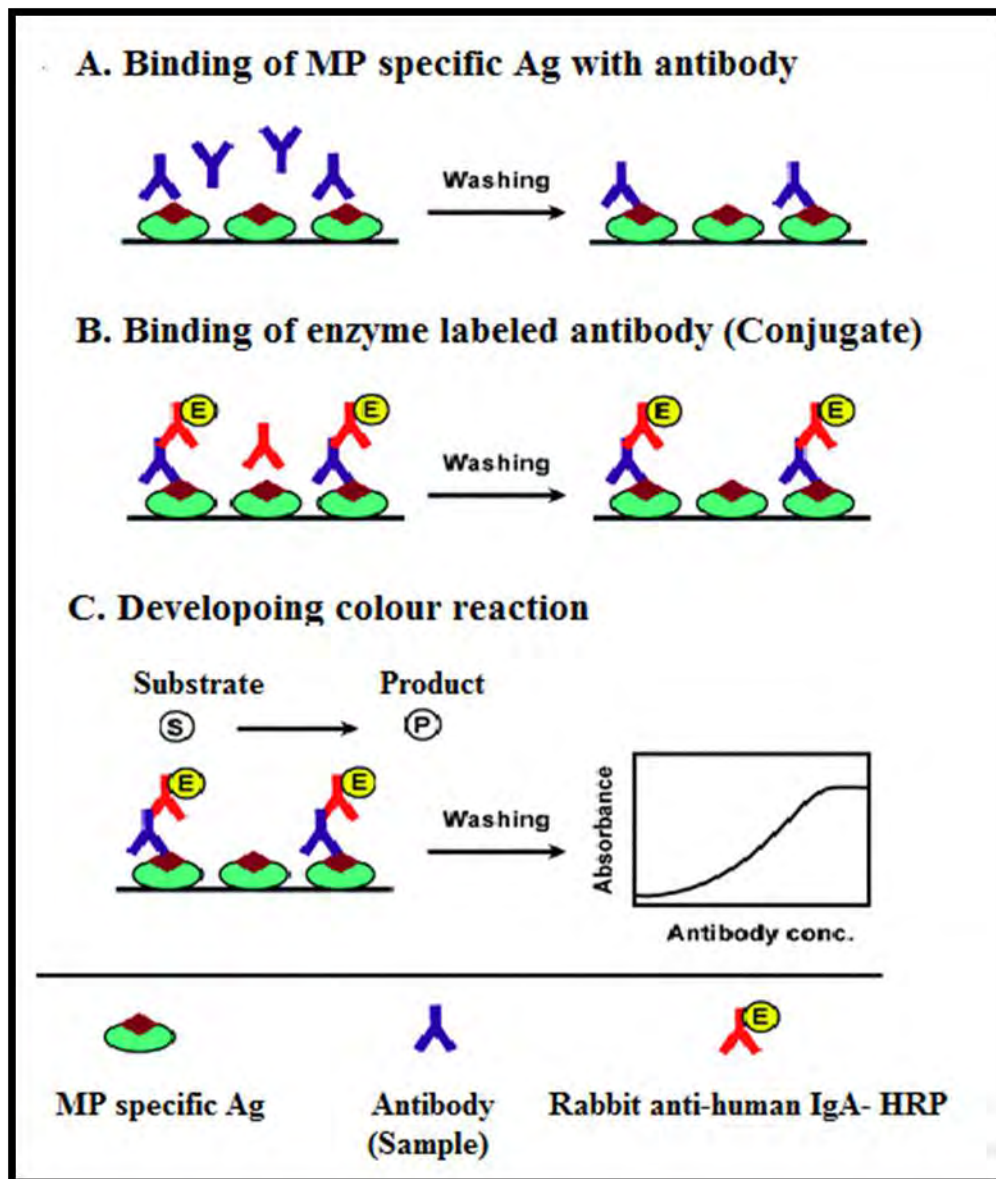


Figure 2.10: The mechanism of MP specific kinetic ELISA from ALS sample.

Conjugate adding:

- The plates were washed three times with PBS-Tween (0.05% Tween) and once with PBS.
- For ALS, the rabbit anti-human IgA, conjugated with horse reddish peroxidase were diluted with 0.1% BSA-PBS-Tween at 1:1000 dilutions and 100 μ L was added in each well. The plates were incubated at room temperature for 90 minutes.

Plate developing:

- The plates were washed three times with PBS-Tween (0.05% Tween) and once with PBS.
- The substrate- H_2O_2 -OPD was prepared by dissolving 10 mg OPD (orthophenylenediamine) in 10 mL of 0.1M sodium citrate buffer (pH 4.5), to which 4 μ L of 30% H_2O_2 was added immediately before use.
- The plates were developed by adding H_2O_2 -OPD 100 μ L in each well.
- Then optical density (O.D.) was measured at 450 nm by the Multiskan Ascent ELISA reader in kinetic mode immediately.

3.2 Statistical Analysis

Comparisons of immunological response for significance were carried out using the Wilcoxon signed rank t-test within a group and Mann Whitney U test among groups. All reported *P* values were two-tailed and $P \leq 0.05$ was considered a threshold for statistical significance. Analyses were performed with GraphPad Prism 5.0 and Microsoft Excel 2010.

Chapter: 3 Results

3.1 Study subjects

A total of 2113 specimens were collected from febrile patients attending ten different study sites in Bangladesh from May 2014 to December 2015. Due to unavoidable problems leading to lack of transportation, all collected blood samples could not be used for both blood culture and TPTest. TPTest was also not carried out for some samples due to blood clot, haemolysis and problems in processing of blood. Blood culture and TPTest was done in 2068 and 2036 specimens respectively. The specimens were analyzed using microbiological, biochemical and immunological techniques for the isolation of *Salmonella* Typhi and Paratyphi A, B, C types. For each specimen the blood culture and TPTest method was used for the laboratory confirmation of enteric fever.

3.2 Prevalence of enteric fever in Bangladesh

3.2.1 Number of cases detected by TPTest method in Bangladesh

In this study, the seven divisions of Bangladesh are representing the overall scenario of enteric fever in Bangladesh (figure 3.2). The results of TPTest ≥ 10.0 is the cut off value for the sample. Three sentinel sites (100 bedded Districts Hospital Narsindgi, Dhaka medical college hospital and Uttara Adhunik medical college hospital) were used for the understanding of enteric fever incidence rate in Dhaka 57.17% (n=303) and found it to be the most prevalent when compared to the other 6 divisions. Medium level of prevalent area was found in Barisal 12.06% (n=64) and Chittagong 10.75% (n=57). The prevalence was comparatively lower in Sylhet 7.92% (42), Rangpur 4.72% (n=25), Khulna 4.34% (n=23) and Rajshahi 3.02% (n=16).

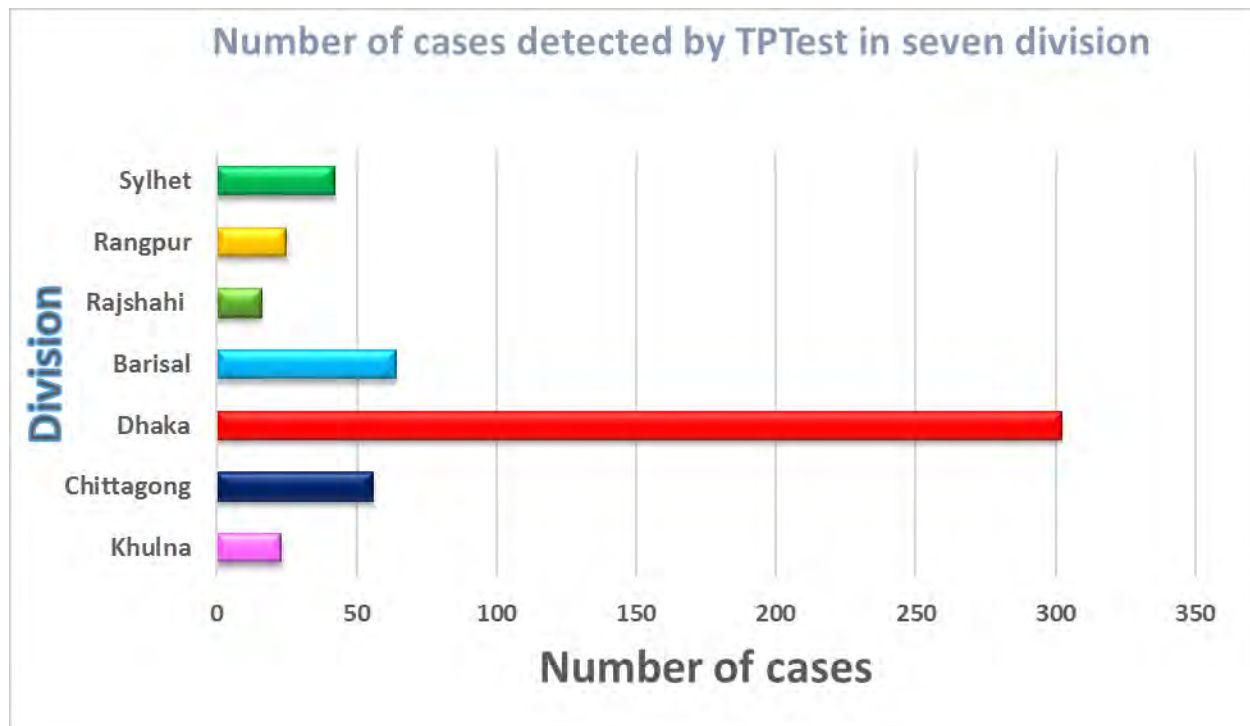


Figure 3.1: Frequency of enteric fever detected by TPTest method in seven divisions of Bangladesh.

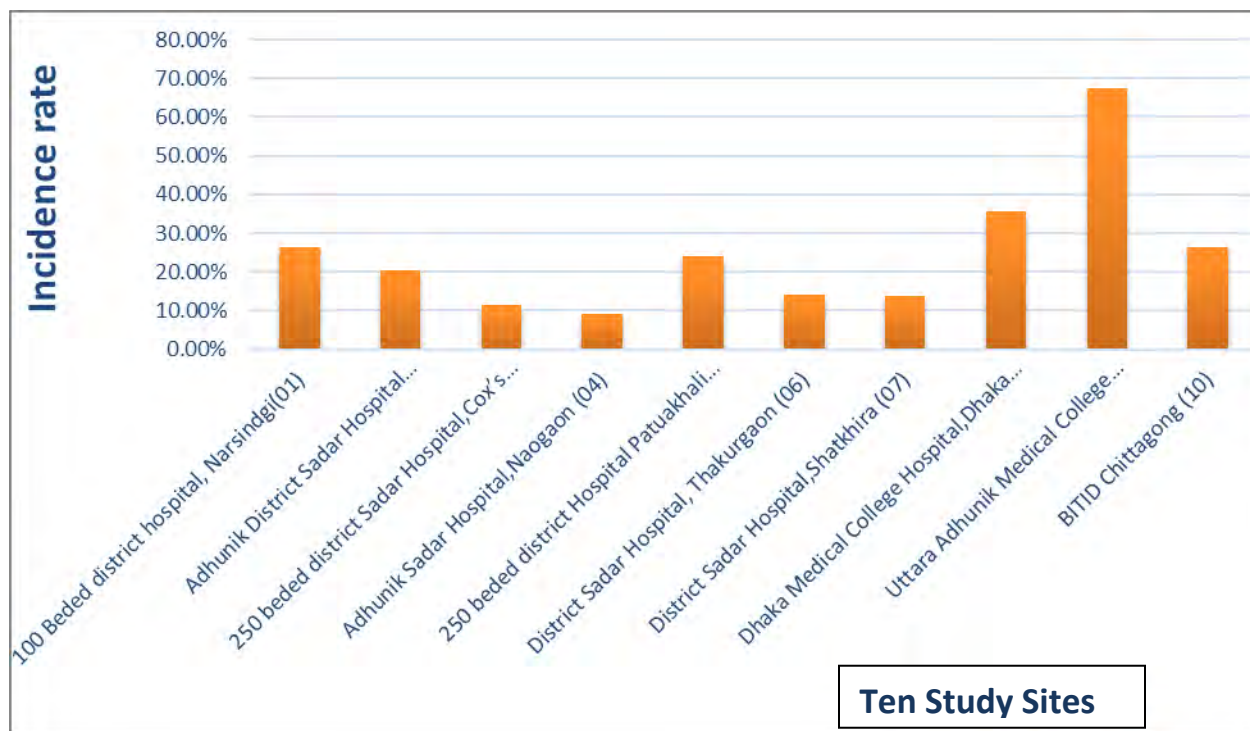


Figure 3.2: Incidence rate of enteric fever detected by TPTest method in the study sites.

3.2.2 Distribution of enteric fever cases in Bangladesh by blood culture method

From 2068 tested samples, 59 culture positive patients were found by using Bactec 9240. Among them 46 were positive for the *S. Typhi* and 13 for *S. Paratyphi A*. Overall 2.85% culture positive specimens were confirmed as enteric fever. The incidence rate of enteric fever in Dhaka was 59.32% (n=33) out of total culture positive sample. The other region comprised 40.68% (n=11) of total organism. Among other divisions, Chittagong was covered by two study sites (250 bedded district sadar hospital, Cox's Bazar and BITID, Chittagong) and the prevalence was found higher 13.56% (n=8). Khulna, Barisal, Rajshahi, Sylhet, Rangpur was less prevalent for enteric fever 10.16% (n=6), 6.77% (n=4), 5.08% (n=3), 23.39% (n=2), 1.69% (n=1) respectively.

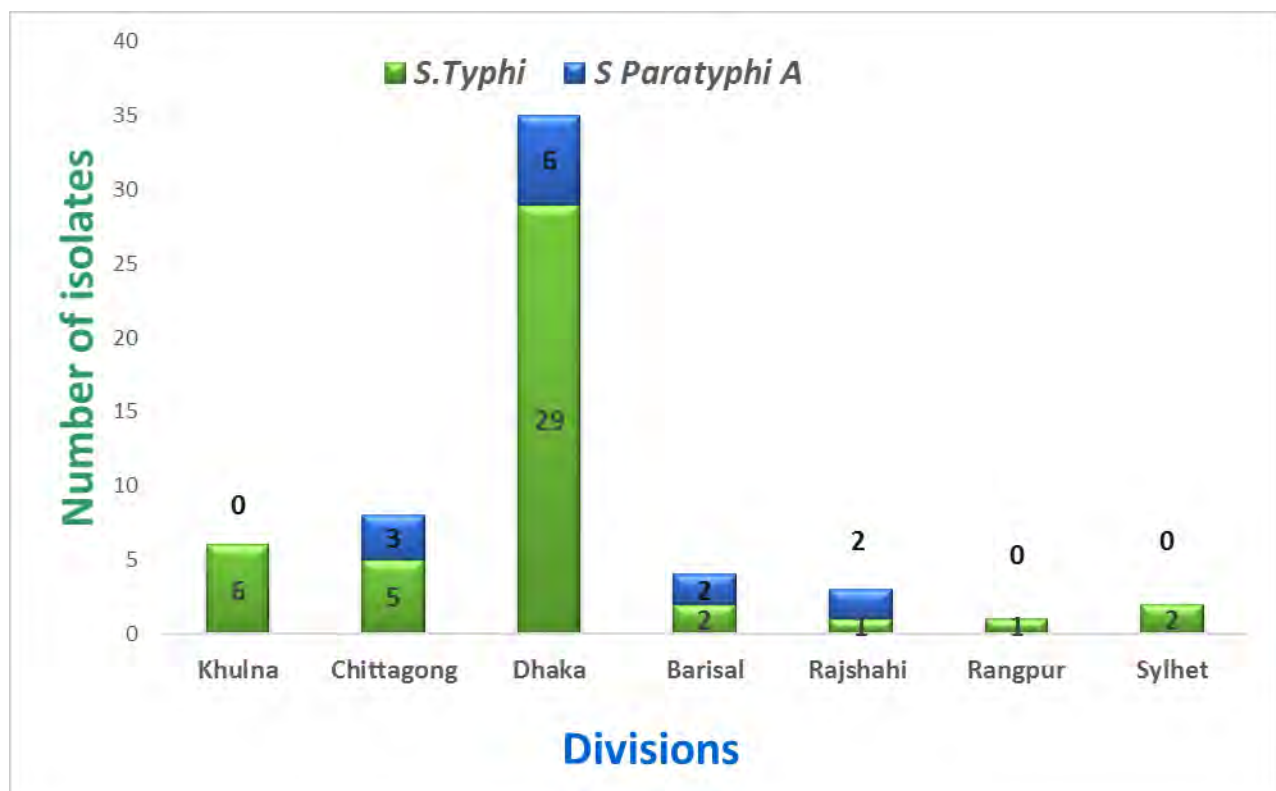


Figure 3.3: Prevalence of enteric fever detected by blood culture method in seven divisions of Bangladesh.

3.2.3 Overall prevalence of enteric fever (TPTest and blood culture) in Bangladesh.

During the study period, the prevalence of enteric fever was positive in 537 out of the total 2113 sample, identified in the blood sample from the patients in ten different sentinel sites around Bangladesh. The number of TPTest positive febrile patients was 530 detected from 2036 sample and the culture confirmed enteric fever patients was 59 out of 2068 sample. In percentage, 26% of the total number of patients was enteric fever positive by the TPTest detection method. In the blood culture method, 2.85 % of the patients were enteric fever positive where 2.22% was infected with *Salmonella* Typhi and *Salmonella* Paratyphi A was 0.62%. But there were no *S. Paratyphi* B and *S. Paratyphi* C isolated

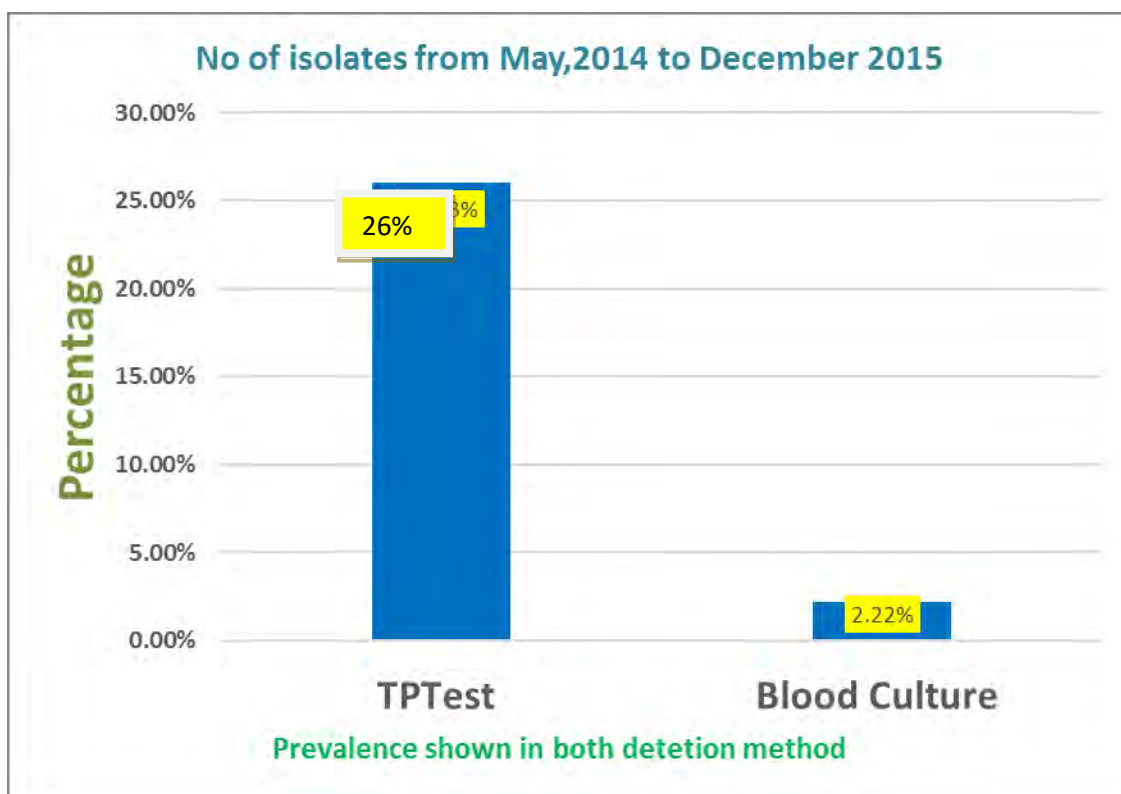


Figure 3.4: Prevalence of enteric fever from May 2014 to December 2015

In this study period, two detection methods were used simultaneously. The number of TPTest positive sample was 530, whereas the culture confirmed isolation of pathogen was 59. The major findings was both detection method revealed Dhaka 57% was the most prevalent division for enteric fever in Bangladesh. TPTest detected more febrile patients in Barisal (12%) and Sylhet (7.92%). The blood culture method showed relatively high prevalence of enteric fever in Khulna 10.16%. Chittagong division remained similarly highly prevalent in both TPTest and blood culture detection method (Table 3.5).

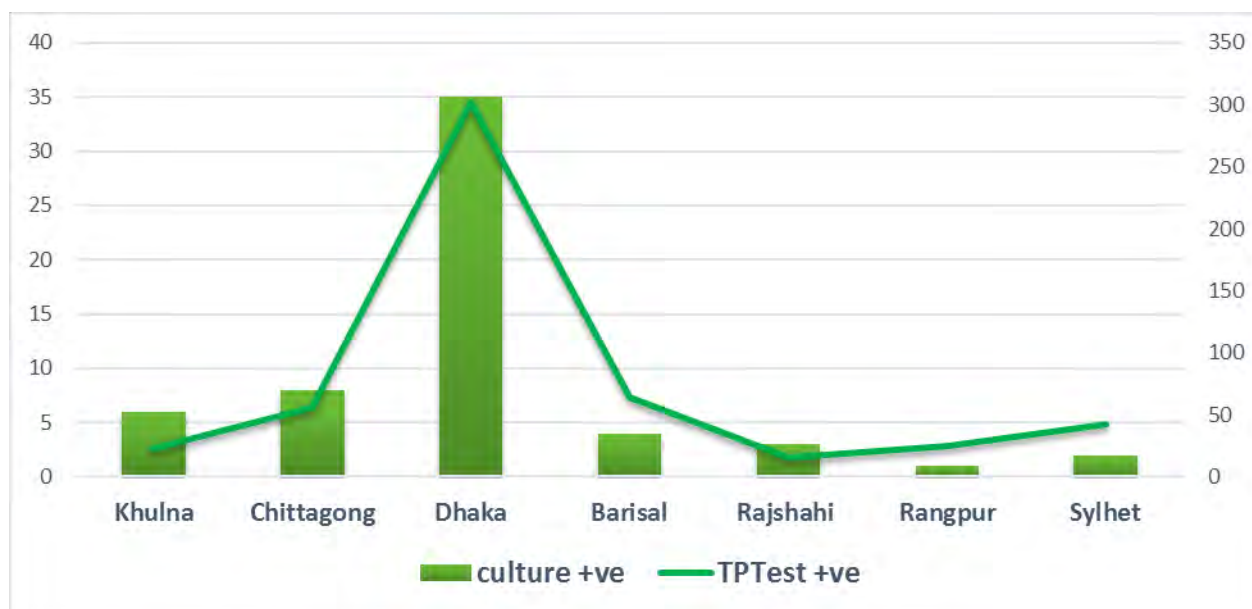


Figure 3.5: Cases of Enteric fever patients detected by TPTest and blood culture method.

3.3 Serotype of Identified Organism

A total of two thousand and sixty eight blood samples of the suspected febrile patients were tested. From 59 culture positive isolates, *Salmonella* Typhi was 46 (78%) and Paratyphi A was 13 (22%). Specific antiserum was used to detect the agglutination with specific Vi-polysaccharide then the colonies were found positive. Biochemical test was also performed and isolates the specific pathogen responsible for sustained fever.

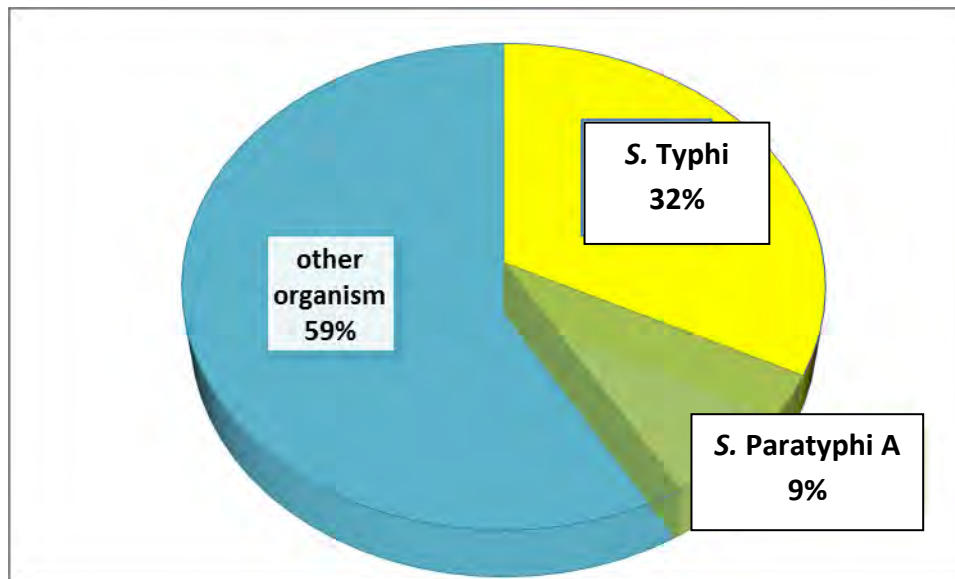


Figure 3.6: Distribution of isolated organism in percentage

3.3.1 Distribution of both serover (Typhoid and Paratyphoid fever) in Bangladesh

The slide agglutination test and biochemical test were using for the identification of *S. Typhi* and *Paratyphi A*, B, C. The patient's sample from seven divisions was analyzed. The findings showed higher incidence rate of typhoid fever in Dhaka 63%. The medium level of typhoid fever incidence rate was observed in Khulna 13.04% and Chittagong 10.86%, Barisal 4.34%, Sylhet 4.34%, Rangpur 2.17% and Rajshahi 2.17% in descending orders.

The number of *S. Paratyphi A* was also significantly higher in Dhaka 46% (n=6), followed by Chittagong 23.08% (n=3), Barisal 15.38% (n=2), Rajshahi 15.38% (n=2). There were no *S. Paratyphi A* found in Sylhet, Rangpur, Khulna.

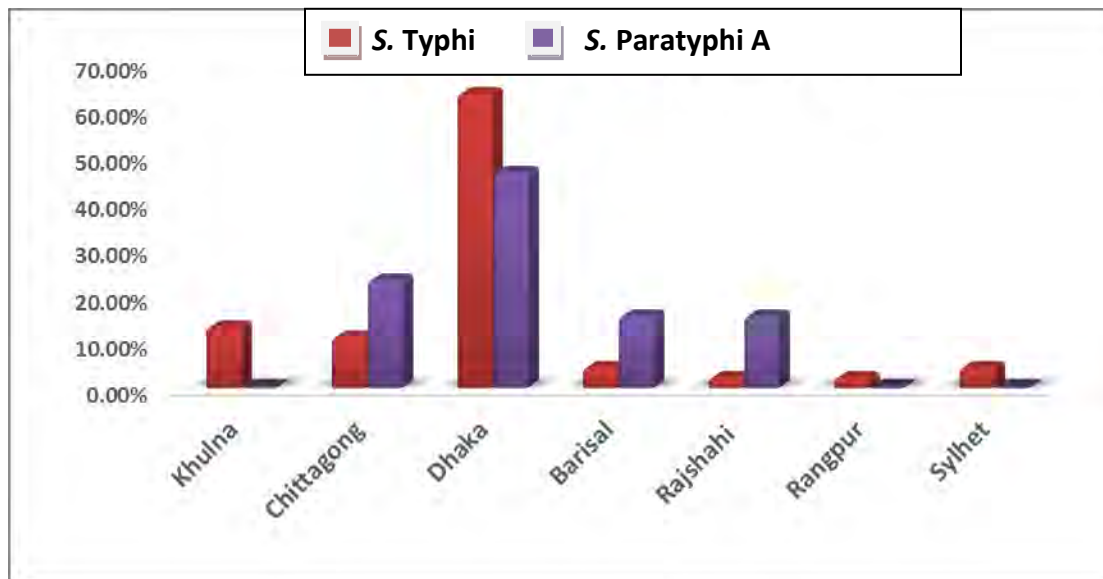


Figure 3.7: Isolation of *Salmonella Typhi* and *Paratyphi A* in different divisions of Bangladesh

3.3.2 Isolation of other organism from blood

Isolated suspected colonies from blood agar plates were also analyzed for other organisms. The number of other organism isolates were 83 and *Pseudomonas spp* and *E. coli* were significantly from May 2014 to December 2015.

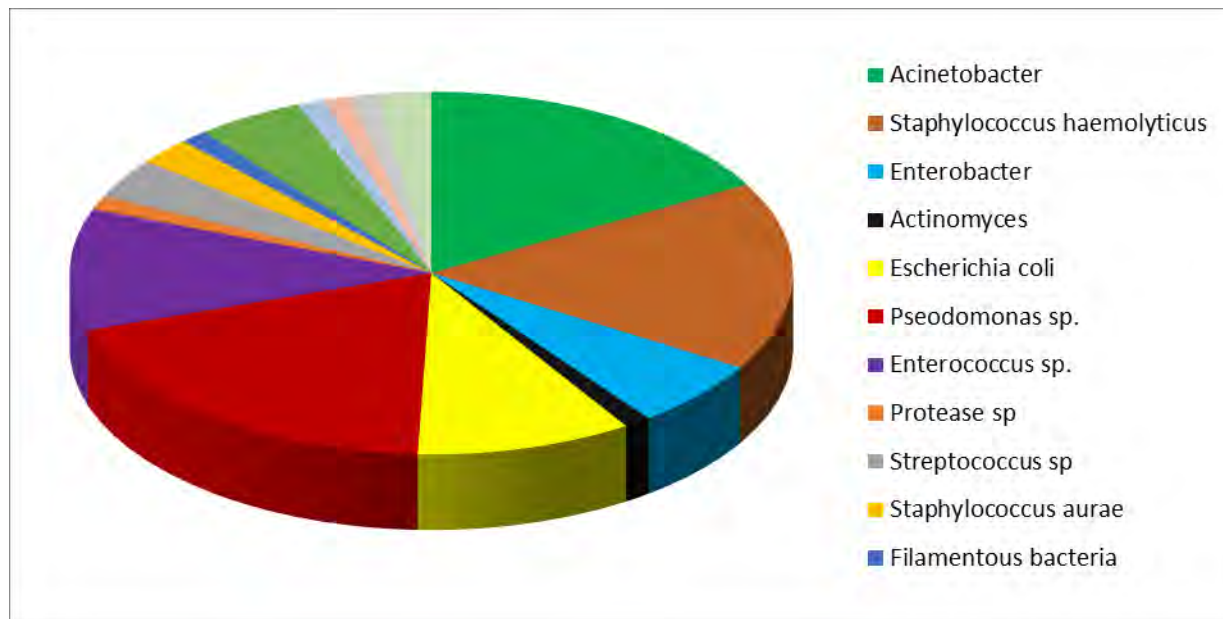


Figure 3.8: The other organisms isolated during the study period.

3.4 Seasonality of Enteric Fever in Bangladesh

From the data of hospitalized patients from May, 2014 to December, 2015, all of the respective information has been collected and compared with different months in a year. For the TPTest method, we have observed two peak seasons. The first peak was observed in the months August 2014 and 2015, the number of positive cases was 34 and 44 patients respectively. The second peak season was observed in November and December of the study period where the number of detected cases was 40, 40 in 2014 as well as 28, 27 in 2015 respectively. The occurrence of enteric fever was low in May (2), 2014 and gradually increased till August, the next two months showed low incidence which gradually rises to highest incidence month. From 2015 onwards, the incidence of enteric fever was quite similar from January to April 2015, 41, 30, 27, 31 respectively, the lower occurrence of

enteric fever took place in May (23), June (23), July (8). The incidence of typhoid and paratyphoid fever were analyzed separately. Typhoid fever incidence rate was significantly higher than paratyphoid fever (figure 3.7).



Figure 3.9: Monthly distribution of enteric fever in Bangladesh

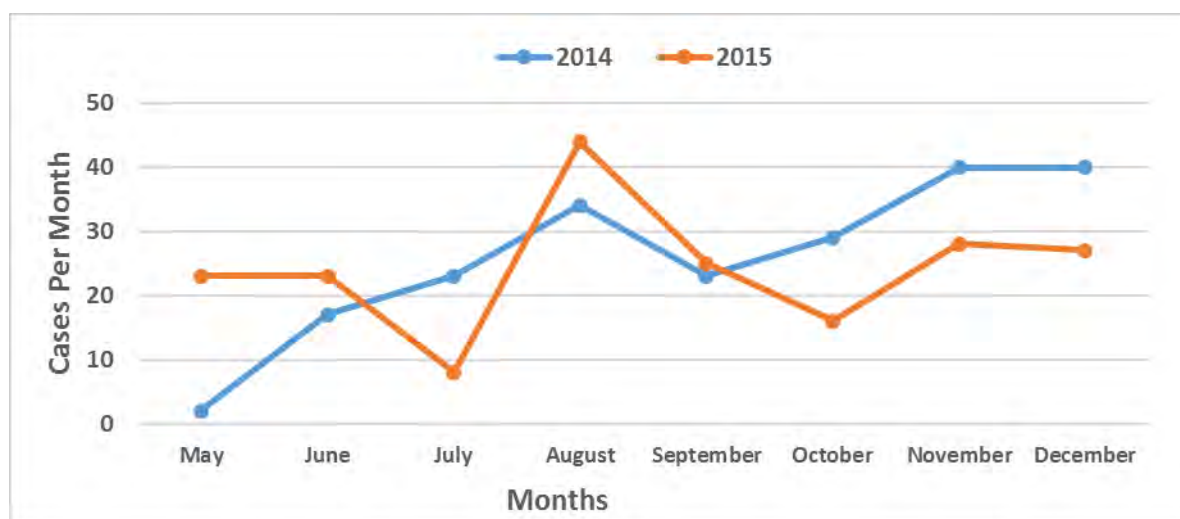


Figure 3.10: Number of TPT test positive infection cases between May 2014 to December 2015.

3.5 Demographic and clinical characteristics of patients in Bangladesh

Some important clinical and demographic data is presented in the following table 2 from the patients attending the sentinel sites during May, 2014 to December, 2015.

Two thousand one hundred and thirteen patients were enrolled the hospital during the study period. The degree of diarrhea, vomiting and clinical severity of the patients were assessed by physician. The median age of patients was 18 years. The Average duration of fever was 7 days. The temperature of the patients during admission was a bit higher (102 °F) while the patients were interviewed (101°F).

Table 2 : Baseline demographic data of the enteric fever patients (Total number of patient=2113)

Features	Median	Percentile (25 th ,75 th)
Median age in year (SD)	20 years	
Temperature During interview	101°F (38.33°C)	100.40 °F (38 °C),102 °F (38.88 °C)
Temperature During admission/Visit	102 °F (38.89 °C)	101.00 °F (38.33°C), 103 °F(39.44°C)
Duration of fever in days	7 days	6, 10
Duration of taking antibiotics before admission	3.57 days	Not available

Table 3: Important clinical features of the enteric fever patients (Total number of patients=2113)

Clinical features	Frequency (%)
Headache	1694 (80%)
Abdominal pain	1185 (56.1%)
Constipation	803 (38%)
Diarrhea	467 (22.1%)
Vomiting	818 (38.7%)
Rash	147 (7%)
Use of antibiotics before admission	711 (33.6%)

On table 3, the highly significant number of patients was suffering from headache (80%). Abdominal pain was a symptomatic problem for enteric fever (56.1%). The number of patients suffering from constipation and vomiting were quite high (38% and 38.7%). Rash was found to be a rare symptom for enteric fever.

Another very important finding about enteric fever was that 33.6% patients were enrolled for current study took antibiotics before admission.

3.5.1 Age and sex distribution of enteric fever:

During the study period three age categories was obtained for disease estimation in Bangladesh. Among 537 patients infected by enteric fever, 278 were adults (age ≥ 18), the second prevalent enteric patient age category was 176 (6-17 years). Enteric fever was highly prevalent among adults. In the blood culture method, *S. Typhi* was isolated mostly from younger child 6-17 years (22) and *S. Paratyphi A* was also isolated mostly from age ≥ 6 (12 out of 13). The prevalent of enteric fever was little bit higher for TPTest positive

(56.6%) males than females (43.4%). Male patients were three times more culture positive than females (Table 4).

Table 4 : Characteristics of enteric fever positive patient's enrolled in the study sites from May,2014 to Dec, 2015.

Feature	TPTest positive	Blood culture positive	
		S.Typhi	S.Paratyphi A
0-5 years	76 (14.3%)	8	1
6-17 years	176 (33.2%)	22	6
≥18 years	278 (52.5%)	16	6
No. of males (%)	300 (56.6%)	36(78.3%)	9(69.2%)
No. of females (%)	230(43.4%)	10(21.7%)	4(30.8%)

3.5.2 Source of water

Contaminated water was the major reason for any outbreak (outbreak) that took place prior to the occurrence. So the current study was focused on the drinking water source of participants. But the results showed a significantly higher number of patients were using tubewell water (1558; 73.7%). Only 0.1% (n=2) people were using pond water. The use of tap water was second most common after tubewell 511 (24.2%).

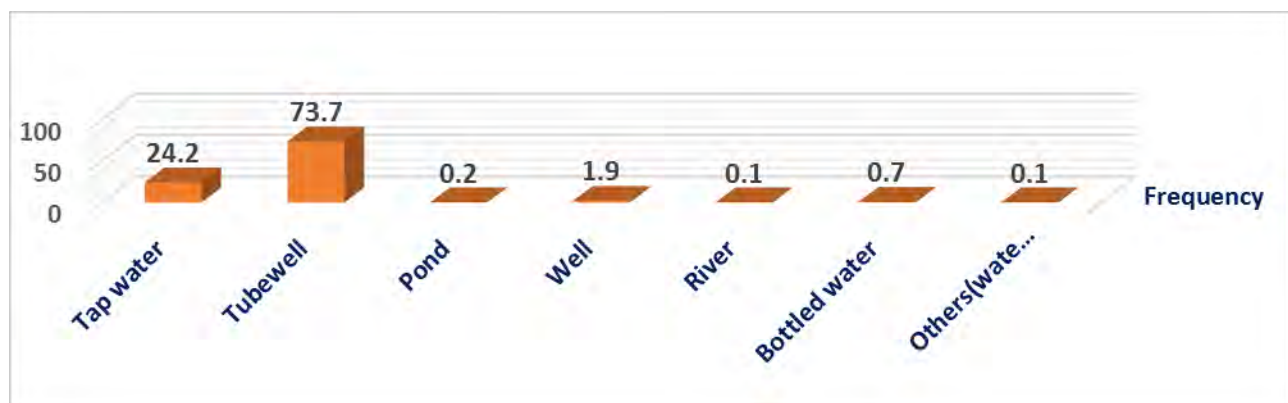


Figure 3.11: Source of water distribution among the participants of study.

3.6 Antibiotic Resistance Pattern of the *Salmonella* Typhi and Paratyphi A

The entire culture positive sample was analyzed for the identification of antibiotic resistance pattern of isolated organism. The most significant results were 100% resistant to Nalidixic acid and 100% patients reduced susceptibility to ciprofloxacin. The resistance to Azithromycin (21%), Clotrimazole (21%), Cloramphenicol (21%) was also significant.

Table 5: Antibiotic susceptibility pattern of isolated *S. Typhi* strains (n=46)

Antibiotics	Number (%)
Resistance to Ampicillin	10(21)
Resistance to Azithromycin	10(21)
Resistance to Nalidixic acid	46(100)
Reduce susceptibility to Ciprofloxacin	46(100)
Resistance to Clotrimazole	10(21)
Resistance to Cloramphenicol	10(21)
Resistance to Ceftriaxone	0

Table 6: Antibiotic susceptibility pattern of isolated *S. Paratyphi A* strains (n=13)

Antibiotics	Frequency (%)
Resistance to Ampicillin	0
Resistance to Azithromycin	7(54)
Resistance to Nalidixic acid	11(85)
Reduced susceptibility to Ciprofloxacin	13(100)
Resistance to Clotrimazole	1(8)
Resistance to Cloramphenicol	0
Resistance to Ceftriaxone	0

3.7 Private health care enhances disease surveillance:

The private health sector provides a major health care facility in developing countries.[2] A recent study was conducted in Pakistan and it was found that private practitioners play an important role in determining the disease burden. Thus the current study had included one private (Uttara Adhunik Medical College) sentinel sites out of 10 sentinel sites. The number of total samples collected in nine sentinel site was 1901 and TPTest positive was 20.41%. In Uttara Adhunik Medical College, The number of total sample was 212 and TPTest positive was 67%. The results show a significant number of people went to private health care facilities.

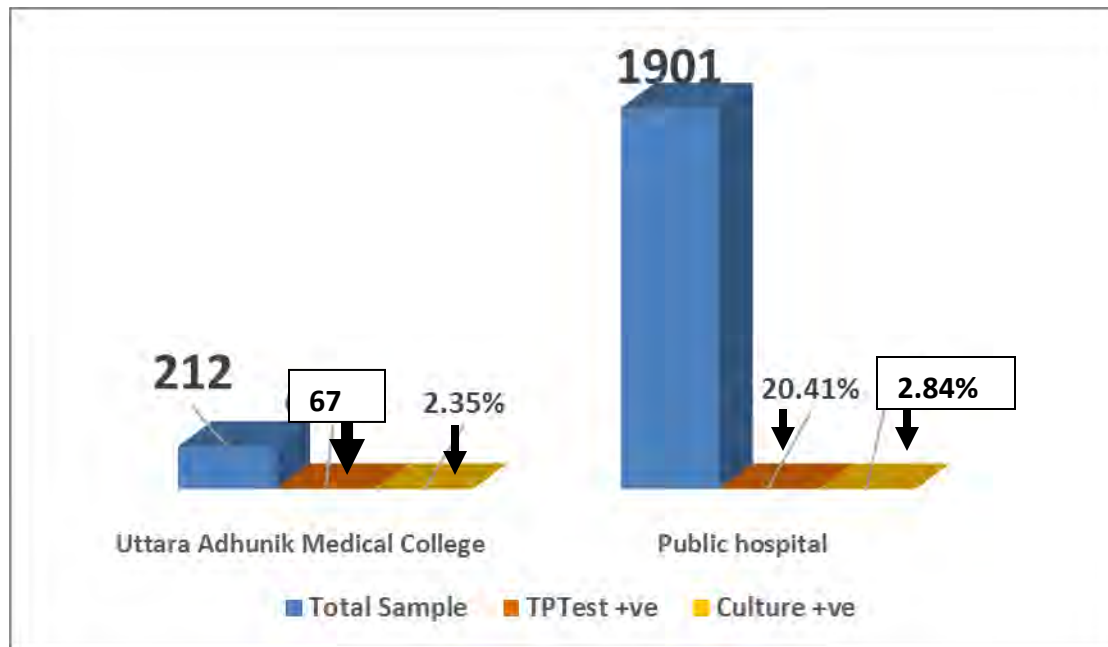


Figure 3.12: Distribution of total sample in private and Government health facilities.

Chapter: 4 Discussion

This study, is based on specimens collected from enteric fever patients, first of its kind prospective, multicenter incorporated and laboratory based surveillance of enteric fever in Bangladesh. Enteric fever is a common febrile illness affecting a significant number of populations in Bangladesh. One of the most threatening challenges is ensuring public health safety by incorporating proper vaccination program in endemic areas within countries. No previous surveillance was conducted in our country for the estimation of enteric fever. Taking preventive immunopropylactic measures is very difficult due to insufficiency of data on disease burden of enteric fever in different regions of Bangladesh. Government and Non-Government organizations are working together to overcome disease outbreak for infectious diseases for minimization of infection and hospitalization rates. The aim of the current study was to conduct surveillance in selected sites in Bangladesh with the collaboration of IEDCR to estimate the prevalence of enteric fever (typhoid and paratyphoid). This study was designed also to overcome the limitations of enteric fever detection techniques by incorporating an immunological assay using lymphocyte secretions involving the TPTest along with blood culture method.

Current study was conducted from May 2014 to December 2015 to screen enteric fever among 2113 people from ten different study sites. The study sites were divided into seven divisions except Dhaka and Chittagong which were covered by more than one study sites because of the higher number of population residing in these two areas.

Total 2036 specimens were analyzed by the TPTest method and the study detected 530 positive patients. Besides, from 2068 total specimens 59 patients were positive in blood culture method. Overall about 26% prevalence was found using TPTest technique and 2.85% by the blood culture method. The sensitivity of TPTest was 100% and specificity was 78-97% whereas the sensitivity and specificity of blood culture was 40-60% [5]. Moreover 33.6% people took antibiotics before seeking treatment from study hospitals and the duration of taking antibiotics was 3-4 days. These findings might have affected the blood culture detection of enteric fever found in similar studies conducted in Dhaka [4] where the intake of antibiotics was found to reduce the number of blood culture positive cases.

In the TPTest method, it was found that enteric fever was highly prevalent in Dhaka (57%) followed by Barisal (12.06%) and Chittagong (10.75%). A study was carried out in 2005 where it was found that people living in urban slum of Dhaka was highly susceptible for typhoid and paratyphoid fever [2]. In the Dhaka division, the highest incidence rate was observed in Uttara Adhunik medical college hospital (67%) which might have resulted from contaminated municipal supply water and street-vended foods, which have been previously reported as risk factors of typhoid fever [6] [8]. On the other hand, the least prevalent area was found in Rajshahi (Adhunik Sadar hospital Naogaon). The specimen received from that site was only 3% TPTest positive. This results give an indication for selection of areas for future vaccination program [7].

Similar prevalence rate was observed by the blood culture method. Dhaka was the most prevalent zone for typhoid (63%) and paratyphoid (46%) fever. This finding also showed that people in Bangladesh are more susceptible to typhoid fever rather than paratyphoid fever. TPTest and blood culture method showed similar patterns of enteric fever positive cases in similar zones of Bangladesh.

A total number of 59 *Salmonella* species were identified by serological analysis during this study period. Of these 46 were positive for *S. Typhi* and 13 were positive for *S. Paratyphi A*. Paratyphoid fever was not found in febrile patients from Sylhet, Rangpur, and Khulna. As a result of this study, it was possible to locate typhoid and paratyphoid fever endemic areas where preventive measure should be taken immediately.

One of the previous studies [1] had shown three peak seasons for typhoid fever which were April, August and December. But the current study has shown two peak seasons which are August and November-December. A decreased number of febrile patients were detected in April in this study. Seasonality of febrile illness in the study period indicates that there is a higher chance of getting infected by the pathogens in winter and late rainy season in the study areas. The spreading of the pathogen may occur by the overflow of river and pond water during rainy season. Other environmental factors such as lower levels of surface water during winter and summer seasons also may enhance the transmission of the organism by the fecal oral route. This observation will be helpful for the understanding of seasonal variation of enteric fever. Thus improving infrastructure of banks and increasing the density of river may result in minimizing the hospitalization rate in different times of a year.

Another previous study had shown that children of 0-5 years of age were more prone to enteric fever [2]. But the current study revealed that younger children and adolescent (6 - 17 years) and adults (age ≥ 18 years) are infected more. This updated data of the prevalent age of typhoid and paratyphoid fever suggests that we should give consideration about vaccination or necessary preventive measures among people of all ages to reduce the disease burden. The prevalence of enteric fever was much higher in males (78.3%) compared to females (21.7%).

Almost all febrile patients were suffering from headache (80%). Constipation (38.0 %) and vomiting (39%) were also very common among the hospitalized patients. About 56% patients had abdominal pain. Significant rise of temperature was observed in almost all patients. The body temperature of the patients were 101°F and 102°F during interview and hospitalization time respectively. In 2011, use of supply water without boiling was found significant for enteric fever prevalence [3]. The present study revealed that 73% of participants were using tubewell as a source of drinking water which is much higher than the previous report. This findings probably attributes to the fact that the majority of the participants in this study were from the rural areas. The sources of drinking water of the patients with typhoid and paratyphoid could not be ascertained and it might be sources other than tap and tubewell water.

The current study incorporated Uttara Adhunik Medical College as a private hospital with nine other Governmental hospitals. As a result we were able to enroll a significant number of patients attending private practitioners which increased the sample size of our surveillance. From Uttara Adhunik Medical College 212 samples were collected and 69% were TPTest positive specimens.

The isolated organisms were tested for susceptibility to various antibiotics. *S. Typhi* and paratyphi A showed 100% resistance to nalidixic acid and reduced susceptibility to ciprofloxacin. In a previous study, similar results were reported [4]. As a result we found that multi drug resistance strains are emerging and causing disease severity [9].

The limitation of the study was that a number of samples could not be analyzed due to the inadequate quality of the specimen. Overall this study resulted in giving an updated result of prevalence of enteric fever and the antibiotic resistance pattern in Bangladesh; it will help to estimate the disease burden of febrile diseases caused by *S. Typhi* and *S. Paratyphi* A. This will also help in characterization of the enteric pathogens and thus lead to planning for vaccine intervention. The designing and proper choice of vaccine for the people particularly for Bangladesh and other enteric fever endemic countries to minimize the prevalence of disease.

The present study may be regarded as a glimpse of the extent of the dreadfulness of two common but very serious infections in Bangladesh due to improper and unhygienic use of water as these are waterborne diseases. Reduction of incidence of the disease can be ensured though preventive measures in terms of proper food and water intake and such reduction will result in reduced use of antibiotics thus reducing the problem of resistance to antimicrobials. The study also confirmed the efficacy of TPTest developed by the ICDDR,B compared to blood culture test for detection of typhoid and Paratyphoid fevers in patients under antibiotic treatment. Understanding the prevalence of the disease and the causative agents would help in development of proper vaccine against these diseases.

Recommendations for future work

- i. More elaborate study needs to be done in the areas covered with much larger sample number to understand the root cause of prevalence of the disease conditions.
- ii. Detailed survey should be made covering family hygiene, living environment and other parameters which may have impact on the disease incidence.
- iii. The lifestyle of the people under study may be undertaken to relate the prevalence of disease condition.
- iv. The relationship between the different sources of water with the disease incidence needs further study to determine the type water suitable for drinking to avoid infection.

Chapter: 5 References

1. Taibur Rahman *1, I.H., Sajib Chakraborty2, *A Rapid Glimpse on Typhoid Fever: An Updated Mini Review*. Journal of life medicine, 2013. 1(3): p. 71-82.
2. Date, K.A., et al., *Typhoid Fever surveillance and vaccine use-South-East Asia and Western Pacific regions, 2009-2013*. MMWR. Morbidity and mortality weekly report. 63: p. 855-60.
3. Crump, J.A. and E.D. Mintz, *Global trends in typhoid and paratyphoid Fever*. Clin Infect Dis. 50(2): p. 241-6.
4. Connor, B.A. and E. Schwartz, *Typhoid and paratyphoid fever in travellers*. Lancet Infect Dis, 2005. 5(10): p. 623-8.
5. Srikantiah, P., et al., *Population-based surveillance of typhoid fever in Egypt*. Am J Trop Med Hyg, 2006. 74(1): p. 114-9.
6. Kanungo, S., S. Dutta, and D. Sur, *Epidemiology of typhoid and paratyphoid fever in India*. J Infect Dev Ctries, 2008. 2(6): p. 454-60.
7. Khan, M.I., et al., *Enhanced disease surveillance through private health care sector cooperation in Karachi, Pakistan: experience from a vaccine trial*. Bull World Health Organ, 2006. 84(1): p. 72-7.
8. Ochiai, R.L., et al., *A study of typhoid fever in five Asian countries: disease burden and implications for controls*. Bull World Health Organ, 2008. 86(4): p. 260-8.
9. Date, K.A., et al., *Typhoid Fever surveillance and vaccine use - South-East Asia and Western Pacific regions, 2009-2013*. MMWR Morb Mortal Wkly Rep. 63(39): p. 855-60.
10. *Hospital Surveillance Progress Report*. 2013.
11. Brooks, W.A., et al., *Bacteremic typhoid fever in children in an urban slum, Bangladesh*. Emerg Infect Dis, 2005. 11(2): p. 326-9.
12. Khanam, F., et al., *Evaluation of a typhoid/paratyphoid diagnostic assay (TPTest) detecting anti-Salmonella IgA in secretions of peripheral blood lymphocytes in patients in Dhaka, Bangladesh*. PLoS Negl Trop Dis. 7(7): p. e2316.
13. Brenner, F.W., et al., *Salmonella nomenclature*. J Clin Microbiol, 2000. 38(7): p. 2465-7.
14. Fierer, J. and D.G. Guiney, *Diverse virulence traits underlying different clinical outcomes of Salmonella infection*. J Clin Invest, 2001. 107(7): p. 775-80.
15. Forest, C.G. and F. Daigle, *Molecular Armory of S. Typhi: Deciphering the Putative Arsenal of Our Enemy*: INTECH Open Access Publisher.
16. Parkhill, J., et al., *Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18*. Nature, 2001. 413(6858): p. 848-52.
17. Kaur, J. and S.K. Jain, *Role of antigens and virulence factors of Salmonella enterica serovar Typhi in its pathogenesis*. Microbiol Res. 167(4): p. 199-210.
18. Adamu Ishaku Akyala1, *, Selwa Alsam1, *Extended Spectrum Beta Lactamase Producing Strains of Salmonella species - A Systematic Review*. Journal of Microbiology Research, 2015.
19. Ram, P.K., et al., *Risk factors for typhoid fever in a slum in Dhaka, Bangladesh*. Epidemiol Infect, 2007. 135(3): p. 458-65.
20. Kothari, A., A. Pruthi, and T.D. Chugh, *The burden of enteric fever*. J Infect Dev Ctries, 2008. 2(4): p. 253-9.

21. Rahman, A., et al., *Prevalence of typhoid fever among the children in a semi urban area of Bangladesh*. Journal of Dhaka Medical College. 20(1): p. 36-43.
22. Kanungo, S., S. Dutta, and D. Sur, *Epidemiology of typhoid and paratyphoid fever in India*. The Journal of Infection in Developing Countries, 2008. 2(06): p. 454-460.
23. Bhan, M.K., R. Bahl, and S. Bhatnagar, *Typhoid and paratyphoid fever*. Lancet, 2005. 366(9487): p. 749-62.
24. Parry, C.M., et al., *Typhoid fever*. N Engl J Med, 2002. 347(22): p. 1770-82.
25. Geoffrey C. Buckle, Christa L. Fischer Walker, and R.E. Black, *Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010*. Journal of global health, 2012. 2.
26. Waddington, C.S., T.C. Darton, and A.J. Pollard, *The challenge of enteric fever*. J Infect. 68 Suppl 1: p. S38-50.
27. Wain, J., et al., *Specimens and culture media for the laboratory diagnosis of typhoid fever*. J Infect Dev Ctries, 2008. 2(6): p. 469-74.
28. Wasihun, A.G., et al., *Diagnosis and Treatment of Typhoid Fever and Associated Prevailing Drug Resistance in Northern Ethiopia*. Int J Infect Dis. 35: p. 96-102.
29. Jesudason, M.V. and S. Sivakumar, *Prospective evaluation of a rapid diagnostic test Typhidot for typhoid fever*. Indian J Med Res, 2006. 123(4): p. 513-6.
30. Butler, T., *Treatment of typhoid fever in the 21st century: promises and shortcomings*. Clin Microbiol Infect. 17(7): p. 959-63.
31. Afroj, S., et al., *Prevalence of ciprofloxacin and nalidixic acid resistant Salmonella enterica serovar Typhi in Bangladesh*. Bangladesh Journal of Microbiology. 28(1): p. 7-11.
32. WHO, *Background document: The diagnosis, treatment and prevention of typhoid fever*. p. 25-26.
33. Hardy, D.J., B.B. Hulbert, and P.C. Migneault, *Time to detection of positive BacT/Alert blood cultures and lack of need for routine subculture of 5- to 7-day negative cultures*. J Clin Microbiol, 1992. 30(10): p. 2743-5.
34. Schwabe, L.D., et al., *Evaluation of BACTEC 9240 blood culture system by using high-volume aerobic resin media*. J Clin Microbiol, 1995. 33(9): p. 2451-3.
35. Edwards, P.R.a.E., W.H., *Identification of Enterobacteriaceae*, 1986. 4th
36. Naheed, A., et al., *Burden of typhoid and paratyphoid fever in a densely populated urban community, Dhaka, Bangladesh*. Int J Infect Dis. 14 Suppl 3: p. e93-9.
37. Farhana Khanam¹, M.A.S., Feroza Kaneez Choudhury¹, Alaullah Sheikh^{1,2}, et al., *Typhoid Fever in Young Children in Bangladesh: Clinical Findings, Antibiotic Susceptibility Pattern and Immune Responses*. PLoS Negl Trop Dis, 2015.
38. Bhutta, Z.A. *Current concepts in the treatment and diagnosis of typhoid fever*. BMJ 2006;333 (7558);P 78-82.
39. Ram.P.K. *Risk factors for typhoid fever in a slum in Dhaka, Bangladesh* Epidemiol. Infect. (2007), 135, 458-465.

