MULTI-HOSPITAL SURVEILLANCE AND ANTIBIOTIC SENSITIVITY AMONG BACTERIAL ISOLATES OF ACUTE RESPIRATORY AND ENTERIC INFECTION IN UNDER-FIVE CHILDREN IN DHAKA, BANGLADESH

THESIS SUBMITTED TO THE
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Declaration of Authenticity

I, the undersigned, Asifuzzaman Rahat, declare that the research work embodying the results reported in this thesis entitled "Multi-hospital surveillance and antibiotic sensitivity among bacterial isolates of acute respiratory and enteric infection in under-five children in Dhaka, Bangladesh", is my original work, gathered and utilized especially to fulfill the purposes and objectives of this study, and has not been previously submitted to any other institution for a higher degree or diploma. It is further declared that this work has been carried out under joint supervision of Professor Firdausi Qadri, Senior Scientist and Executive Director, Institute for Developing Science and Health Initiatives (ideSHi), and Professor Naiyyum Choudhury, Biotechnology Program, Department of Mathematics and Natural Sciences (MNS), BRAC University. I also declare that the publications cited in this work have been personally consulted.

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I dedicate this work to my loving parents and my three sisters.

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Abstract

Acute lower respiratory tract infection (ARI), enteric fever and diarrhea remain the prevalent causes for mortality and morbidity of under-five children in Bangladesh. However, little information is available on true prevalence of the pathogens responsible for these diseases in under-five of Bangladesh and their susceptibility to antibiotics. In this work, we evaluated the bacterial causes of infection in children seeking care in two hospitals in Dhaka. This study utilized specimens collected as part of routine diagnostic purpose encompassing, nasal swab for ARI, stool for diarrhea, and blood for enteric fever. For isolation of the bacterial pathogens, conventional microbiological culture methods were used, followed by detection using appropriate biochemical and serological tests. For enteric fever, an additional immunological detection of *Salmonella* Typhi and Paratyphi specific IgA immune response in lymphocyte secretions by Typhoid-Paratyphoid Test (TPTest) was done and results compared with that of blood culture. For diarrheal samples, ETEC isolates were tested for specific toxins using multiplex PCR. Antibiotic susceptibility tests were performed according to the Clinical and Laboratory Standard Institute (CLSI) guidelines. Of 100 ARI samples analyzed, 30 bacteria of different species were isolated, of which *S. pneumoniae* (50%) and *K. pneumoniae* (26.7%) were the most prevalent. The overall antibiotic sensitivity test of the isolates showed Meropenem, Impenem, Ceftriaxone, Ciprofloxacin, Penicillin and Ampicillin as the most potent antibiotic against respiratory isolates. Of 47 samples tested for enteric fever, 1 (2.1%) was culture positive for *Salmonella* Typhi, while 23 (49%) were TPTest positive. The *S*. Typhi isolate was only resistant to Nalidixic acid. 5 of 17 (29.4%) diarrheal samples were culture positive, 3 of which were identified as ETEC and the other two V. cholerae, and *Shigella dysenteriae* respectively. The findings from this study elucidate the major aetiologies causing infection in under-five children in Dhaka. The antimicrobial sensitivity pattern of the relevant pathogens unraveled will help guide physicians to prescribe appropriate antibiotics in settings where diagnostic facilities are limited or not available.

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List of Abbreviations

ADP	Adenosine Diphosphate
ALS	Antibody in Lymphocyte Supernatant
AMC	Amoxicillin+ Clavulanic acid
AMP	Ampicillin
API	Analytical Profile Index
ARI	Acute Respiratory Infection
AST	Antimicrobial Sensitivity Test
ATP	Adenosine Triphosphate
AZI	Azithromycin
BAP	Blood Agar Plate
BMRC	Bangladesh Medical Research Council
BSA	Bovine Serum Albumin
C	Chloramphenicol
cAMP	Cyclic Adenosine Monophosphate
CAP	Chocolate Agar Plate
CF	Colonization Factor
CFA	Colonization Factor Antigens
CFM	Cefixime
CLSI	Clinical Laboratory Standard Institute
CMV	Cytomegalovirus
CoNS	Coagulase Negative Staphylococcus aureus
COT	Cotrimoxazole
CRO	Ceftriaxone

CSA	Coli Surface Antigens
CT	Cholera Toxin
dNTPs	Deoxynucleotide Triphosphates
E	Erythromycin
EAggEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
GM1	Monosialosyl Ganglioside
GTP	Guanosine Triphosphate
HSV	Herpes Simplex Virus
ideSHi	Institute for Developing Science and Health Initiatives
IMP	Impenem
IVIAT	In Vivo Induced Antigen Technology
LEV	Levoxine
LRI	Lower Respiratory Tract Infections
LT	Labile Toxin
LPS	Lipopolysaccharide
MDG	Millennium Development Goal
MEM	Meropenem
MERS	Middle East respiratory syndrome
MIU	Motility Indole Urea
MP	Membrane Preparation

NA	Nalidixic Acid
NAD	Nicotinamide Adenine Dinucleotide
OPD	Ortho Phenylene Diamine
P	Penicillin
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCF	Putative Colonization Factors
PCR	Polymerase Chain Reaction
RMA	Respiratory MultiCode–PLx Assay
RSV	Respiratory Syncytial Virus
RPMI	Roswell Park Memorial Institute Medium
SARS	Sever Acute Respiratory Syndrome
SS	*Salmonella-Shigella*
STGG	Skim milk, Tryptone, Glucose, and Glycerin
STh	Stable Toxin Human
STp	Stable Toxin Porcine
SXT	Trimethoprim + Sulphamethoxazole (Sxt)
T	Tetracyclin
TCBS	Thiosulfate Citrate Bile Salts Sucrose
THY	Todd-Hewitt Broth with Yeast Extract
TPTest	Typhoid Paratyphoid Test
TSI	Triple Sugar Iron
TTGA	Taurocholate Tellurite Gelatin Agar
UNICEF	United Nations Children's Fund
URI	Upper Respiratory Tract Infections

UV	Ultra-Violet
VZV	Varicella Zoster Virus
WC	Whole Cell

Introduction

Chapter 1

1.1 General Overview

One of the millennium development goals (MDG) as stated in the United Nations millennium declaration in the year 2000 resolves to reduce under-five child mortality rate by two thirds by the year 2015 (MDG4). Disregarding population growth, the worldwide mortality in under-five children has decreased from a staggering 12.7 million a year in 1990 to almost a half of 6.3 million in 2013, dropping from 90 to 48 deaths per thousand live births (The United Nations Millennium development goal report, 2014). The global annual rate of reduction in under-five mortality has accelerated steadily from 1.2 per cent between 1990 and 1995 to 3.9 per cent between 2005 and 2012. However, despite significant progress, there is still some way to go before the goal is achieved. World regions including Central Asia, Southern Asia, Oceania, sub-Saharan Africa and Caucasus are still lagging behind the 2015 target. It will take until 2028 to reach Goal 4 globally at the current rate. The main causes of under-five mortality are preventable diseases. A majority of these deaths can be attributed to pneumonia, diarrhea, malaria, measles and HIV/AIDS. Pneumonia till date remains the leading infectious cause of under-five child death in the world, killing close to 2600 children a day (UNICEF, 2014). In 2013, pneumonia killed 940000 under-five children, constituting 17% of all under-five mortality.

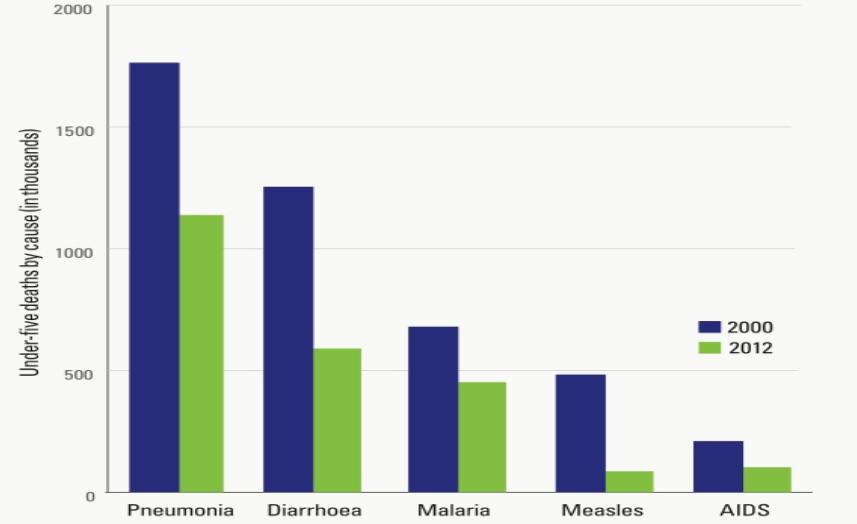


Figure 1.1: Global under-five deaths from five infectious diseases, 2000 and 2012 – UNICEF (2013)

Infectious diseases including acute respiratory infections (ARI)/pneumonia, diarrhea and enteric fever contribute to more than two-thirds of all deaths in children aged less than one year in Bangladesh (Baqui, et al. 2001); (Kabir, et al. 2009) also identified ARI as the leading cause of under-five mortality in Bangladesh with 77.5% of infant mortality caused by pneumonia alone.

Enteric pathogens such as *Vibrio cholerae*, enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* are the predominant aetiological agents of bacteria induced acute watery diarrhea in developing countries including Bangladesh and a large proportion of cases require hospitalization (Qadri, et al. 2005); (Sack, et al. 2003); (Khan, et al. 1988).

Similarly, typhoid and paratyphoid fever also known as enteric fever cause mucosal as well as systemic diseases of human. In a fever surveillance study in an urban area at Kamalapur in Dhaka, Bangladesh it was reported that children <5 years of age were at 8.9-fold increased risk of developing typhoid fever compared to all other age groups (Brooks et al. 2005).

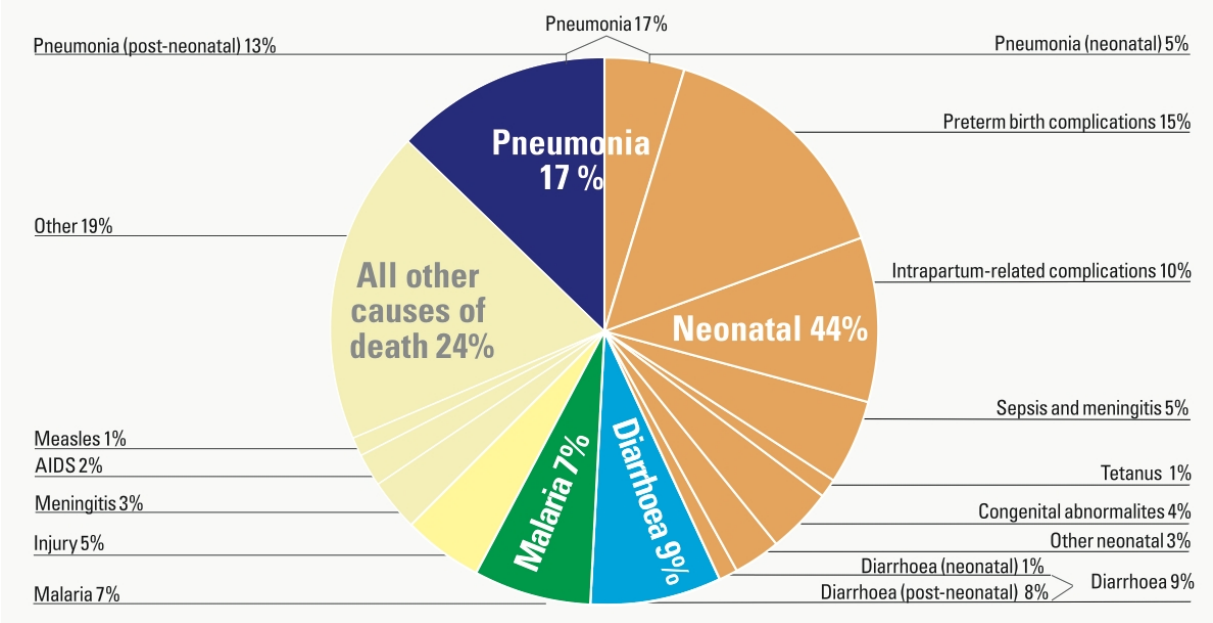


Figure 1.2: Global distribution of deaths among children under age 5, by cause, 2012 – UNICEF (2013)

1.2 Acute Respiratory and Enteric Infection

1.2.1 Acute Respiratory Infection: Acute respiratory infection can be classified into two types. These are upper respiratory tract infections (URIs), or, lower respiratory infections (LRIs). While the upper respiratory tract constitutes of the passage from the nostrils to the vocal cords in the larynx including the paranasal sinuses and the middle ear, the airways from the trachea and bronchi to the bronchioles and alveoli are identified as the lower respiratory tracts. Often, ARIs are not confined to the respiratory tract and have systemic effects because of possible extension of infection or microbial toxins, inflammation, and reduced lung function. Diphtheria, pertussis (whooping cough), and measles are vaccine-preventable diseases that may have a respiratory tract component but also affect other systems.

1.2.1.1 Upper Respiratory Tract Infections: Rhinitis, sinusitis, ear infections, acute pharyngitis, epiglottitis and laryngitis are some common URIs. A significant fraction of URIs is caused by viruses. 25 to 30 percent of URIs are caused by Rhinoviruses; 25 to 35 percent by respiratory syncytial viruses (RSVs), parainfluenza and influenza viruses, human metapneumovirus, and adenoviruses; corona viruses are responsible for 10 percent; and unidentified viruses for the remainder (Denny, 1995). Also, often viral infection leaves children prone to bacterial infection of sinuses leading to development of LRIs from infected secretions and cells (Berman, 1995)

1.2.1.1.1 Acute Pharyngitis: In young children, acute pharyngitis is caused by virus in more than 70% of cases, typically causing pharyngeal redness, swelling, and tonsil enlargement. For under-five children, streptococcal infection is rare when compared to older children. In countries with high population density and crowded living conditions, post-streptococcal sequelae such as acute rheumatic fever and carditis are common in school-age children, but may also occur in those under-five years of age. In developing countries, acute pharyngitis in conjunction with the development of a membrane on the throat is nearly always caused by *Corynebacterium diphtheriae*.

1.2.1.1.2 Acute Ear Infection: At least one third of all URIs are accompanied by acute ear infection. Failure to receive appropriate medical treatment may lead to perforation in eardrums and chronic ear discharge in later childhood and ultimately to hearing impairment or deafness (Berman, 1995). Repeated bouts of acute ear infection developing into chronic ear infection are common in developing countries, affecting 2 to 6 percent of school-age children.

1.2.1.2 Lower Respiratory Tract Infections: In children, the most common types of LRIs are pneumonia and bronchiolitis. One valuable clinical sign for diagnosis of acute LRI in children who are breathing and coughing rapidly is the breathing rate, while lower chest wall indrawing indicates more severe form of the disease (Mulholland et al. 1992); (Shann, Hart, and Thomas, 1984). RSVs, the leading causative agents for LRIs, unlike parainfluenza viruses are seasonal. A major portion of the developing countries are still lacking epidemiological data on influenza infection in children which prevents their access to the safe and effective vaccines that are already available.

1.2.1.2.1 Pneumonia: Can be both bacterial and viral. *Streptococcus pneumoniae*, or, *Haemophilus influenzae*, mostly type b (Hib), and sometimes *Staphylococcus aureus* are generally the bacterial causes of pneumonia. Atypical pneumonias are caused by bacterial pathogens like *Mycoplasma pneumoniae* and *Clamydia pneumoniae.* Because of the lack of sensitivity and specificity of the techniques in practice to establish bacterial etiology, it is hard to determine the disease burden of LRIs caused by Hib or *S. pneumoniae.* Pharyngeal cultures do not always reveal the pathogen causing the LRI. Culturing lung aspirate even though is considered a gold standard, is not practical for field conditions. Vuori-Holopainen and Peltola's (2001) review of several studies indicates that *S. pneumoniae* and Hib account for 13 to 34 percent and 1.4 to 42.0 percent of bacterial pneumonia respectively, whereas studies by (Adegbola et al. 1994); (Shann et al. 1984), and (Wall et al. 1986) suggest that Hib accounts for 5 to 11 percent of pneumonia cases. Infection of upper respiratory tract with potentially pathogenic organisms and aspiration of contaminated secretions have been suggested to be responsible for the pathogenesis of bacterial pneumonia in young children. Infection

of the upper respiratory tract by influenza virus or RSVs has been shown to increase the binding of both *H. influenzae* (Jiang et al. 1999) and *S. pneumoniae* (Hament et al. 2004; McCullers and Bartmess, 2003) to cells lining the nasopharynx. (Fiddian-Green and Baker, 1991) proposed infection of lungs after bacterial entry through the gut and spreading via circulation as the pathogenesis of Gram-negative organisms. However, such bacteria are uncommon aetiological agents of pneumonia in immune-competent children, although Gram-negative pneumonia is not uncommon in neonates.

Viruses are responsible for 40 to 50 percent of infection in infants and children hospitalized for pneumonia in developing countries (Hortal et al. 1990; John et al. 1991; Tupasi et al. 1990). Measles virus, RSVs, parainfluenza virus, influenza type A virus, and adenoviruses are the most important causes of viral pneumonia.

1.2.1.2.2 Bronchiolitis: Fast breathing and lower chest wall indrawing, fever in some cases, and wheezing are the clinical features of bronchiolitis (Cherian et al. 1990). It occurs mostly in the first year of life and with diminished frequency in the second and third years. It can also cause segments of lungs to collapse as a consequence of hyperinflation of lungs resulted from inflammation obstructing small airways. The signs and symptoms of bronchiolitis resemble that of pneumonia; hence physicians often find it difficult to differentiate between the two. Two ways that may provide a solution out of this dilemma are a definition of the seasonality of RSVs in the locality, and the skill to detect wheezing. RSVs are the main cause of bronchiolitis worldwide and can cause up to 70 or 80 percent of LRIs during high season (Simoes 1999; Stensballe, Devasundaram, and Simoes 2003).

1.2.2 Enteric Fever: Enteric fever, more commonly known as typhoid and paratyphoid fever is a group of multi-systemic diseases whose aetiological agents are *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovars Paratyphi A, B and C (Halt et al. 2007). In south-central and south-east Asia, enteric fever remains a significant cause of morbidity and mortality often due to unavailability of proper sanitation and lack of hygienic food and water supply (Crump et al. 2007). Despite the wide array of studies

done on enteric fever, little is known about the patho-physiology of this human adapted pathogen and the complexity of the disease in areas where it is endemic (Wain et al. 2014). The major drawbacks for control of enteric fever are the development of multidrug resistance in the pathogens effecting the efficiency of antibiotic treatment and lack of vaccines that are immunogenic in young children (Wain et al. 2014).

1.2.3 Diarrhea: It is the condition characterized by having at least three loose or liquid bowel movements a day. The mortality caused by it is due to the severe dehydration caused by fluid loss. Worldwide 4 billion diarrheal episodes occur every year, accounting for 4% of all deaths (Marteau, 2002).

Acute diarrhea is resulted due to bacterial, viral, or, parasitic infections. Chronic diarrhea however, is associated with some form of functional disorder such as irritable bowel syndrome and Crohn's disease

1.2.3.1 Diarrheagenic *Escherichia coli*: All forms cause disease in children in the developing world, but enterohemorrhagic *E. coli* (EHEC, including *E. coli* O157:H7) causes disease more commonly in the developed countries. Different pathogenic *E. coli* causing diarrhea include:

• Enterotoxigenic *E. coli* (ETEC) — causes traveler's diarrhea, also responsible for diarrhea in infants and children in developing countries.

• Enteropathogenic *E. coli* (EPEC) — affects children <2 years; causes chronic diarrhea in children; rarely causes disease in adults.

• Enteroinvasive *E. coli* (EIEC) —causes bloody mucoid diarrhea; fever is common.

• Enterohemorrhagic *E. coli* (EHEC) — causes bloody diarrhea.

• Enteroaggregative *E. coli* (EAggEC) — causes watery diarrhea in young children; also causes persistent diarrhea in children and adults with human immunodeficiency virus (HIV).

Shigella species, *Salmonella* and *Campylobacter are* also responsible for causing diarrheal diseases. *Vibrio cholerae* is the causative agent of another form of acute diarrhea, called cholera.

1.3 Epidemiology of ARI, Enteric fever and Diarrhea

1.3.1 Epidemiology of Pneumonia

1.3.1.1 Global Epidemiology of Pneumonia: Every year, pneumonia causes 4 million deaths worldwide, constituting a staggering 7% of the world's yearly total (Ruuskanen et al. 2011; Kabra, Lodha, and Pandey, 2010). It affects approximately 450 million people a year and occurs in all parts of the world (Ruuskanen et al. 2011). However, incidence is greatest in children under five years of age and adults over 75 years of age (Ruuskanen et al. 2011). It occurs about five times more frequently in the developing world compared to the developed world (Ruuskanen et al. 2011). Viral pneumonia accounts for about 200 million cases (Ruuskanen et al. 2011).

In the United Kingdom the yearly incidence rate is 6/1000 people in 18-39 years of age. The rate rises to 75/1000 people for age above 75 years. 20-40% of these individuals require hospital admission with 5-10% cases getting critical. The fatality rate in UK is around 5-10% (Hoare and Lim, 2006).

Pneumonia is the 6th among the leading causes of death in the United States, affecting 5.6 million people annually (Anevlavis and Bouros, 2010). In 2011, pneumonia was the second-most common reason for hospitalization in the U.S., with approximately 1.1 million stays, a rate of 36 stays per 10,000 populations (Pfuntner, Wier and Stocks, 2011)

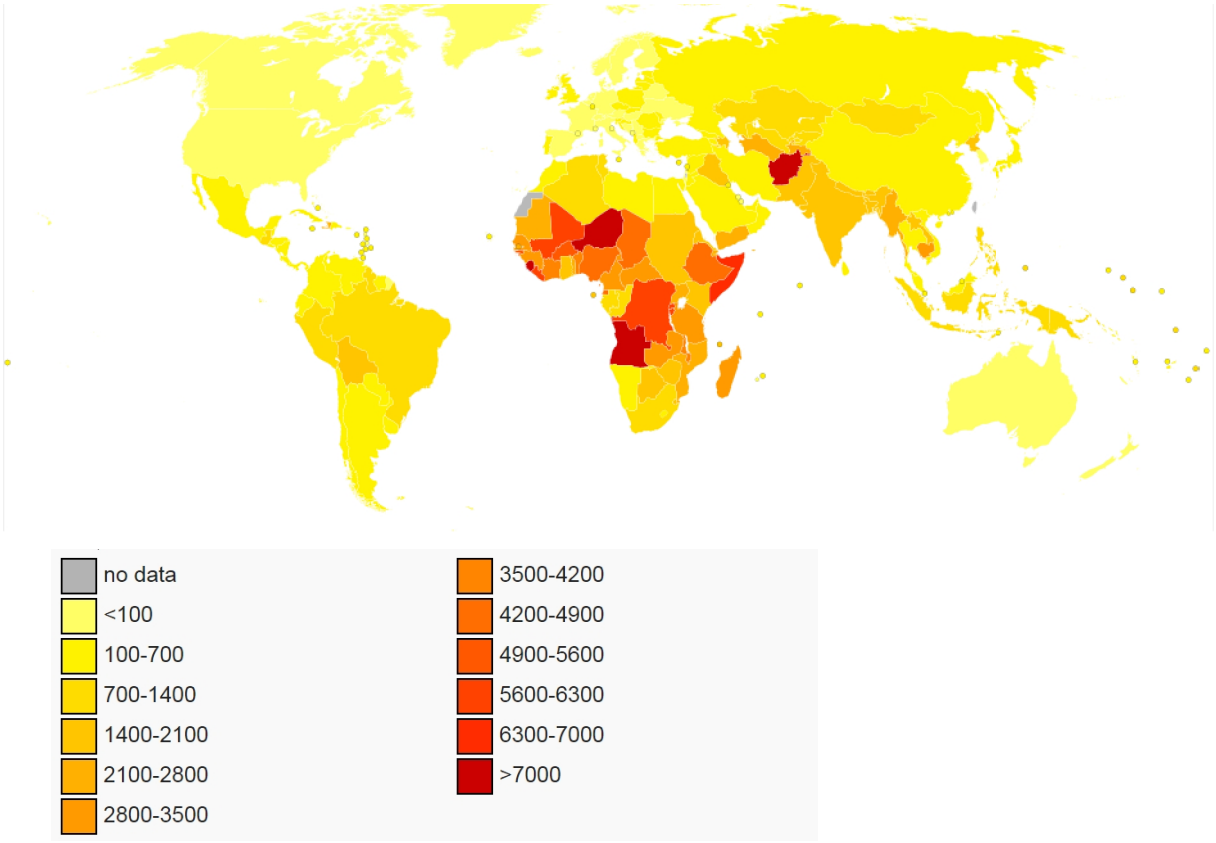


Figure 1.3: Age standardized death from lower respiratory tract infections per 100,000 inhabitants in 2004.

Pneumonia is responsible for 1.6 million deaths per year constituting 28-34% of all under-five mortality, 95% of which occur in the developing countries (Ruuskanen, et al. 2011); (Singh and Aneja, 2011). Worldwide, approximately 156 million children were infected with pneumonia in the year 2008, of which 151 million infections took place in the developing world and the remaining in the developed world (Ruuskanen et al. 2011). India with an incidence of 43 million, China with 21 million, and Pakistan with 10 million are the countries with the greatest burden of pneumonia (Rudan, et al. 2008). Many of these deaths caused by pneumonia occur in the newborn period. The World Health Organization estimates that one in three newborn infant deaths is due to pneumonia (Garenne, Ronsmans and Campbell, 1992)

1.3.1.2 Epidemiology of Pneumonia in Bangladesh: Pneumonia is the leading cause for 90% of all childhood and infant death in Bangladesh (Ahmad, Lopez and Inoue,

2000), (Baqui, et al. 2001). In Bangladesh near to 52% of all under-five child death are caused by infection in lower respiratory tract (Mitra, et al. 2012). Demographic studies in Bangladesh reveal a high incidence of pneumonia in under-five children living in rural areas (0.23 episodes per child-year) and urban areas (0.56 episodes per child year) (Brooks, et al. 2005), (Zaman, et al. 1997). However, a lot still remains to be known regarding the severity of and risk factors associated with pneumonia among young children in this region.

1.3.2 Epidemiology of Enteric Fever

1.3.2.1 Global Epidemiology of Enteric Fever: In the year 2000, typhoid fever was responsible for causing an estimated 21.7 million illnesses and 217,000 deaths, while paratyphoid fever caused an estimated 5.4 million illnesses throughout the world (Crump, Luby and Mintz, 2004). In 2013 it resulted in about 161,000 deaths, down from 181,000 in 1990 (Naghavi, et al. 2015). Infants, children, and adolescents in south-central and Southeastern Asia experience the greatest burden of illness (Crump, Luby and Mintz, 2004).

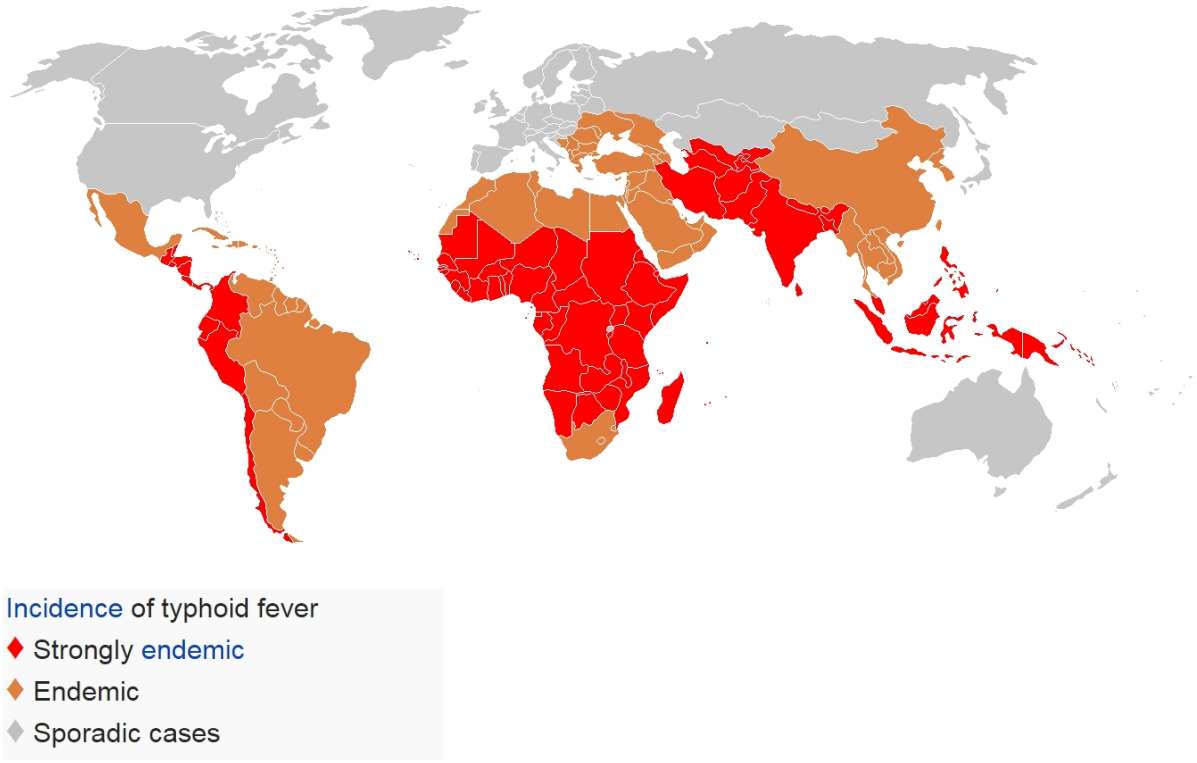


Figure 1.4: Global incidence of Typhoid fever

1.3.2.2 Epidemiology of Enteric Fever in Bangladesh: In Bangladesh, the annual incidence of typhoid fever is 2.0 episodes/1000 individuals, with children under five years of age having a higher annual incidence of 10.5/1000 individuals when compared to individuals >5 years of age. The annual incidence of paratyphoid fever is 0.4/1000 individuals without variation by age group (Naheed, et al. 2010). In Bangladesh the incidence of typhoid happens to be at peak in the monsoon and summer seasons and lowest in the winter (Saha, et al. 2001).

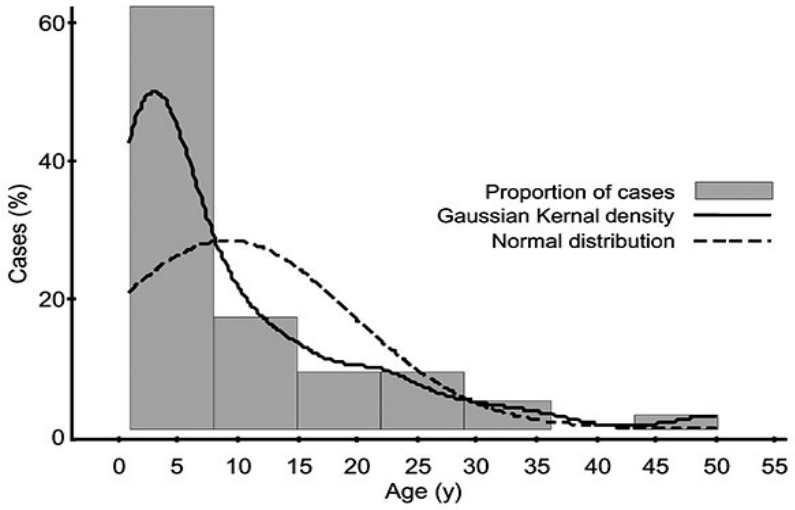


Figure 1.5: Distribution of Typhoid fever by age (Brooks, et al. 2005).

1.3.3 Epidemiology of Diarrhea

1.3.3.1 Global Epidemiology of Diarrhea: Diarrhea is the second biggest cause of infant mortality after pneumonia. Worldwide, there were 2.5 billion incidences of diarrhea in 2004 that caused death of at least 1.5 million under-five children. A significant portion of these cases occur in the developing world, with over half of the recorded cases of childhood diarrhea occurring in Africa and Asia with 696 million and 1.2 billion cases respectively, compared to only 480 million in the rest of the world. In 2011, diarrhea caused about 0.7 million under-five deaths worldwide, and 250 million children lost their schooldays (walker, et al. 2013).

Cholera first emerged from the Ganges Delta of the Indian subcontinent as early as the nineteenth century, and later swept across the world in form of seven pandemics since 1817. The current seventh pandemic was caused by the El Tor biotype of *V. cholerae* serogroup O1 which began in Indonesia in 1961, and subsequently spread to Africa and the Americas in 1970 and 1991 respectively. In 1992, a novel serotype, O139, emerged in India and spread rapidly into Bangladesh and neighboring countries in Asia. The strain was classified as O139 Bengal and was later shown to be a genetic derivative of

the seventh pandemic O1 strains clone with its replacement of the O antigen (Sanchez and Taylor, 1997). So far, the spread of *V. cholerae* O139 Bengal is still restricted to Asia ('Cholera vaccines: WHO position paper'). Recently, the emergence of El Tor variants appears to be more virulent and cause more severe illness than the original El Tor strains. These variant strains that previously caused outbreaks in Bangladesh and India are now predominant in parts of Africa and Asia and Southeast Asia (Nair, et al. 2006).

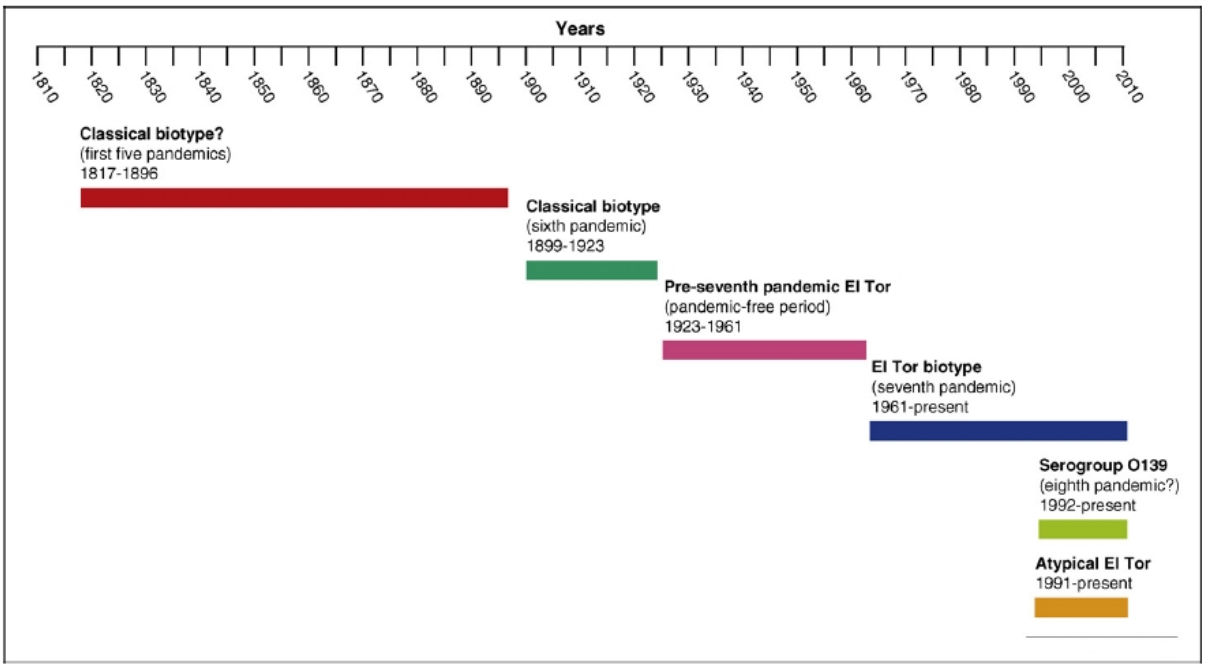


Figure 1.6: Key evolutionary events in cholera epidemiology since 1817 (Safa, Balakrish and Richards, 2010)

1.3.3.2 Epidemiology of Diarrhea in Bangladesh: In Bangladesh, the average incidence rate of diarrhea is 1.8/child-year, and the average number of diarrheal days is 3.7 days/child-year over an average observation period of 2.8 years/child (Haque, et al. 2003). Three-fourth of the diarrheal episodes is ≤ 2 days in duration (Haque, et al. 2003). Persistent diarrhea is relatively uncommon (0.2% of the children) and chronic diarrhea is observed in only one episode. Compared with malnourished and/or stunted children, better-nourished children experience significantly fewer diarrheal episodes (Haque, et al. 2003).

1.4 Pathophysiology of ARI, Enteric fever and Diarrhea

1.4.1 Pathophysiology of ARI: The primary aetiological agents of pneumonia are bacteria and viruses and less commonly fungi and parasites. Even though more than 100 strains of infectious agents have been identified, only a selective few are responsible for causing the disease. 45% of infections in children may occur due to co-infection by both bacteria and viruses, while only 15% infections in adults are resulted by mixed infections (Ruuskanen et al. 2011).

1.4.1.1 Bacterial Pneumonia

1.4.1.1.1 Gram-positive: *Streptococcus pneumoniae,* a Gram-positive bacterium often living as part of normal microbiota in the throat of healthy people is the most common bacterial cause of pneumonia. Other Gram-positive causative agents include *Staphylococcus aureus* and *Bacillus anthracis.*

1.4.1.1.2 Gram-negative: Among Gram-negative Bacteria, *Haemophilus influenzae*, *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Bordetella pertussis,* and *Moraxella catarrhalis* are the most common causes of pneumonia. They mostly live in the gastrointestinal tracts and enter lungs when contents of the gut (such as vomit or faeces) are inhaled.

1.4.1.1.3 Pathophysiology: Bacterial entry in lungs can occur through bloodstream from infected parts of the body and more commonly through inhalation. Often, bacteria live in parts of the upper respiratory tract and are continuously being inhaled into the alveoli. Once inside the alveoli, bacteria translocates into inter-cellular spaces and also between adjacent alveoli through connecting pores. This invasion instigates the immune system to respond by sending white blood cells to the lungs for fighting off the infection. The neutrophils engulf and kill the offending organisms but also release cytokines that result in a general activation of the immune system. This results in the fever, chills, and fatigue common for bacterial and fungal pneumonia. The neutrophils, bacteria, and fluid leaked from the surrounding blood vessels fill the alveoli and result in impaired oxygen

transportation. Bacteria often travel from lung into the blood stream and can result in serious illness such as sepsis and eventually septic shock, in which there is low blood pressure leading to damage in multiple parts of the body including the brain, kidney and heart. They can also travel to the pleural cavity which is the area between the lungs and the chest wall.

1.4.1.2 Viral Pneumonia: After bacteria, viruses are the second major cause of pneumonia. In children viruses are the most common cause of pneumonia, though in adults bacteria are the more common cause.

1.4.1.2.1 Causes: Some common causes of viral pneumonia are:

- Influenza virus A and B
- Respiratory syncytial virus (RSV)
- Human parainfluenza viruses (in children)

Some rarer viruses that can also cause pneumonia include:

- Adenoviruses
- Metapneumoviruses
- Sever acute respiratory syndrome virus (SARS coronavirus)
- Middle East respiratory syndrome virus (MERS)

Viruses that primarily cause other diseases, but can sometimes cause pneumonia are:

- Herpes simplex virus (HSV)
- Varicella-zoster virus (VZV)
- Measles virus
- Rubella virus
- Cytomegalovirus (CMV)

1.4.1.2.2 Pathophysiology: Usually a virus will reach the lungs by travelling in droplets through the mouth and nose with inhalation. There, the virus invades the cells lining the

airways and the alveoli. This invasion often leads to cell death either through direct killing by the virus, or by self-destruction through apoptosis.

Further damage to the lungs occurs when the immune system responds to the infection. White blood cells, in particular lymphocytes, are responsible for activating a variety of chemicals (cytokines) which cause leaking of fluid into the alveoli. The combination of cellular destruction and fluid-filled alveoli interrupts the transportation of oxygen into the bloodstream. In addition to the effects on the lungs, many viruses affect other organs and can lead to illness affecting many different bodily functions. Viruses also make the body more susceptible to bacterial infection; for this reason, bacterial pneumonia often complicates viral pneumonia.

1.4.2 Pathophysiology of Enteric Fever

Studies using murine and *Salmonella typhimurium* provide the best model for *Salmonella* Typhi infection in humans (Pasetti, Levine and Sztein, 2003). Being an enteric pathogen, *S.* Typhi infects the host through the gastro-intestinal tract after being ingested in contaminated food or drinks. It enters the small intestine through microfold cells (M cells) of the Peyer's patches. The bacteria next migrate to the mesenteric lymph nodes and multiply following being endocytosed by the M cells (Janeway, et al. 2001). From here, the bacteria gains access to the bloodstream getting a leeway to circulate systemically and cause a systemic disease (Janeway, et al. 2001). Subsequently, the S. typhi is taken up from blood by antigen presenting macrophages that line sinusoids of the liver, spleen and bone marrow. While inside the macrophages, the S. typhi is still capable of replication and stay in the spleen, liver and bone marrow (Daigle, Graham and Curtiss, 2001). The macrophages can lose their ability to kill the intracellular bacteria. The eventual death of macrophages by the T helper 1 (Th1) cell mediated caspase pathway releases the bacteria from macrophages allowing them to re-enter circulation (Brennan and Cookson, 2000). This is when the clinical manifestation of the disease including fever, nausea, constipation and diarrhea occur. Then, the S. typhi is removed from blood via the gall bladder to the small intestine. The S. typhi gets exposed to

Peyer's patches for the second time as a result of its localization to the small intestine, causing inflammation, ulceration, and typhoid ulcers. The ulcers can hemorrhage and this usually occurs during this time. The ulcerations are caused by the production of the enzyme, matrix metalloproteinases and this causes the loss of the mucosal membranes in the small intestine (Macdonald et al. 2000). The loss of the mucosal membranes leads to ulcerations. The Peyer's patches perforate causing generalized peritonitis and septicemia, the commonest cause of death in typhoid fever (Butler, et al. 1991).

1.4.3 Pathophysiology of Diarrhea

1.4.3.1 Pathophysiology of Cholera: Transmission of *V. cholerae* takes place through the feco-oral route. After ingestion, most of the bacteria are killed by gastric acid. Organisms that can survive colonize the small intestine and secrete cholera toxin (CT), the major virulence factor for pathogenic strains. CT is the principal virulence factor responsible for the pathogenicity of *Vibrio cholerae*. It all starts when the CT irreversibly binds to the cell surface monosialosyl ganglioside (GM1) receptor present on the intestinal mucosal cells resulting in the internalization of the CT into the cells by endocytosis (Scott, 2004); (Voet, 2005). The CT is then activated through proteolytic cleavage and reduction of disulfide bonds of the A subunit to form two fragments A1 (~195 amino acid residues) and A2 (~ 45 amino acid residues) where upon A1 is released into the cytosol. Once in the cytosol, the A1 subunit catalyzes the transfer of an adenosine diphosphate (ADP)-ribose unit from nicotinamide adenine dinucleotide $(NAD)^+$ to an arginine side-chain of $G_S\alpha$. This ADP ribosylated $G_S\alpha GTP$ can activate adenylate cyclase but is incapable of hydrolyzing its bound GTP, thus leaving adenylate cyclase locked in its active state (Voet, 2005). The resultant uncontrolled and elevated production of intracellular cAMP leads to release of chloride ions through the apical chloride ion channels causing diarrhea (Gill, 1975) (Cassel and Pfeuffer, 1978) (Gill and Meren, 1978). A second major virulence factor of *V. cholerae* is the toxin-coregulated pillus, TCP, another colonization factor whose expression is regulated in parallel to cholera toxin (Taylor, et al. 1987) (Matson, Withey and DiRita, 2007)

1.4.3.2 Pathophysiology of ETEC Diarrhea: Two major virulence factors are responsible for the pathogenesis of ETEC strains. These are:

a. Enterotoxins: ETEC strains produce heat-labile (LT) and/or heat-stable (ST) enterotoxins. These are released by ETEC after successful fimbrial adhesion to mammalian intestinal cells.

b. Colonization factors: Colonization of the host intestinal epithelium is an essential step in ETEC pathogenesis, and is mediated by proteinaceous surface structures that are referred to as colonization factors (CFs), colonization factor antigens (CFAs), coli surface antigens (CSAs), or, putative colonization factors (PCFs) (Kaper, Nataro and Mobley, 2004). Most of them are plasmid-encoded. They are antigenically and structurally diverse and confer the species specificity of the pathogen. At least 25 different CFs have been described to date (Fleckenstein, et al. 2010)

ETEC infection causes a noninflammatory toxin-mediated diarrhea after initial adherence to the surface of small intestinal enterocytes through ligand–receptor interaction via colonization factors. The mechanism of action of ETEC enterotoxins resembles that of Vibrio cholerae CT.

1.5 Review of Literature:

1.5.1 Pneumonia: Developed countries have been able to significantly reduce death from pneumonia through improved nutrition, hygiene and air quality. The same however cannot be said about the developing countries till today. Even though many under-five mortality from pneumonia can be prevented by immunization or simple effective treatment, what stand in the way are critical gaps in our understanding of the epidemiology, etiology and pathophysiology of pneumonia (Scott, et al. 2008)

1.5.1.1 Etiology and Diagnosis of Pneumonia: The accepted convention for pneumonia diagnosis includes two stages: first, determination of the condition by history, clinical examination, and chest radiology; and second, determination of the etiology by microbiological, serological and molecular tests. In a developing country

setting, there are several hurdles that are faced in achieving an accurate and timely diagnosis of pneumonia. First, it is hard to obtain appropriate specimens from the lower respiratory tract of children as they generally can't produce sputum. Lung aspirates are a possible option; however, they require invasive methods and are often conducted in only a small number of facilities in developing countries (Shann, et al. 1984); (Adegbola, et al. 1994). Second, many of the pathogens that cause pneumonia are fastidious and require sophisticated laboratory culture systems for growth. Third, the existing tests for pneumonia pathogens are far from perfect and therefore there is no gold standard against which new diagnostic methods can be tested.

Microbiological isolation from blood specimens of suspected patients is a highly specific method of bacterial pneumonia detection; however, the sensitivity of this method is less than 15% (Cutts, et al. 2005). Detection of viral pneumonia by cell culture or immunofluorescence techniques also has poor sensitivity (Lee, et al. 2007). PCR is a much more sensitive approach, and multiplex PCR can improve throughput (Lee, et al. 2007; Mahony, et al. 2007). The Respiratory MultiCode–PLx Assay (RMA; EraGen Biosciences) integrates multiplex PCR with microsphere flow cytometry which allows simultaneous identification of eight groups of respiratory virus (respiratory syncytial virus [RSV]; parainfluenza virus; influenza A and influenza B; human rhinovirus; enteroviruses; metapneumovirus; adenovirus B, adenovirus C, and adenovirus E; and coronaviruses). Compared with conventional diagnostic methods, this technique increases the number of pathogen-positive samples roughly 3-fold (Lee, et al. 2007). Similarly, the mass tag technique links multiplex PCR for both bacterial and viral pathogens to the detection of stable organic molecules with unique molecular weights, known as mass tags (Briese, et al. 2005). These molecules are coupled to the oligonucleotides used in PCR through UV-cleavable linkers and are detected with mass spectrometry following purification of amplification products. However, these methods are still limited by the determination of the causative organisms identified in blood or nasal secretion.

1.5.1.2 Pneumonia with Multiple Aetiologies: Although pneumonia can be caused by bacteria and virus independently, cross-talking between influenza and *S. pneumoniae* can exacerbate severe pneumonia (Schawrzmann, et al. 1971; O'Brien, et al. 2000). A study conducted in rural Thailand reported the detection of influenza virus in 10% of all patients hospitalized with pneumonia, with infants and those aged 75 years or older being at greatest risk (Katz, et al. 2007). In 26% of older patients admitted with pneumonia caused by infection with the influenza virus, an underlying respiratory disease was found. However, underlying disease was uncommon in children under five years of age (Katz, et al. 2007). Study with animal model provides evidence in favor of this synergism (McCullers, 2006). The influenza neuraminidase protein strips off sialic acid from receptors on epithelial cells lining the respiratory tract which aids pneumococcal invasiveness. Pneumococci can attach to these receptors, enhancing their invasiveness. This viral contribution to the initiation of pathology often goes unrecognized. With the advent of molecular methods and their increased usage in diagnosis it's been discovered that patients with acute respiratory infections (ARIs), often have multiple viruses detected in the respiratory tract (Jennings, et al. 2004).

1.5.1.3 Pneumonia of Unknown Aetiology: 25%-33% of all pneumonia cases are not attributed to any pathogen, as even with sophisticated laboratory techniques it's not always possible to determine the causative agent (Lee, et al. 2007; Templeton, et al. 2005). An unresolved issue in the research of pneumonia is the extent to which cases with no definable cause either reveal the low sensitivity of current diagnostic methods for known pathogens or point to novel uncharacterized pneumonia pathogens. The recent association of novel coronaviruses and rhinoviruses with respiratory illness in humans (van-der-Hoek, et al. 2004; Lamson, et al. 2006) highlights the need of an adaptable approach to the aetiological investigation of pneumonia.

1.5.2 Enteric Fever

1.5.2.1 Identification of Immunogenic Antigens: (Charles, et al. 2013) reported a study aimed at the identification of immunogenic Salmonella enterica serovar Typhi

antigens expressed in vivo in chronic billiary carriers. It was the largest study involving immunoproteomic screening of in-vivo expressed S. typhi antigens. They used a technique called in vivo-induced antigen technology (IVIAT) which identifies humorally immunogenic bacterial antigens expressed uniquely in the in vivo environment and hypothesized that in vivo *Salmonella* Typhi may express a different antigenic profile. Genomic DNA expression library was created from in-vitro grown *S.* Typhi which was then adsorbed using pooled sera from convalescent phase patients with bile culture positive for *S.* Typhi. Gene expression profiling was done to identify the antigens expressed. Thirteen *S.* Typhi antigens that were immunoreactive in carriers, but not in healthy individuals from a typhoid endemic area, were identified. The identified antigens included a number of putative membrane proteins, lipoproteins, and hemolysin-related proteins. YncE (STY1479), an uncharacterized protein with an ATP-binding motif, gave prominent responses in their screen. The response to YncE in patients whose biliary tract contained *S.* Typhi was compared to responses in patients whose biliary tract did not contain *S.* Typhi, patients with acute typhoid fever, and healthy controls residing in a typhoid endemic area (Charles, et al. 2013).

1.5.2.2 *Salmonella* Typhi and Paratyphi Specific Antibody Response in ALS: (Sheikh, et al. 2009) investigated the serum and antibody in lymphocyte supernatant (ALS) responses to serovar Typhi among individuals with suspected typhoid fever in Bangladesh. Serovar Typhi-specific responses using three antigen preparations—Lipopolysaccharides (LPS), formalin inactivated whole cell (WC), and membrane preparation (MP) were analyzed. It was found that both IgG and IgA against LPS and WC were present across the 5 clinically characterized groups, but that MP-specific responses were restricted to the IgA isotype (Sheikh, et al. 2009).

1.5.2.3 Development of Novel Diagnostic Test for Typhoid Detection: (Khanam, et al. 2013) developed a novel immunodiagnostic assay for rapid detection of enteric fever. IgA is the vital antibody response in mucosal immunity. Since serovar Typhi interacts with both the mucosal and the systemic immune systems, activated mucosal lymphocytes migrate from intestinal tissue and circulate within peripheral blood. This

migration peaks 1 to 2 weeks after intestinal infection. The available diagnostic methods take time and have low sensitivity and specificity as well. They described an immunodiagnostic assay, the TPTest, which is based on the use of secretions of antibodies from peripheral blood lymphocytes using ELISA.

1.6 Rationale of Study

Though acute respiratory and enteric infection related morbidity is high among under-five children, the actual burden is still unknown. Lack of proper diagnostic facilities is one of the major barriers. The study under question will give emphasis on isolation and identification of infectious agents with their antimicrobial susceptibility patterns. The outcome of this study can determine the prevalence of pathogens associated with ARI and enteric infection. Moreover, the antimicrobial susceptibility patterns can guide health care providers regarding use of appropriate antibiotics where diagnostic facilities are limited or not available. This will enhance the development in the existing health facilities and laboratories as well as health personnel and creating awareness of prevalent infectious diseases.

1.7 Objectives of Study

1) To determine the actual burden of respiratory pathogens such as *Streptococcus pneumoniae, Haemophillus influenzae, Klebsiella pneumoniae* and their antimicrobial sensitivity patterns.

2) To determine the pathogens and their antimicrobial susceptibility associated with acute watery diarrhea (*V. cholerae*, ETEC and shigella spp.)

3) To determine the prevalence of enteric fever.

Materials and Methods

Chapter 2

2.1 Place of Study

This study was conducted in the laboratory facility of Institute for Developing Science and Health Initiatives (ideSHi), Dhaka, Bangladesh. The study subjects were enrolled into the study from two hospitals in Dhaka, namely, Shaheed Suhrawardy Medical College Hospital; and Dhaka Medical College Hospital.

2.2 Ethical Approval

The study was approved by the Ethical Review Committee of Bangladesh Medical Research Council (BMRC). Written consent was obtained from the patients' parents and/or legal guardians since the eligible participants of the study were under five years of age. The risks and benefits had been clearly stated in the consent form.

2.3 Study Participants

The participants enrolled in the study were pediatric patients who were divided into three separate groups. These groups included children with symptoms of acute respiratory tract infection, watery diarrhea, and fever.

Nasal swabs were tested for ARI cases. Stool swabs or rectal swab (RS) specimens were tested for detection of diarrheal pathogens. Blood were tested for enteric fever.

2.3.1 Inclusion Criteria

Acute Respiratory Infection:

- Subjective fever
- Rapid, labored or noisy breathing
- Cyanosis.

Enteric Fever:

- High Temperature (100-104 °F)
- Diarrhea or constipation.
- Abdominal pain
- Muscle aches.

Diarrheal Disease:

- Frequent (2-3 times or more a day) loose, watery stools.
- Abdominal cramps and pain.
- Nausea and vomiting

2.4 Study Design for ARI Pathogen Detection

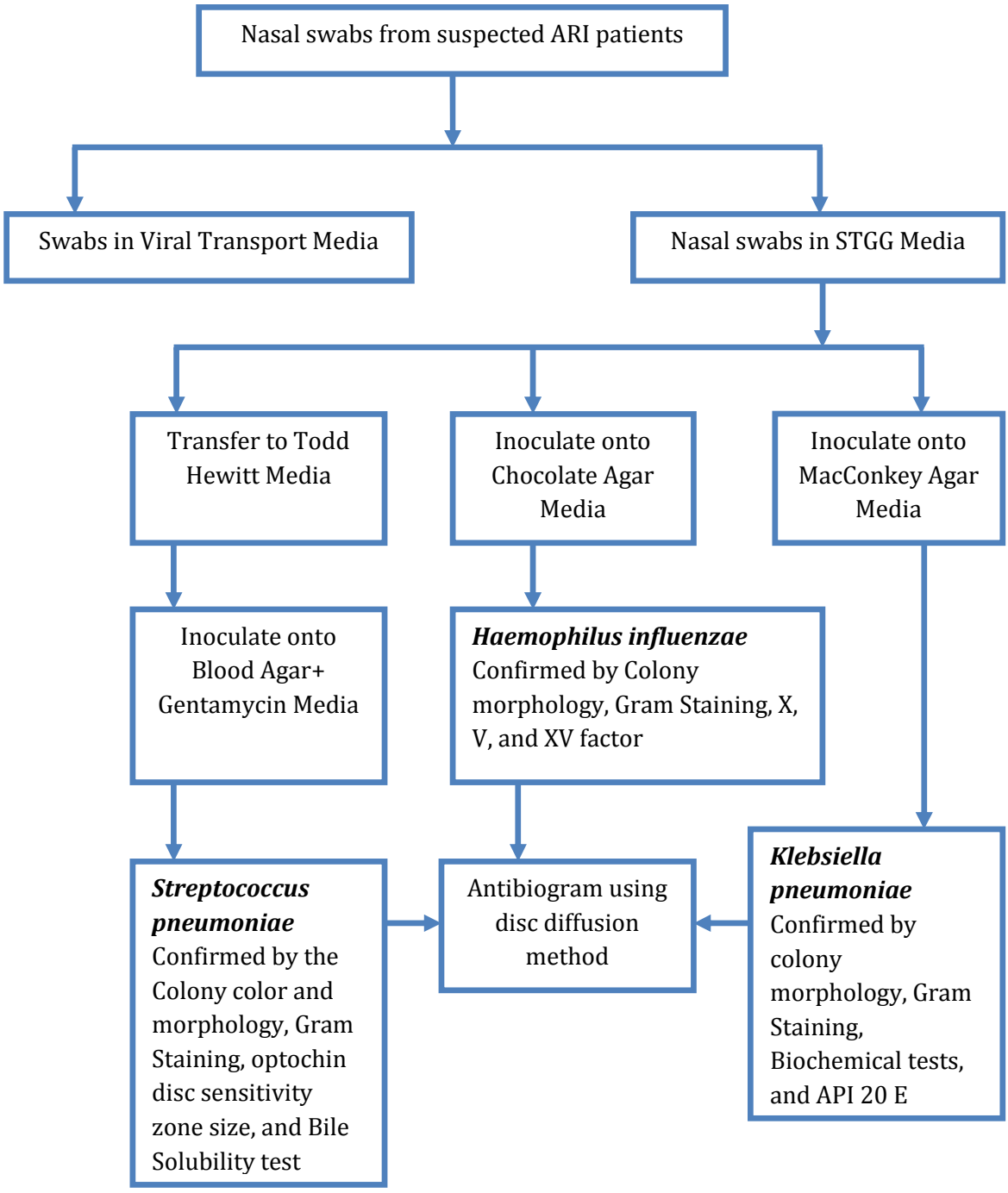


Figure 2.1: Flow-chart depicting study design for detection of ARI pathogen

2.4.1 Identification and Characterization of *Streptococcus pneumoniae*

The following specialized tests were used to identify colonies on a BAP that resemble pneumococci. *Streptococcus pneumoniae* was identified using Gram stain, catalase, and optochin tests simultaneously, with bile solubility as a confirmatory test. When these tests indicated that the isolate is *S. pneumoniae*, serological tests were performed to identify the serotype.

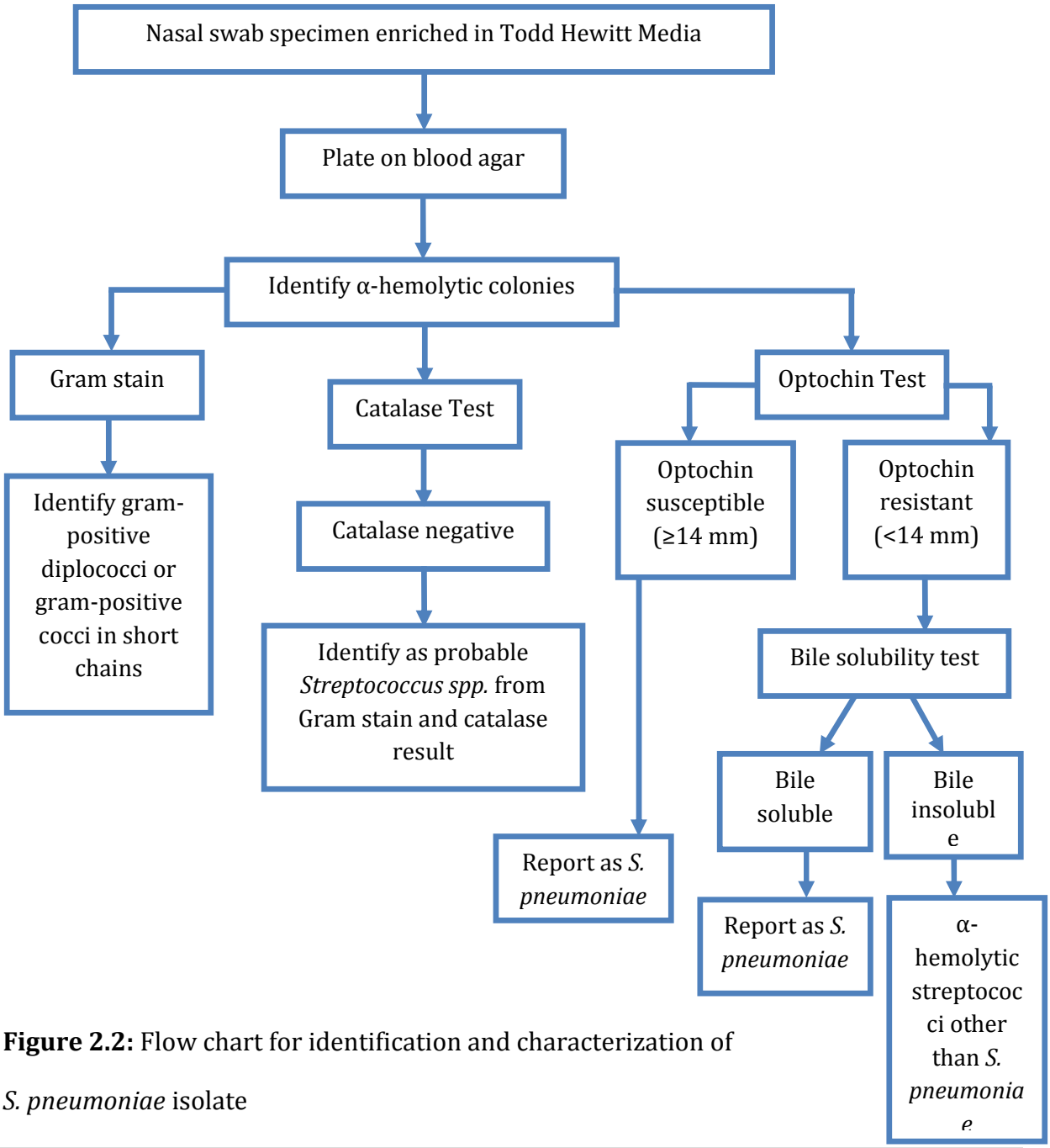


Figure 2.2: Flow chart for identification and characterization of *S. pneumoniae* isolate

2.4.1.1 Materials

1) STGG Media

2) Todd Hewitt Media

3) 0.5% sheep blood (BAP) with 5 µg of gentamicin per ml.

4) Optochin disc.

5) Saline.

6) Rabbit serum.

7) Sterile distilled water.

8) Wire or sterile disposable plastic loops.

9) Screw capped 1.5 ml vials.

2.4.1.2 Method

2.4.1.2.1 Nasal Swab Specimen Enrichment in Todd Hewitt Media

1) Within 4-6 hours after collection, 200 µl of the NP-STGG was transferred to 5 ml enrichment broth that had been combined with 1 ml rabbit serum. The enrichment broth was made by preparing 100 ml Todd Hewitt broth with 0.5 g dissolved yeast extract (THY).

2) The inoculated enrichment broth was vortexed and incubated for 4 hours at 35-37°C in a CO_2 incubator or candle jar.

3) Following enrichment the THY was vortexed and one loop (~10 µl) of the THY enriched culture was used to inoculate on blood agar with 5 µg of gentamicin per ml, streaked for isolated colonies and incubated for 18-24 hours at 35-37°C in a CO_2 incubator or candle jar.

4) 1.0 ml of the THY enriched growth was transferred into screw-cap 1.5 ml vials (cryotube) and stored at -70°C.

2.4.1.2.2 Pneumococcal Isolates Detection and Identification

1) After overnight incubation, the BAP growth was carefully examined for typical pneumococcal colonies surrounded by a greenish zone of alpha-hemolysis.

2) Suspected pneumococcal colonies were in parallel subjected to Gram stain, catalase test and optochin test.

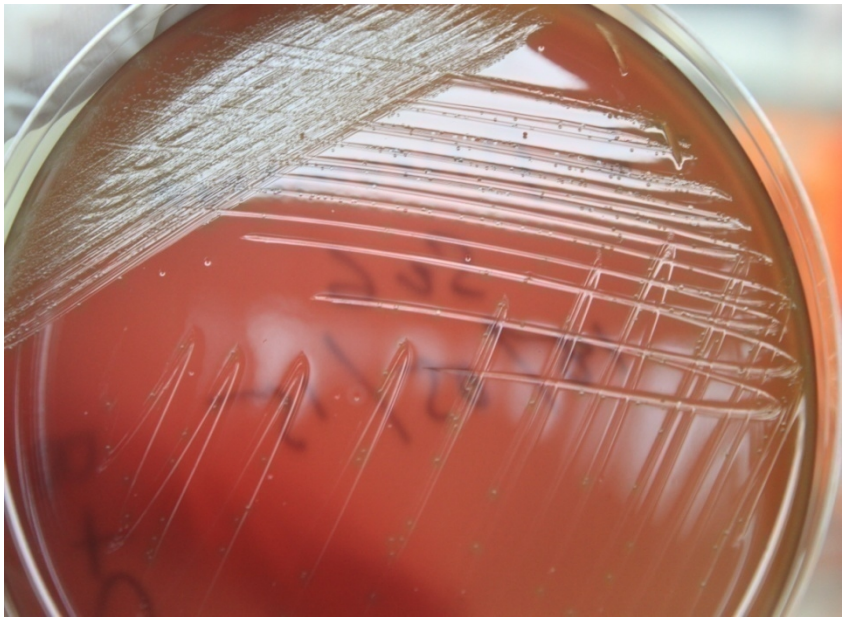


Figure 2.3: *Streptococcus pneumoniae* colony on BAP.

2.4.1.2.3 Gram Staining

1) Using a sterile loop, suspected pneumococcal colonies were picked and a thin smear prepared on a glass slide.
2) The smear was then air dried and heat fixed by passing the slide through flame several times.
3) The smear was stained with crystal violet and allowed to rest for 30 seconds.
4) The crystal violet was then decanted and the slide gently washed with water.
5) The smear was stained with Gram's iodine solution and allowed to rest for 30 seconds.
6) The iodine solution was then decanted and the slide gently washed with water.
7) The smear was immediately decolorized with 20% acetone ethanol.
8) Without waiting for too long, the smear was gently rinsed with tap water.
9) The smear was then stained with saffranin and allowed to rest for 30 seconds.

10) Finally, the slide was gently rinsed with tap water, blotted dry with clean tissue paper, air dried and examined under oil immersion using 100X objective lense.

2.4.1.2.4 Catalase Test: Catalase is the enzyme that breaks down hydrogen peroxide (H_2O_2) into H_2O and O_2. The oxygen is given off as bubbles in the liquid. The catalase test is primarily used to differentiate between gram-positive cocci. Members of the genus *Staphylococcus* are catalase-positive, and members of the genus *Streptococcus* and *Enterococcus* are catalase-negative.

1) From overnight growth on the BAP, a disposable loop was used to carefully remove a colony and place it on a glass slide. Special care was taken not to transfer any of the blood agar to the slide as erythrocytes in the blood agar will cause a false-positive reaction.

2) 1.0 ml of 3% H_2O_2 was added to the slide and mixed with the bacteria.

3) The bacterial suspension on the slide was observed immediately for vigorous bubbling.

2.4.1.2.5 Optochin Test: *Streptococcus pneumoniae* strains are sensitive to the chemical optochin (ethylhydrocupreine hydrochloride). Optochin sensitivity allows for the presumptive identification of alpha-hemolytic streptococci as *S. pneumoniae*.

1) To perform the optochin susceptibility test, the suspect alpha-hemolytic colony was streaked into Blood Agar plates (BAP) in confluent lines.

2) A 5 µg optochin disk with 6 mm diameter was placed in the streaked area.

3) The plate was then incubated in a CO_2 incubator or candle jar at 35-37°C for 18-24 hours.

4) After incubation the growth on the BAP near the optochin disk was observed and the zone of inhibition measured.

5) A zone of inhibition of diameter 14 mm or greater indicated sensitivity and allowed for presumptive identification of pneumococci. A smaller zone of inhibition (< 14 mm) or no zone of inhibition indicated that the bile solubility test was required.

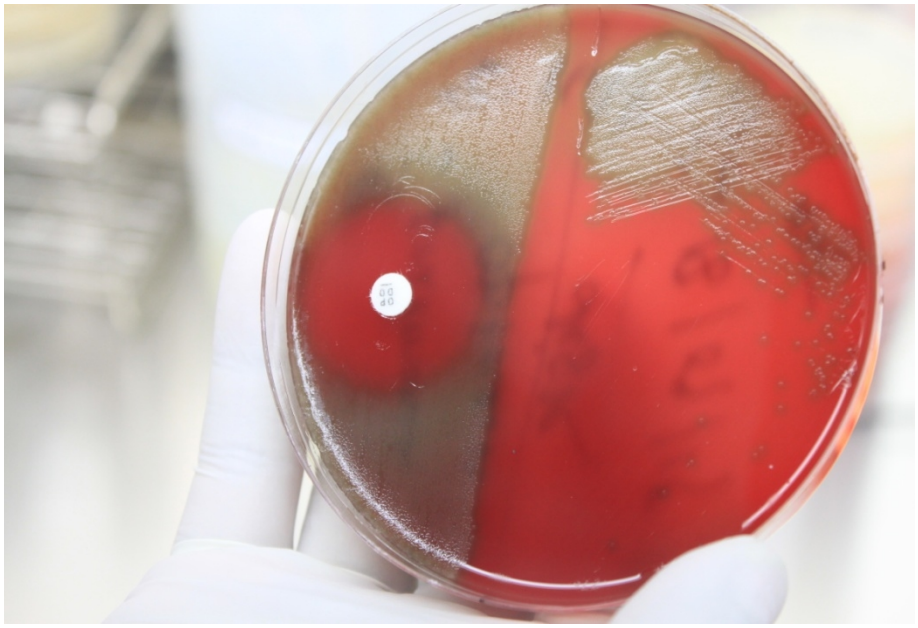


Figure 2.4: Optochin test of suspected pneumococcus on BAP

2.4.1.2.6 Bile Solubility Test: The bile (sodium deoxycholate) solubility test distinguishes *S. pneumoniae* from all other alpha hemolytic streptococci. *S. pneumoniae* is bile soluble, whereas all other alpha-hemolytic streptococci are bile resistant. Sodium deoxycholate (2% in water) will lyse the pneumococcal cell wall.

1) A suspension (McFarland No.1) was prepared from an overnight culture in 1 ml of 0.5% saline.

2) The suspension was divided in two tubes (test and control) of 0.5 ml.

3) 0.5 ml of 2% sodium deoxycholate (bile salts) was added to the test tube and 0.5 ml of normal saline was added to the control tube.

4) The suspensions were then vortexed and incubated in CO_2 incubator or candle jar at 35-37°C for upto 2 hours.

5) *S. pneumoniae* test tube turned completely transparent without any turbidity, while any other alpha-hemolytic streptococci test tube remained turbid after 2 hours of incubation.

2.4.1.2.7 Anti-Microbial Susceptibility Test: The antimicrobial sensitivity of the isolated pathogen was determined by the disc diffusion technique of the modified Kirby-

Bauer (1966) method using Mueller-Hinton agar and commercially available antimicrobial discs (Oxoid, Hampshire, United Kingdom). Following antibiotics and their concentration per discs were used for the sensitivity test:

Table 2.1: List of antibiotics for the AST of *Streptococcus pneumoniae*

Antibiotic	Concentration per Disc
Penicillin (P)	10 µg
Ampicillin (Amp)	10 µg
Erythromycin (E)	15 µg
Azithromycin (Azi)	15 µg
Trimethosporine+ Sulphamethoxazole (Sxt)	1.25/ 23.75 µg
Ceftriaxone (Cro)	30 µg
Cefixime (Cfm)	5 µg
Levoxine (Lev)	5 µg

2.4.1.2.7.1 Method

1) Fresh Mueller- Hinton + 5% Blood agar plates, or, Blood Agar plates were dried in an incubator at 37^0C for 30 minutes before use.

2) With a sterile wire loop, half of one well isolated colony from a pure culture was suspended in 2 ml of Mueller- Hinton broth in a sterile screw capped tube. The inoculated media was then incubated at 37^0C in an aerophilic incubator for 2 hours.

3) The turbidity of the inoculum was adjusted to 0.5 McFarland standard by adding more organisms or more broth, thus achieving a log phase containing approximately $1.5x10^8$organisms/ml.

4) A sterile cotton swab was immersed into the bacterial suspension and the excess suspension was removed by rotating the swab with a firm pressure against the inner side of the tube above the fluid levels. The inoculum was then lawned evenly on the entire surface of a Mueller- Hinton + 5% Blood agar plate, or, Blood Agar plate in three

different planes (by rotating the plates approximately 60˚ angle each time) to get a uniform distribution of the organism.

5) The inoculum was then allowed to dry for 15 minutes at room temperature with lids closed.

6) The discs were then placed 15 mm away from the edge of Petri dish on the lawned surface of the Mueller- Hinton + 5% Blood agar, or, Blood Agar using a sterile needle while keeping at least 25 mm gap in between the discs. The discs were gently pressed down to ensure contact.

7) The plate was then incubated at 37^0C for 18-24 hours in an anaerobic incubator.

2.4.1.2.7.2 Measurement of Inhibition Zone: After overnight incubation, each plate was examined and the diameter of complete inhibition zone was measured with the help of a scale placed beneath the surface of the petri dish without opening the lids. Zone of inhibition was measured in mm in two directions at right angle to each other through the center of each disc and the average of the two readings was taken.

2.4.1.2.7.3 Interpretation of Zone: The zone of inhibition of growth produced by each antimicrobial agent on the test organisms was compared with that produced on control organisms. Depending on the diameter of the clear zone of inhibition around the discs the test organisms were categorized into sensitive (S), intermediate sensitive (IS) and resistant (R) to the respective anti microbial agents.

2.4.2 Identification and Characterization of *Haemophilus influenzae*

The following tests were performed to confirm the identity of cultures that morphologically appeared to be *Haemophilus influenzae*. *H. influenzae* can be identified using Kovac's oxidase test and by determining the necessity of hemin and NAD as growth requirements. When the oxidase test was positive, hemin and NAD growth factor requirement testing were performed.

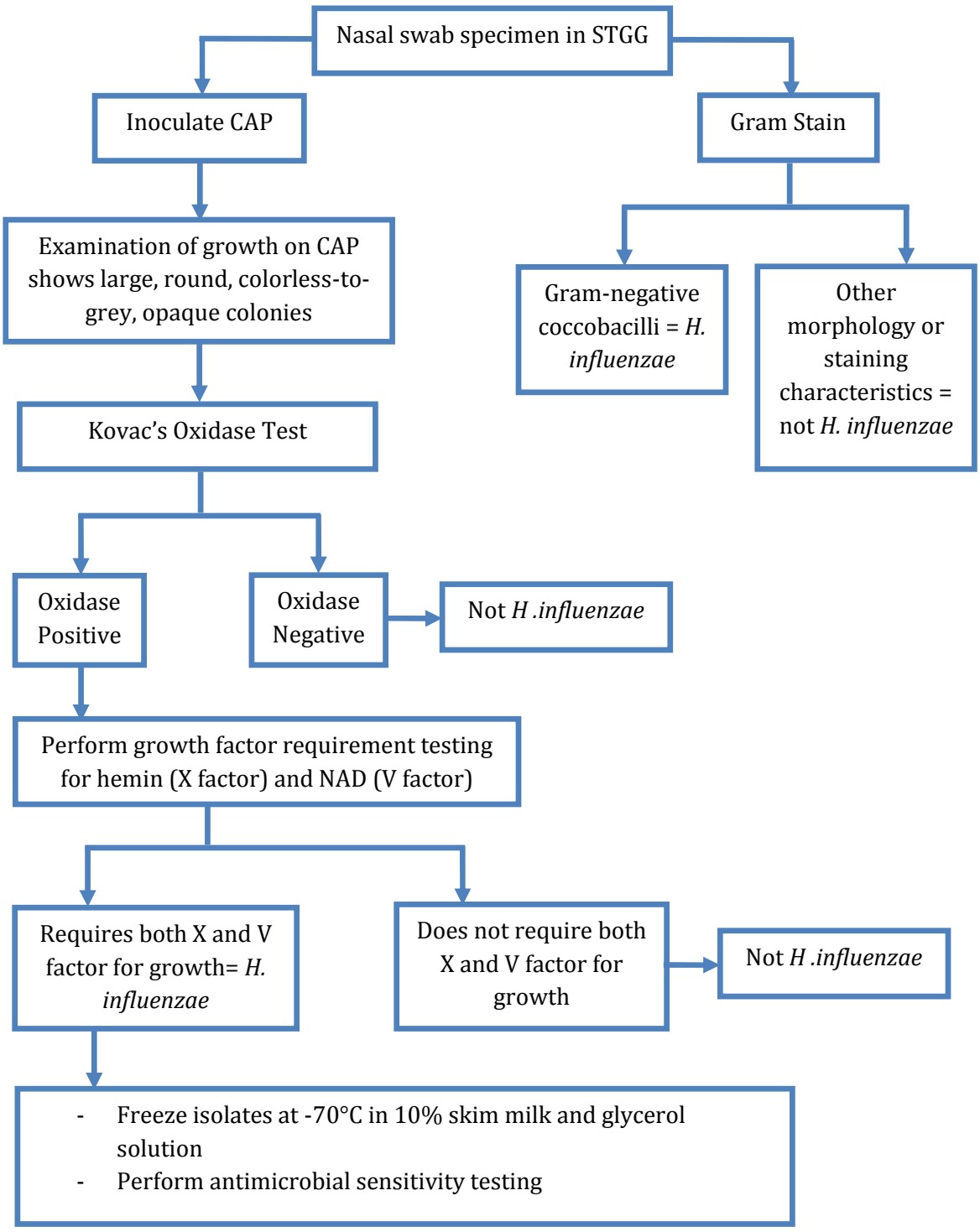


Figure 2.5: Flow chart for identification and characterization of *Haemophilus influenzae* isolate.

2.4.2.1 Method

2.4.2.1.1 Nasal swab culture in Chocolate Agar Media

1) Within 4-6 hours after collection, one loop (~10 µl) of the NP-STGG was used to inoculate on Chocolate Agar Plate (CAP), streaked for isolated colonies and incubated for 18-24 hours at 35-37°C in a CO_2 incubator or candle jar.
2) 0.5 ml of the NP-STGG was transferred into screw-cap 1.5 ml vials (cryotube) and stored at -70°C.

2.4.2.1.2 *Haemophilus influenzae* Isolates Detection and Identification

1) After overnight incubation, the BAP growth was carefully examined for typical *H. influenza* colonies.
2) *H. influenzae* appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on a CAP. Encapsulated strains appear more mucoidal than non-encapsulated strains, which appear as smaller, compact grey colonies. No hemolysis or discoloration of the CAP is apparent.

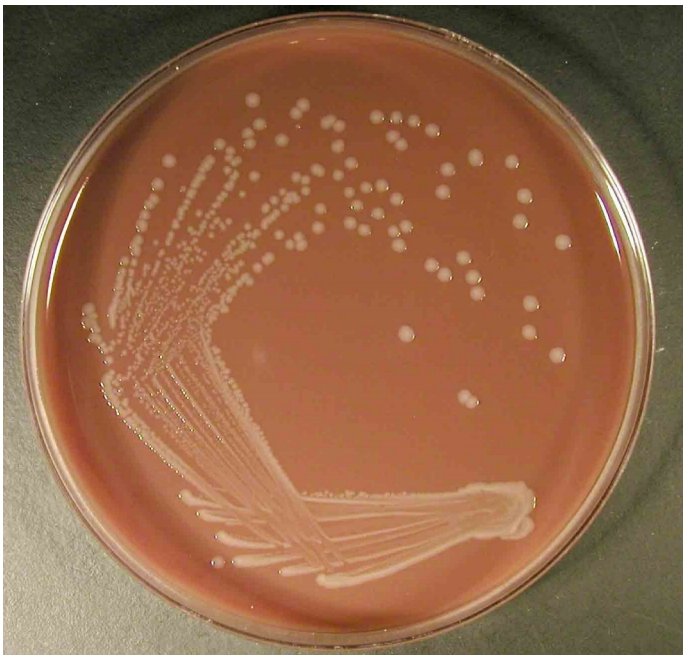


Figure 2.6: *H. influenzae* colonies on a CAP.

2.4.2.1.3 Gram Staining: Gram staining was done in the same process described in section 2.4.1.2.3

2.4.2.1.4 Kovac's Oxidase Test: Kovac's oxidase test is used to determine the presence of cytochrome oxidase. Kovac's oxidase reagent, tetramethyl-p-phenylenediamine dihydrochloride, is turned into a purple compound by organisms containing cytochrome c as part of their respiratory chain.

1) The isolates to be tested were grown for 18-24 hours on CAP at 37°C in a CO_2 incubator or candle jar.

2) A strip of filter paper was wetted on a non-porous surface with a few drops of Kovac's oxidase reagent. The strip was then allow to air dry

3) An wooden applicator stick was used to pick a portion of a colony from overnight growth on the CAP and rub it onto the treated filter paper.

4) The filter paper was observed for color change to purple.

5) Positive reactions developed within 10 seconds in the form of a purple color where the bacteria were applied to the treated filter paper. Negative reactions did not produce any color change.

2.4.2.1.5 Identification of Hemin (X) and NAD (V) as Growth Requirements: *H. influenzae* can be identified on the basis of growth requirements for hemin and NAD

Table 2.2: Identification of *Haemophilus spp*. by their growth requirements for hemin (X factor) and NAD (V factor) and β-hemolysis on blood agar

Organism	Requirement for hemin (X factor)	Requirement for NAD (V factor)	β-hemolysis on blood agar
H. influenzae	+	+	-
H. parainflenzae	-	+	_
H. haemolyticus	+	+	+
H. parahaemolyticus	-	+	+
H. aphrophilus	+	-	-
H. paraphrophilus	-	+	-

1) The isolates to be tested were grown for 18-24 hours on a Chocolate Agar Plate (CAP) at 35-37 °C in a CO_2 incubator or candle jar.
2) A moderately heavy suspension of cells was prepared from the overnight growth on the CAP in 1 ml of Mueller-Hinton broth which was then mixed well by vortexing and incubated at 35-37 °C in a CO_2 incubator or candle jar for 2 hours.
3) A sterile cotton swab was immersed into the enriched bacterial suspension and the excess suspension was removed by rotating the swab with a firm pressure against the inner side of the tube above the fluid levels. The inoculum was then lawned evenly on the entire surface of a Mueller- Hinton agar plate. The inoculum was then allowed to dry.
4) After the inoculum had dried, commercially available paper disks containing hemin, NAD, and hemin+NAD were placed on the inoculated plate.
5) The plate was then incubated at 35-37 °C in a CO_2 incubator or candle jar for 18-24 hours.
6) After overnight incubation, the plate was observed for growth around the paper discs.

2.4.2.1.5.1 Reading the hemin and NAD paper disk results

1) H.influenzae only grew around the paper disc containing both hemin and NAD.
2) Other Haemophilus spp grew around the disc containing both hemin and NAD and either the individual hemin or the NAD disc.

2.4.2.1.6 Anti-Microbial Susceptibility Test: The anti-microbial sensitivity testing was done in the same method except using Mueller-Hinton Agar plate as described in section **2.4.1.2.7** The following antibiotics with their concentration per disc were used for the sensitivity testing:

Table 2.3: List of Antibiotics for the AST of *Haemophilus influenzae*

Antibiotic	Concentration per Disc
Amoxicillin+ Clavulanic acid (AMC)	20/10 µg
Ampicillin (AMP)	10 µg
Azithromycin (AZI)	15 µg
Chloramphenicol (C)	30 µg
Ceftriaxone (CRO)	30 µg
Trimethosporine+ Sulphamethoxazole (SXT)	1.25/23.75 µg

2.4.3 Identification and Characterization of *Klebsiella pneumoniae*

The following tests were performed to confirm the identity of cultures that morphologically appeared to be *Klebsiella Pneumoniae*. *Klebsiella Pneumoniae* can be identified using biochemical tests including Triple Sugar Iron agar (TSI), Motility Indole Urea (MIU), and Citrate. When the biochemical tests confirmed *Klebsiella spp,* further confirmation was achieved using analytical profiling index (API 20 E X 100).

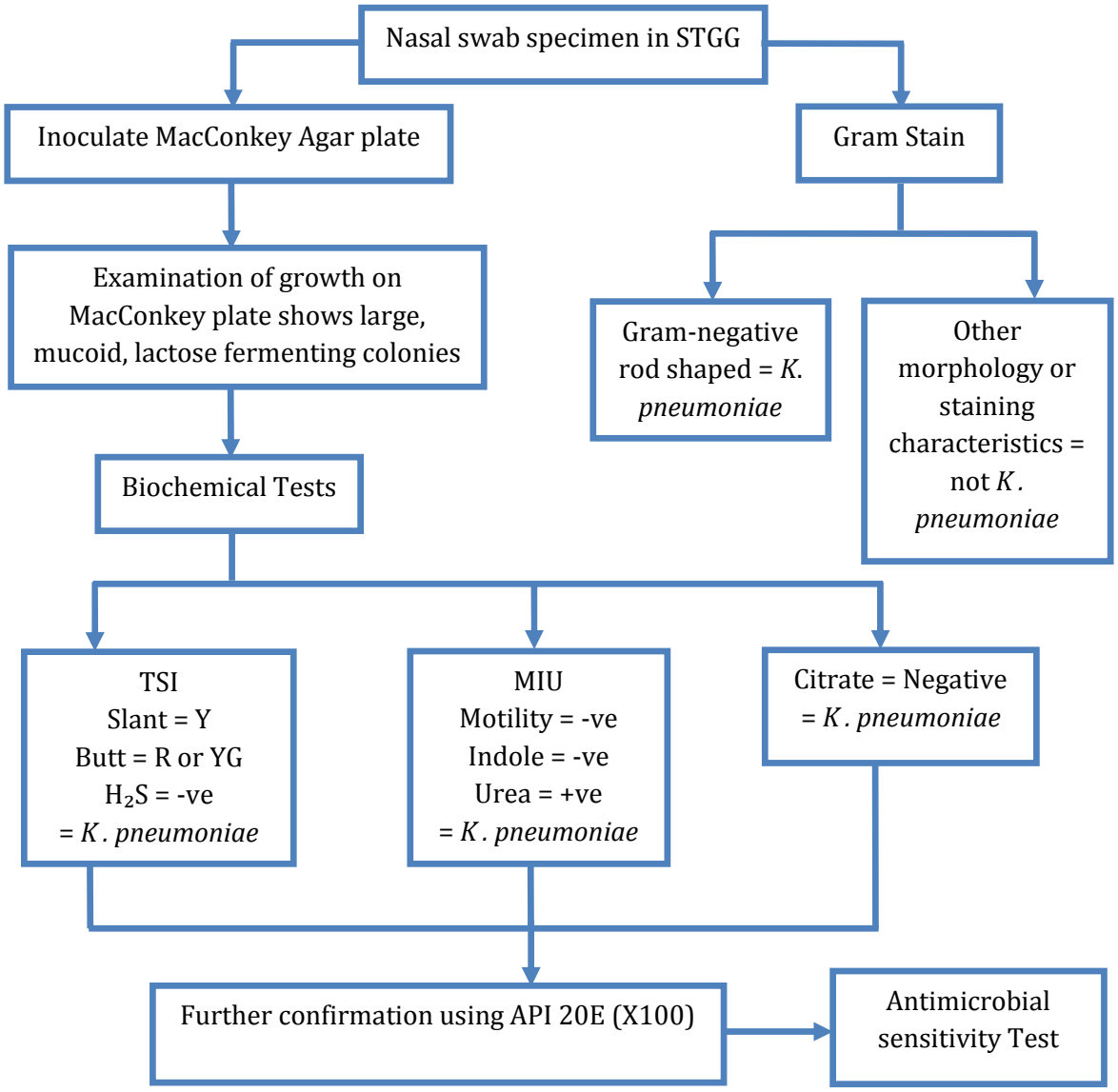


Figure 2.7: Flow chart for identification and characterization of Klebsiella *pneumoniae* isolate.

2.4.3.1 Method

2.4.3.1.1 Nasal swab culture in MacConkey Agar Media

1) Within 4-6 hours after collection, one loop (~10 µl) of the NP-STGG was used to inoculate on MacConkey Agar media, streaked for isolated colonies and incubated for 24-48 hours at 35-37°C in an aerophilic incubator.
2) 0.5 ml of the NP-STGG was transferred into screw-cap 1.5 ml vials (cryotube) and stored at -70°C.

2.4.3.1.2 *Klebsiella pneumoniae* Isolates Detection and Identification

1) After overnight incubation the MacConkey Agar plate growth was carefully examined for typical *Klebsiella pneumoniae* colonies.
2) *K . pneumoniae* appear as large, usually mucoidal lactose fermenting colonies on a MacConkey Agar plate.

Figure 2.8: *Klebsiella pneumoniae* colonies on MacConkey agar Plate.

2.4.3.1.3 Gram Staining: Gram staining was done in the same process as described in section 2.4.1.2.3.

2.4.3.1.4 Biochemical Tests

2.4.3.1.4.1 Triple Sugar Iron Agar

2.4.3.1.4.1.1 Principle: Triple Sugar Iron Agar (TSI Agar) was used for the differentiation of gram negative enteric bacilli based on carbohydrate fermentation and the production of hydrogen sulfide. TSI Agar contains three sugars (dextrose, lactose and sucrose), phenol red for detecting carbohydrate fermentation and ferrous ammonium sulfate for detection of hydrogen sulfide production. Carbohydrate fermentation is indicated by the production of gas and a change in the color of the pH indicator from red to yellow. To facilitate the detection of organisms that only ferment dextrose, the dextrose concentration is one-tenth the concentration of lactose or sucrose. The small amount of acid produced in the slant of the tube during dextrose fermentation oxidizes rapidly, causing the medium to remain red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube because it is under lower oxygen tension. After depletion of the limited dextrose, organisms able to do so will begin to utilize the lactose or sucrose.

2.4.3.1.4.1.2 Procedure

1) To prepare the TSI Agar, 6.5 g of the TSI Agar powder (Difco) was suspended in 100 ml of purified water, mixed thoroughly, and then heated with frequent agitation and boiled for1 minute to completely dissolve the powder. The solution was then dispensed into screw cap glass test tubes and autoclaved at 121°C for 15 minutes following which they were allowed to cool in a slanted position so that deep butts were formed.

2) To inoculate, a cool sterile needle was touched at the centre of an isolated colony which was then used to stab the medium in the butt of the tube, and then streak back and forth along the surface of the slant.

3) The inoculated TSI Agar tube was then incubated with loosened cap at 34°C in an aerophilic incubator for 18-24 hours for carbohydrate fermentation, gas production and hydrogen sulfide production.

4) To enhance the alkaline condition of the slant, free exchange of air was permitted by closing the tube cap loosely. Also, the incubation was not continued any longer than 24

hours as the acid reaction in the slant of lactose and sucrose fermenters may revert to an alkaline reaction.

2.4.3.1.4.1.3 Result Interpretation: Following incubation the reactions produced by the isolate under question was compared with those produced by known control organisms.

1) Carbohydrate fermentation was indicated by a yellow coloration of the medium. If the medium in the butt of the tube turned yellow (acidic), but the medium in the slant turned red (alkaline), it meant the organism only ferments dextrose (glucose).

2) A yellow (acidic) color in the slant and butt indicated that the organism ferments dextrose, lactose and/or sucrose.

3) A red (alkaline) color in the slant and butt indicated that the organism is a non-fermenter.

4) Hydrogen sulfide production resulted in a black precipitate in the butt of the tube

5) Gas production was indicated by splitting and cracking of the media.

Figure 2.9: Triple Sugar Iron Agar.

2.4.3.1.4.2 Motility Indole Urea

2.4.3.1.4.2.1 Principle: Motility Indole Urea Agar (MIU) is a semisolid medium designed for detection in Enterobacteriaceae of urease activity, motility, and indole production. Its components include tryptone, sodium chloride, potassium dihydrogen phosphate, phenol red and agar. Tryptone is a pancreatic digest of casein. Casein is the main protein of milk and is a rich source of amino acid and nitrogen. This hydrolysate has high tryptophan content and is therefore used in media for testing the indole reaction. Sodium chloride maintains the osmotic balance. Potassium dihydrogen phosphate buffers the medium, while phenol red is a pH indicator. The small amount of agar makes the medium semisolid. Bacterial motility can be observed directly from examination of the tubes following incubation. Growth spreads out of the line of inoculation if the organism is motile. Highly motile organisms provide growth throughout the tube. Growth of non motile organisms only occurs along the stab line. Urease activity can be observed by a change of color to red. When organisms utilize urea, ammonia is formed during incubation which makes the reaction of these media alkaline, producing a red-pink color. Consequently, urease production may be detected by the change in the phenol red indicator. Organisms that possess the enzyme "tryptophanase" degrade the amino acid tryptophan to indolepyruvic acid, from which indole can be formed through deamination.

2.4.3.1.4.2.2 Procedure

1) To prepare the MIU agar, 4.3 g of MIU Agar powder was suspended in 100 ml of deionized water, heated until completely dissolved, and then autoclaved at 121°C for 15 minutes**.** The preparation was then allowed to cool to 50°C following which 5 ml of urea 40% supplement was aseptically added to it.

2) To inoculate, a cool sterile needle was touched at the centre of an isolated colony which was then used to stab the center of the column of medium to greater than half the depth

3) The inoculated MIU Agar tube was then incubated with loosened cap at 35°C in an aerophilic incubator for 18-48 hours.

2.4.3.1.4.2.3 Result Interpretation

1) Motility was observed by growth extending from the line of inoculum or diffuse turbidity of the medium. Non-motile organisms grew only along the line of inoculation.
2) Urease activity was observed by a change of color to red.
3) Indole production was indicated by the formation of a pink to red color after the addition of few drops of Kovac's reagent to the surface of the medium. A negative reaction was indicated by the development of a yellow color.

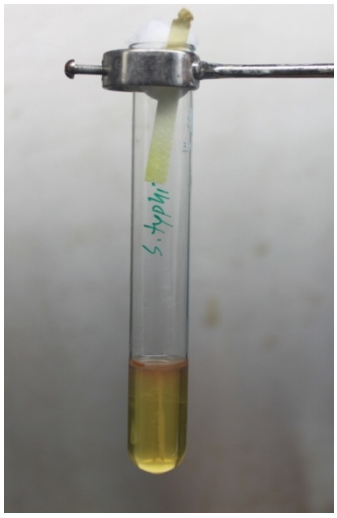


Figure 2.10: Motility Indole Urea

2.4.3.1.4.3 Citrate Agar Test

2.4.3.1.4.3.1 Principle: Simmons Citrate Agar is used for the differentiation of gram-negative bacteria on the basis of citrate utilization. Organisms able to utilize ammonium dihydrogen phosphate and sodium citrate as the sole sources of nitrogen and carbon, respectively, grow on this medium and produce an alkaline reaction as evidenced by a change in the color of the bromthymol blue indicator from green (neutral) to blue (alkaline).

2.4.3.1.4.3.2 Procedure

1) For preparing the Citrate agar media, 2.3 g of Simmons Citrate Agar powder (oxoid) was suspended in 100 ml of deionized water, mixed thoroughly, heated with frequent

agitation and boiled for 1 minute to completely dissolve the powder. The solution was then dispensed into screw cap glass test tubes and autoclaved at 121°C for 15 minutes following which they were allowed to cool in a slanted position.

2) Using a cold sterile inoculation loop a pure culture was picked up and used for inoculating the slant by streaking.

3) The inoculated Citrate Agar tube was then incubated at 35 ± 2°C in an aerophilic incubator for up to 4 days.

2.4.3.1.4.3.3 Result Interpretation

1) A positive reaction was indicated by growth with an intense blue color in the slant

2) A negative reaction was evidenced by no growth to trace growth with no change in color (medium remained dark green)

Figure 2.11: Citrate Agar Test

2.4.3.1.4.3.4 Typical Biochemical Reactions of *Klebsiella pneumoniae*

Table 2.4: Biochemical Test References for identification of *Klebsiella pneumoniae*

Organism	Slant	Butt	H_2S	Motility	Indole	Urea	Citrate
Klebsiella pneumoniae	Y	R or YG	-ve	-ve	-ve	+ve	+ve

R= Red Y= Yellow YG= Gas formed

2.4.3.1.4.4 Analytical Profile Index

Analytical Profile Index (API) 20E is a biochemical panel used for identification of members of the family Enterobacteriaceae. The API 20E consists of a plastic strip that holds twenty mini-test chambers containing dehydrated media having chemically defined compositions for each test. The tests include:

1. ONPG: test for β-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside
2. ADH: decarboxylation of the amino acid arginine by arginine dihydrolase
3. LDC: decarboxylations of the amino acid lysine by lysine decarboxylase
4. ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase
5. CIT: utilization of citrate as only carbon source
6. H2S: production of hydrogen sulfide
7. URE: test for the enzyme urease
8. TDA (Tryptophan deaminase): detection of the enzyme tryptophan deaminase: Reagent to put- Ferric Chloride.
9. IND: Indole Test-production of indole from tryptophan by the enzyme tryptophanase.
10. VP: the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway
11. GEL: test for the production of the enzyme gelatinase which liquefies gelatin
12. GLU: fermentation of glucose (hexose sugar)

13. MAN: fermentation of mannose (hexose sugar)
14. INO: fermentation of inositol (cyclic polyalcohol)
15. SOR: fermentation of sorbitol (alcohol sugar)
16. RHA: fermentation of rhamnose (methyl pentose sugar)
17. SAC: fermentation of sucrose (disaccharide)
18. MEL: fermentation of melibiose (disaccharide)
19. AMY: fermentation of amygdalin (glycoside)
20. ARA: fermentation of arabinose (pentose sugar)

2.4.3.1.4.4.1 Principle: API test strips consists of microtubes (cupules) containing dehydrated substrates to detect the enzymatic activity or the assimilation/ fermentation of sugars by the inoculated organisms. During incubation, metabolism produces colour changes that are either spontaneous, or, revealed by the addition of reagents. When the carbohydrates are fermented, the pH within the cupule changes and is shown by an indicator. Assimilation tests are inoculated with a minimal medium (API AUX medium) and the bacteria grow if they are able to utilize the corresponding substrate. A positive result is indicated by growth.

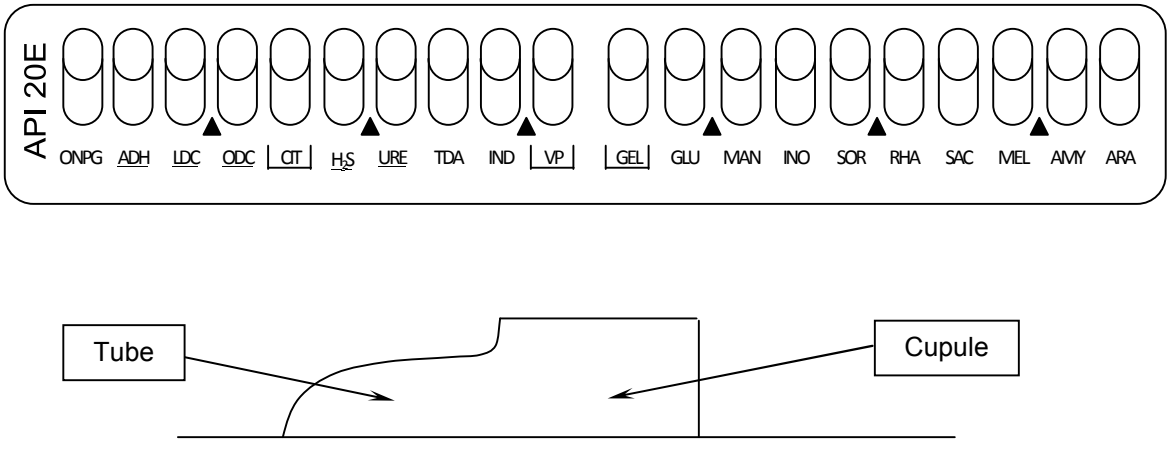


Figure 2.12: Schematic representation of an API 20E strip and a microtube

2.4.3.1.4.4.2 Procedure

2.4.3.1.4.4.2.1 Preparation of the strip

1) An incubation box with tray and lid was prepared and about 5 ml of demineralized water was distributed into the honey combed wells of the tray to create a humid atmosphere.
2) The test strip was then placed in the incubation box.

2.4.3.1.4.4.2.2 Preparation of the inoculum

1) 5 ml of API NaCl 0.85% medium was taken.
2) Using a pipette, a single well isolated colony was removed from an isolation plate. Cultures older than 24 hours were avoided.
3) Careful emulsification was done to achieve a homogeneous bacterial suspension which was used immediately after preparation.

2.4.3.1.4.4.2.3 Inoculation of the Strip

1) Using the same pipette, both tube and cupule of the tests CIT, VP and GEL were filled with the bacterial suspension.
2) For the other tests only the tube and not the cupule were filled.
3) For the tests ADH, LDC, ODC, H_2S and URE, anaerobic condition was created by overlaying with mineral oil.
4) The incubation box was then closed and incubated at 36°C ± 2°C for 18-24 hours.

2.4.3.1.4.4.3 Interpretation of Results

1) Identification was obtained with the numerical profile.
2) On the result sheet, the tests remain separated into groups of 3 and a value 1, 2 or 4 remains indicated for each. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number was obtained for the 20 tests of the API 20 E strip. The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive.

3) The identification was done by looking up the numerical profile in the list of profiles in the database.

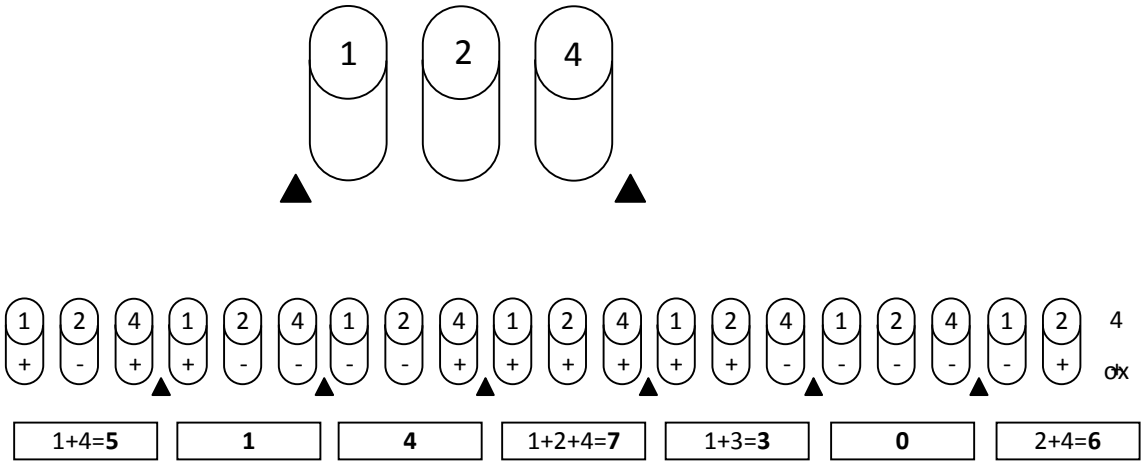


Figure 2.13: Representation of the numbers designated to tubes on API strip used for generating a numerical profile.

Table 2.5: API 20E test references			
Test	Reaction	Negative	Positive
ONPG	β-galactosidase	Colourless	Yellow
ADH	Arginine dihydrolase	Yellow	Orange or red
LDC	Lysine decarboxylase	Yellow	Orange or red
ODC	Ornithine decarboxylase	Yellow	Orange or red
CIT	Citrate utilisation	Light green	Blue-green/ blue
H_2S	H_2S production	Colourless	Black
URE	Urea hydrolysis	Yellow	Pink
TDA	Tryptophan deamination	Yellow	Dark brown
IND	Indole production	Colourless reagent	Pink
VP	Acetoin production	Colourless	Pink or red
GEL	Gelatin hydrolysis	Colourless	Black diffuse pigment
GLU	Glucose fermentation	Blue	Yellow
MAN	Mannitol	Blue	Yellow
INO	Inositol	Blue	Yellow
SOR	Sorbitol	Blue	Yellow
RHA	Rhamnose	Blue	Yellow
SAC	Sucrose	Blue	Yellow
MEL	Melibiose	Blue	Yellow
AMY	Amygdalin	Blue	Yellow
ARA	Arabinose	Blue	Yellow
Oxidase	Cytochrome oxidase	Colourless	Purple

2.4.3.1.4.4.4 Typical Reactions of *Klebsiella pneumoniae* in API

Table 2.6: Typical reactions of *Klebsiella pneumoniae* in API										
ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL
+	-	+	-	+	-	V	-	-	V	-
GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	NO_2	N_2
+	+	+	+	+	+	+	+	+	+	-

2.4.3.1.5 Anti-Microbial Susceptibility Test: The anti-microbial sensitivity testing was done in the same method except using Mueller-Hinton Agar plate as described in section **2.4.1.2.7** The following antibiotics with their concentration per disc were used for the sensitivity testing:

Table 2.7: List of antibiotics for the AST of *Klebsiella pneumoniae*

Antibiotic	Concentration per Disc
Gantamicin (GN)	10 µg
Tobramycin (TOB)	10 µg
Ceftriaxone (CRO)	30 µg
Cefixime (CFM)	5 µg
Ciprofloxacine (CIP)	5 µg
Impenem (IMP)	10 µg
Meropenem (MEM)	10 µg
Azithromycin (AZI)	15 µg

2.5 Study Design for Detection of Enteric Fever

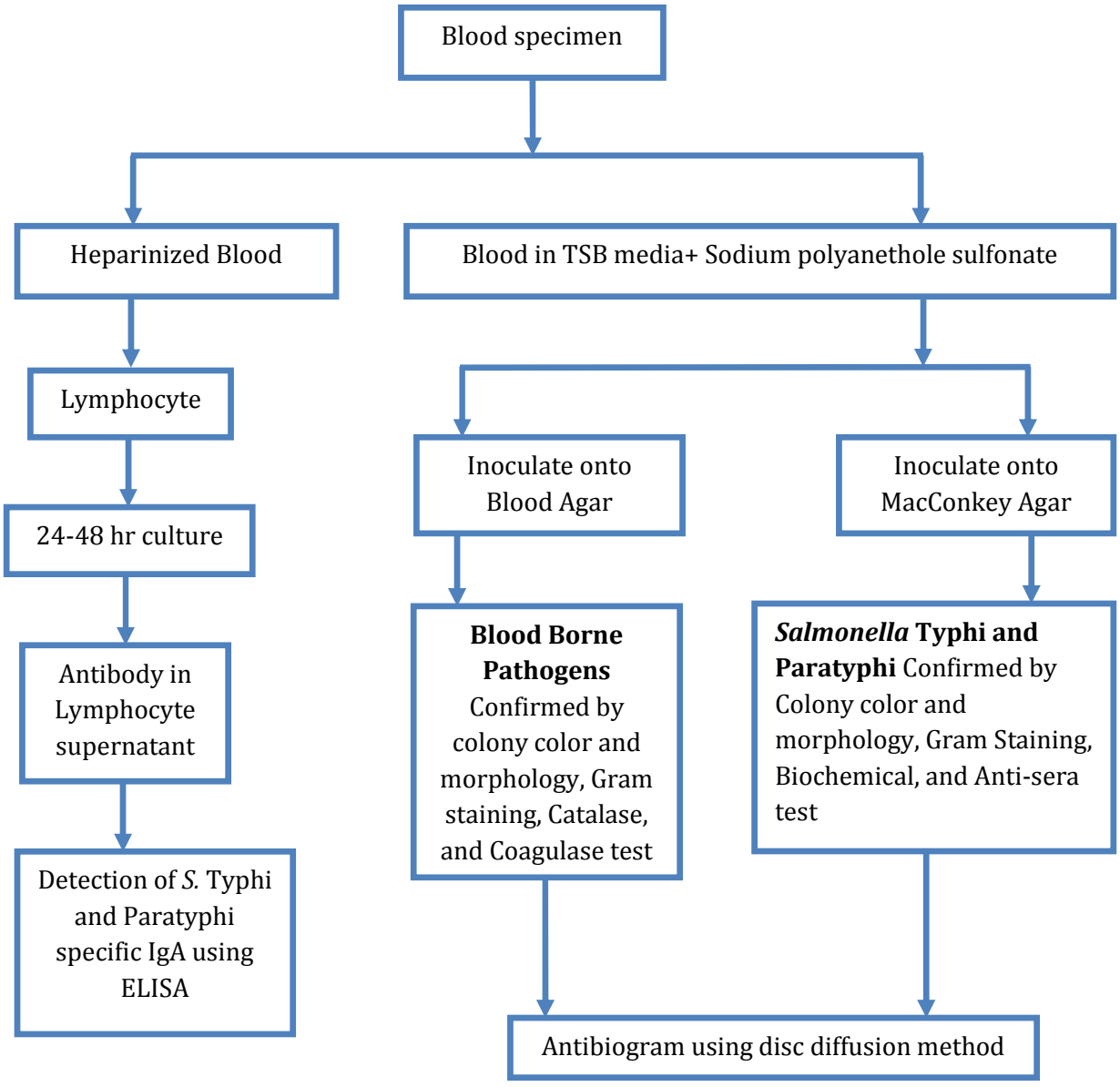


Figure 2.14: Flow-chart depicting study design for detection of Enteric fever and its pathogen.

2.5.1 Blood Culture

1) 3 ml of blood was taken in an aerobic blood culture bottle containing Tryptic Soy Broth with sodium polyanethol sulfonate.

2) The culture was then incubated at 37°C in an aerobic incubator for 48 hours.

3) Following incubation, one loop-full (~10 µl) of the blood culture was inoculated on a MacConkey agar plate and a Blood agar plate, streaked for isolated colonies, and incubated for 24 hours in an aerophilic incubator.

4) After overnight incubation, the plates were observed for growth.

2.5.1.1 *Salmonella* Typhi and *Salmonella* Paratyphi Isolates Detection and Identification

1) Salmonella produces non-lactose fermenting smooth colonies on MacConkey agar

2) Salmonella Typhi and Salmonella Paratyphi appear as non-haemolytic smooth white colonies.

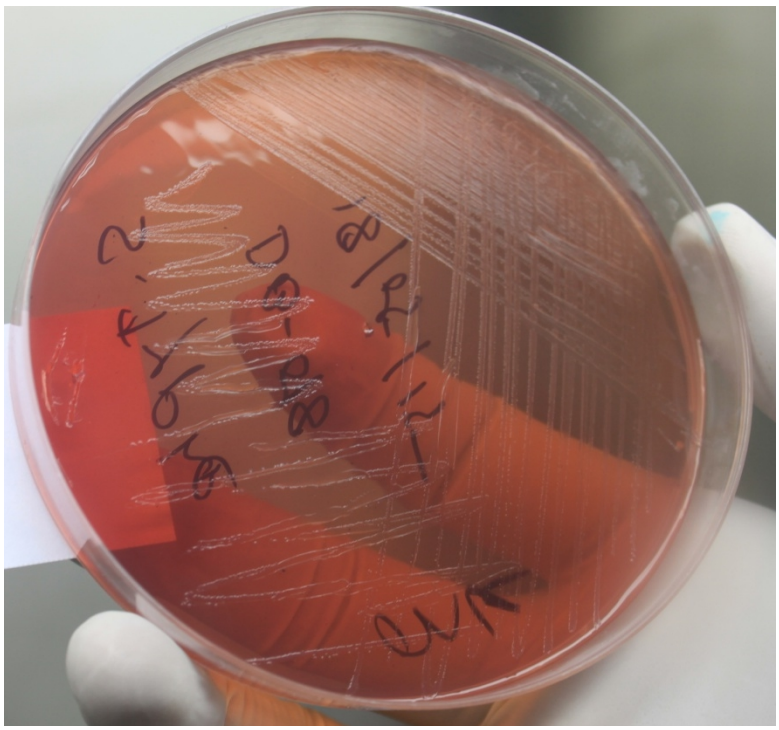


Figure 2.15: *Salmonella* Typhi colonies on MacConkey agar Plate

2.5.2 Gram Staining: Gram staining was done in the same process described in section 2.4.1.2.3.

2.5.3 Catalase Test: The catalase test for Gram-positive blood-borne pathogens was done in the same process as described in section 2.4.1.2.4.

2.5.4 Coagulase Test

Coagulase test is used to differentiate *Staphylococcus aureus* (positive) from Coagulase Negative *Staphylococcus* (CoNS). Coagulase is an enzyme produced by *S. aureus* that converts soluble fibrinogen in plasma to insoluble fibrin. *Staphylococcus aureus* produces two forms of coagulase, bound and free. Slide coagulase test was done to detect bound coagulase or clumping factor. Tube coagulase test was done to detect free coagulase

2.5.4.1 Method

2.5.4.1.1 Slide Coagulase Test

1) A drop of plasma was placed on a clean grease free and dry glass slide
2) A drop of distilled water or saline was placed next to the drop of plasma as a negative control.
3) A portion of the isolated colony being tested was emulsified in each drop to make a smooth milky suspension
4) The slide was gently rocked for 5- 10 seconds and checked for coagulation.
5) For coagulase positive, macroscopic clumping was observed in 10 seconds or less in coagulated plasma drop and no clumping was observed in saline or water drop.
6) For coagulase negative, no clumping was observed in either drop.

2.5.4.1.2 Tube Coagulase Test

1) Several isolated colonies of test organism was emulsified in 0.5 ml of rabbit plasma to give a milky suspension
2) The tube was incubated at 35°C for 4 hours in an aerobic environment.

3) The tube was then checked for clot formation. If at 4 hours the result was negative, the tube was incubated at room temperature overnight and checked again for clot formation.

2.5.5 Biochemical Tests

2.5.5.1 Triple Sugar Iron Agar: The Triple Sugar Iron (TSI) test was done in the same way as described in section 2.4.3.1.4.1

2.5.5.2 Motility Indole Urea: The Motility Indole Urea (MIU) test was done in the same way as described in section 2.4.3.1.4.2

2.5.5.3 Citrate Agar Test: The Citrate Agar test was done in the same way as described in section 2.4.3.1.4.3

2.5.5.4 Typical Reactions of *Salmonella* Typhi and Paratyphi

Table 2.8: Biochemical test references for identification of *S.* Typhi and Paratyphi

Organism	Slant	Butt	H_2S	Motility	Indole	Urea	Citrate
Salmonella Typhi	R	Y	+ve	+ve	-ve	-ve	-ve
Salmonella Paratyphi A	R	YG	-ve	+ve	-ve	-ve	-ve
Salmonella Paratyphi B	R	YG	+ve	+ve	-ve	-ve	+ve

R= Red Y= Yellow YG= Gas formed

2.5.6 Anti-Sera Test

Salmonella Grouping Antisera were used in qualitative slide agglutination tests for the serological differentiation of *Salmonella*. Using these antisera, *Salmonella* isolates may be classified into *S* Typhi and Paratyphi A, B and C. The *Salmonella* O Polyvalent antiserum was used for screening in conjunction with biochemical tests. The *Salmonella* Vi antiserum detects the Vi antigen, a heat-sensitive envelope antigen usually found in

fresh isolates of *S.* Typhi and *S.* Paratyphoid. The following antisera were used for *Salmonella.*

Table 2.9: List of antisera used for identification of *Salmonella* Typhi and Paratyphi	
Salmonella Antisera- Omnivalent	Kauffmann-White group A-067
Salmonella Antisera H-c	Salmonella Flagellum H Antigen c
Salmonella Antisera H-b	Salmonella Flagellum H Antigen b
Salmonella Antisera H-a	Salmonella Paratyphi A
Salmonella Antisera Group O2	Identified Salmonella group O2
Salmonella Antisera Vi	To investigate the presence of Vi capsular antigen
Salmonella Antisera H-d	Salmonella Flagellum H antigen d
Salmonella Polyvalent 4	Verification of genus (Salmonella enterica)

2.5.6.1 Method

1) Using an inoculating loop, needle, sterile applicator stick, or toothpick, portion of the growth from the surface of a nonselective agar medium was removed. Serologic testing was not done on growth from selective media because selective media may yield false-negative serologic results.

2) The bacterial growth was suspended in 0.5 ml of 0.85% saline to prepare an antigenic suspension which was then used for the antisera test.

3) A drop (30 µl) each of polyvalent serum and 0.85% saline as a control was placed on a clean glass slide partitioned into several parts with a glass pencil.

4)5-10 µl of the antigenic suspension was placed onto the serum and 0.85% saline on the glass slide.

5) The reagents were mixed by tilting the glass slide back and forth for 1 minute and the agglutination pattern was observed. The agglutination was grossly observed with light through the slide

6) When a specimen tested positive with a polyvalent serum, steps 3-5 mentioned above were performed using each monovalent serum constituting the polyvalent serum

2.5.6.2 Interpretation of Results

1) It was first confirmed that no agglutination was found on the reaction with antigenic suspension and 0.85% saline.
2) Only strong agglutination observed within 1 minute of the reaction with each serum was regarded as positive. Delayed or weak agglutination was regarded as negative.

2.5.7 Antimicrobial Susceptibility Test: The anti-microbial sensitivity testing was done in the same method except using Mueller-Hinton Agar plate as described in section 2.4.1.2.7. The following antibiotics with their concentration per disc were used for the sensitivity testing:

Table 2.10: List of Antibiotics for the AST of *Salmonella* Typhi and Paratyphi

Antibiotic	Concentration per Disc
Ampicillin (AMP)	10 µg
Chloramphenicol (C)	30 µg
Azithromycin (AZI)	15 µg
Ciprofloxacine (CIP)	5 µg
Ceftriaxone(CRO)	30 µg
Cotrimoxazole (COT)	10 µg
Nalidixic Acid (NA)	30 µg
Cefixime (CFM)	5 µg

2.5.8 Antibody in Lymphocyte Supernatant Assay

2.5.8.1 Separation of Peripheral Blood Mononuclear Cells (PBMC) by The RBC Lysis Method: Since serovar Typhi interacts with both the mucosal and the systemic immune systems, the principal focus was to analyse the mucosal immune response. As activated mucosal lymphocytes migrate from intestinal tissue and circulate within

peripheral blood before re-homing to mucosal tissues, venous blood from the study participants was collected and peripheral blood mononuclear cells (PBMC) were separated using the RBC lysis method using an isotonic solution of ammonium chloride (NH_4Cl). Many of the methods available for isolation of human PBMCs for functional analysis result in a degree of red blood cell contamination. An easy solution to this problem is the removal of RBC by differential lysis using NH_4Cl. RBC membranes are effectively permeable to NH_4Cl and cell lysis occurs due to unbalanced osmotic pressure of their colloid content. The hemolysis happens due to diffusion of free NH3 across the membrane. Once inside the cell, a new equilibrium between NH3 and $NH4^+$ is established and the intracellular OH^- exchanges to the extracellular Cl^- resulting in a net influx of NH4Cl.

2.5.8.1.1 Method

1) The RBC lysis solution constitutes of ammonium chloride (NH_4Cl), potassium bicarbonate ($KHCO_3$), and ehylenediaminetetraacetic acid EDTA ($C_{10}H_{16}N_2O_8$).
2) Using 1X NH_4Cl lysis solution, heparinized venous blood in a falcon tube was diluted at a dilution factor of 1:20.
3) The diluted blood specimen was then incubated at room temperature for exactly 5 minutes while mixing gently by repeatedly inverting the tube.
4) The diluted blood was then centrifuged at 953 Xg for 5 minutes at 20°C.
5) After centrifugation, the supernatant was decanted immediately and the pellet resuspended in 10 ml of ice-cold PBS
6) The cell pellet was properly dissolved in the ice-cold PBS by pipetting bringing the cells in suspension.
7) After proper homogenization of the cell suspension by tapping the tube, 25 µl of the suspension was sampled for cell counting.
8) The cells were washed by centrifuging the cell suspension at 953 Xg for 5 minutes at 20°C.
9) Using a haemocytometer, the number of lymphocytes in the sample was counted.

10) After centrifugation, the supernatant was discarded and the PBMC pellet dried by inverting the tube on fresh tissue papers.

Figure 2.16: Separation of PBMC using RBC Lysis Solution

2.5.8.2 Lymphocyte Culture and Harvesting of Antibody in Lymphocyte Supernatant (ALS)

1) The isolated PBMCs were resuspended in RPMI complete medium to achieve a seeding density of $10x10^6$ cells per ml.
2) The cells were cultured in 96 well flat bottomed cell culture plates (NUNC Costar) at 37°C in a 5% CO_2 incubator for 48 hours.
3) After 48 hours of incubation, the culture supernatant was collected in a fresh microcentrifuge tube and centrifuged at 12,000 rpm (11,600xg) for 5 minutes at 20°C.
4) After centrifugation, the lymphocyte supernatant was carefully collected using a micropipette without drawing any of the cells and debris at the bottom.
5) 1 µl of protease inhibitor (0.2 µM AEBSF, 1µg /ml Aprotinin, 10 µM Leupeptin

Hemisulfate, 1 mg/ml Na-azide) was added per 100 µl of antibody in lymphocyte supernatant (ALS) after which the ALS was stored at -70°C for future analysis.

2.5.8.3 Preparation of Ty21a Membrane Preparation (MP)

1) *Salmonella* Typhi strain Ty21a was cultured on sheep blood agar plates and the bacteria harvested in Tris-$MgCl_2$ buffer (5 mM $MgCl_2$, 10 mM Tris [pH 8.0].
2) The bacterial suspension was then sonicated five times at 60% amplitude and centrifuged at 1400 xg for 10 minutes.

3) The supernatant was transferred to fresh tubes and centrifuged at 14900 xg for 30 minutes.
4) The resultant pellet was resuspended in harvest buffer and the protein content was determined by the Bio-Rad protein assay and stored at -70°C.

2.5.8.4 Analysis of Antibody Response in ALS by Kinetic ELISA

The enzyme linked immunosorbent assay (ELISA) is an analytic biochemistry assay that utilizes a solid-phase enzyme immunoassay for detecting the presence of a substance, usually an antibody or antigen, in a liquid sample. In this method, a specific antigen is coated over solid polystyrene surface. Application of a liquid sample containing the analyte antibody over the surface causes the specific antibodies to bind to the antigen. In the next step, application of a secondary antibody conjugated with an enzyme results in the secondary antibody binding specifically to the primary antibody. Finally, addition of the enzyme's substrate and subsequent reaction produces a detectable signal, most commonly a color change in the reaction mixture that can be read quantitatively using a spectrophotometer and used for determining the concentration of the particular antibody in the respective sample.

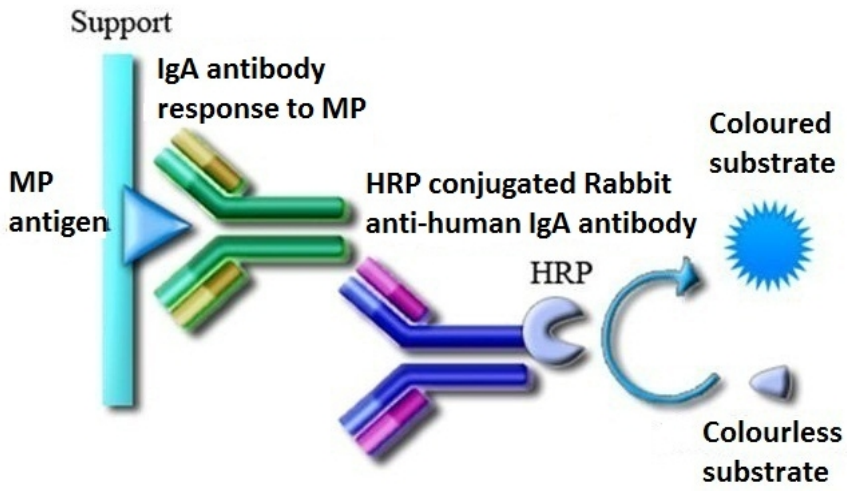


Figure 2.17: Schematic representation of MP specific IgA Response detection by ELISA

2.5.8.4.1 Antigen Coating of ELISA Plates

1) The antigen, Ty21a MP, was diluted with autoclaved PBS at a concentration of 5.0 µg/ml.
2) 96 well microtiter ELISA plates (NuncF) were coated with 100 µl of the antigen suspension in each well.
3) The plates were then allowed to stand at room temperature overnight following which they were immediately used or preserved at 4°C refrigerator.

2.5.8.4.2 Plate Blocking

1) The antigen suspension from the coated plates was decanted and plates washed thrice with Phosphate Buffered Saline (PBS).
2) The coated wells were blocked with 200 µl/well of 1% bovine serum albumin in PBS (BSA-PBS) and incubated at 37°C for 30-60 minutes.

2.5.8.4.3 Loading of Sample and Controls

1) After incubation, the blocking solution was discarded and the plates washed three times with PBS-Tween (0.05% by volume) and once with PBS. The plates were dusted to get rid of any residual PBS.
2) Using 0.1% BSA-PBS-Tween as a diluent, the ALS samples were diluted by a ratio of

1:2, and 100 µl of the samples were added to designated wells.

3) For positive control, an in-house pooled convalescent-phase standard sera of blood culture-confirmed typhoid patients was used, which was diluted with 0.1% BSA-PBS-Tween at a ratio of 1:100 and 100 µl was added to designated wells.

4) For negative control, 100 µl of 0.1% BSA-PBS-Tween was added to designated wells.

5) The Plates were then incubated at 37°C for 90 minutes

2.5.8.4.4 Addition of Conjugate

1) After incubation, the plates were washed three times with PBS-Tween (0.05% by volume) and once with PBS. The plates were dusted to get rid of any residual PBS.

2) For preparation of conjugate, rabbit anti-human IgA conjugated with horseradish peroxidase was diluted in 0.1% BSA-PBS-Tween at a ratio of 1:1000.

3) 100 µl of the preparation was added to each well designated for samples and the positive and negative controls.

4) The Plates were then incubated at 37°C for 90 minutes.

2.5.8.4.5 Plate Development

1) After incubation, the plates were washed three times with PBS-Tween (0.05% by volume) and once with PBS. The plates were dusted to get rid of any residual PBS.

2) The substrate used was ortho-phenylenediamine (OPD) which was prepared by dissolving 0.01 g of OPD in 10 ml of 0.1 M Sodium citrate buffer (pH 4.5) to make a 0.1% solution, to which 4 µl of 30% hydrogen peroxide (H_2O_2) was added immediately before use.

3) The plates were developed by simultaneously adding 100 µl of the substrate to each of the designated wells, immediately after which the plates were read kinetically at 450 nm wavelength for 5 minutes at 19 seconds intervals using EON ELISA Reader.

2.5.8.4.6 Interpretation of Results

1) The maximal rate of change of optical density was expressed as milli optical density absorbance units per minute (mAB/min).

2) The mAB/min kinetic reaction rate of test samples was divided by that of the positive control, and the product was multiplied by 100 and was expressed as ELISA units.

3) The cut-off value of the TPTest was calculated as greater than the geometric mean of ELISA units plus 2 standard deviations of healthy Bangladeshi control.

4) A positive value was defined as >10 ELISA units.

2.6 Study Design for Detection of Diarrheal Pathogens

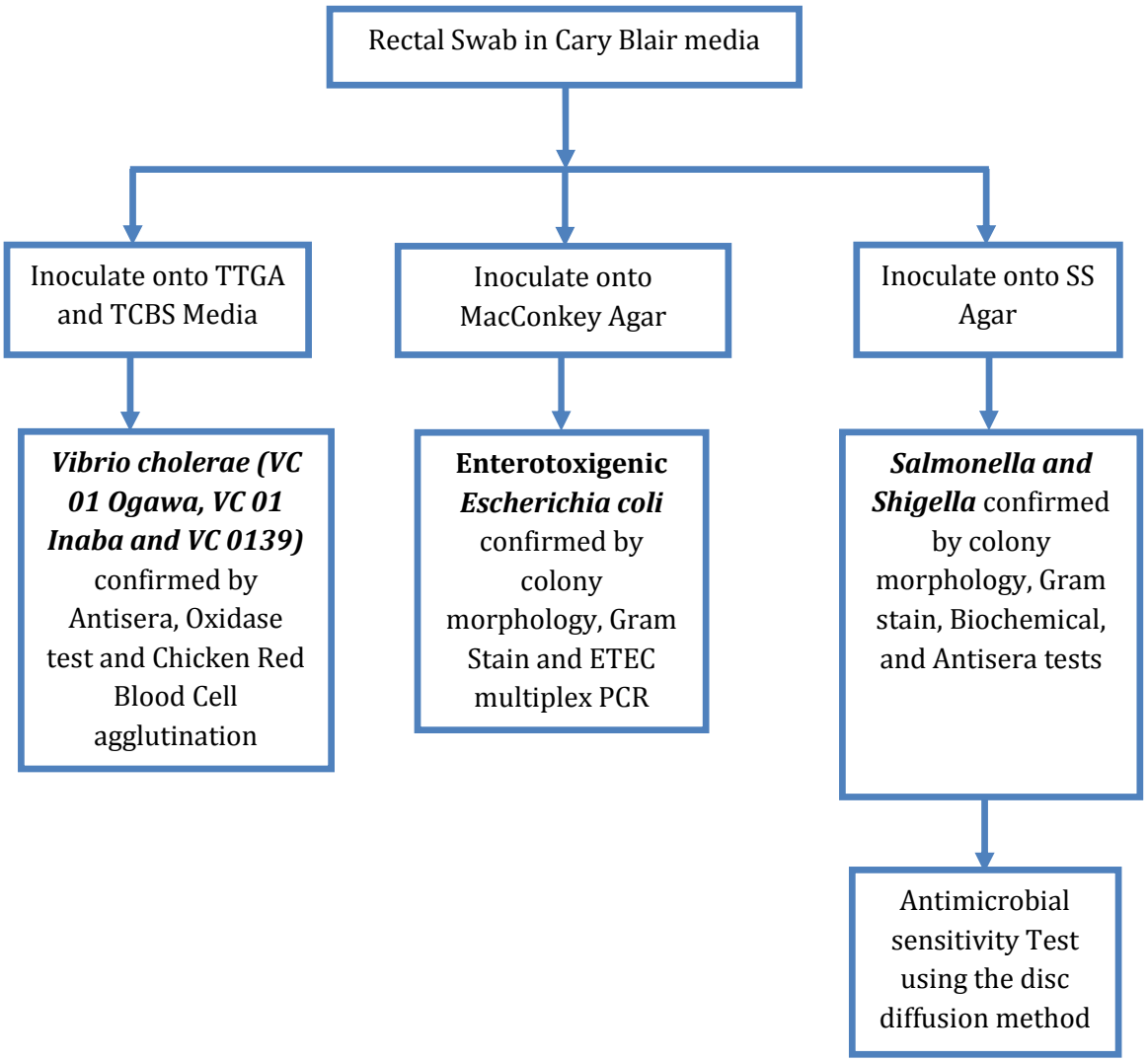


Figure 2.18: Flow-chart depicting study design for detection of Diarrheal pathogens

2.6.1 Identification and Characterization of *Vibrio cholerae*

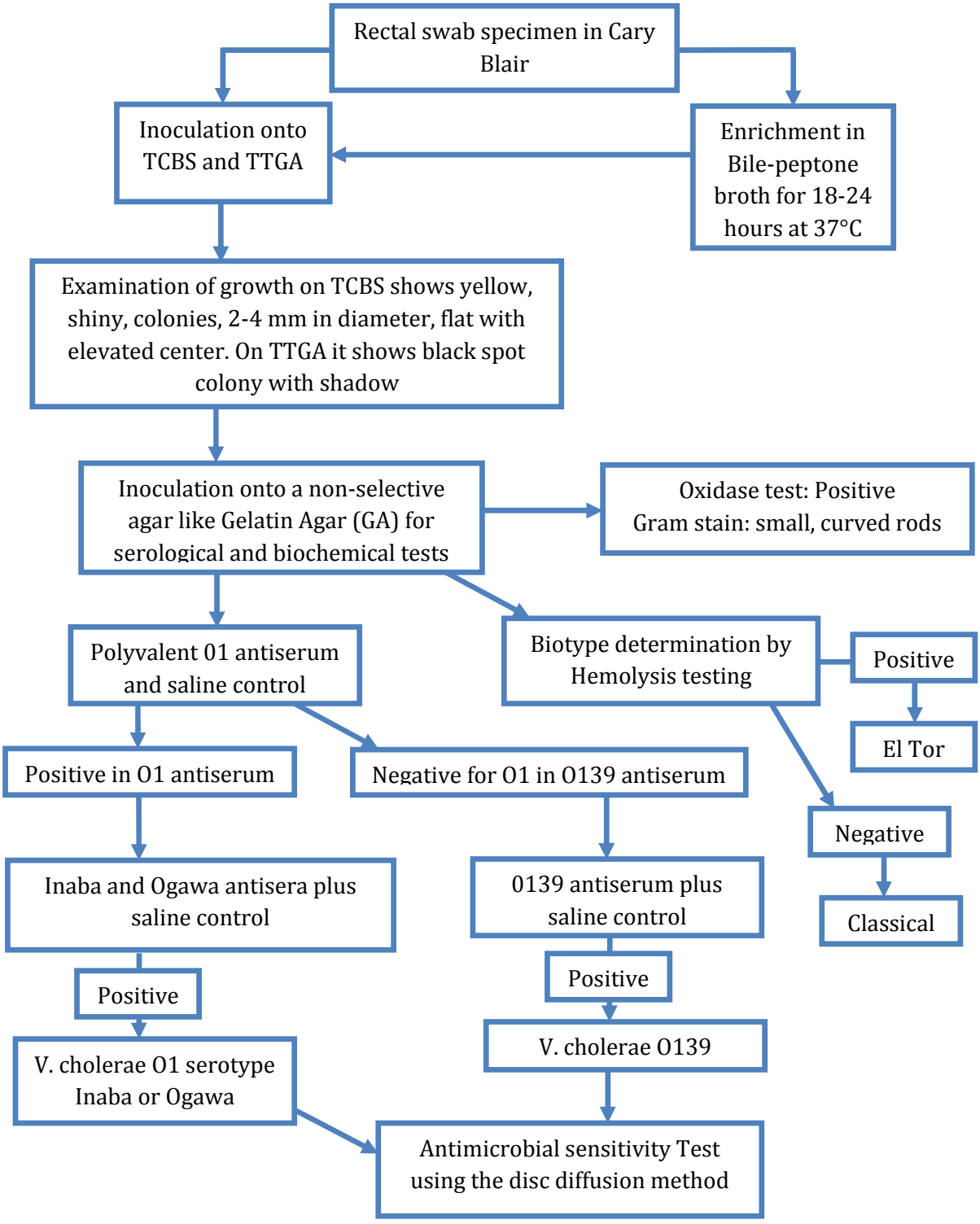


Figure 2.19: Flowchart for Identification and Characterization of *Vibrio cholerae*.

2.6.1.1 Method

2.6.1.1.1 Rectal Swab Specimen Enrichment in Bile Peptone

1) Within 4-6 hours after collection, the rectal swab was taken from Cary Blair media and used to make smears on thiosulfate citrate bile salts sucrose agar (TCBS) and taurocholate tellurite gelatin agar (TTGA) media plates.
2) The swab stick was then used for inoculating 5 ml of Bile Peptone broth in a screw capped drum vial for enrichment.
3) The inoculated enrichment broth was vortexed and incubated with the cap loosened for 18-24 hours at 35-37°C in an aerophilic incubator.
4) Following enrichment only the specimens that were negative in direct culture were subcultured on TTGA plates.

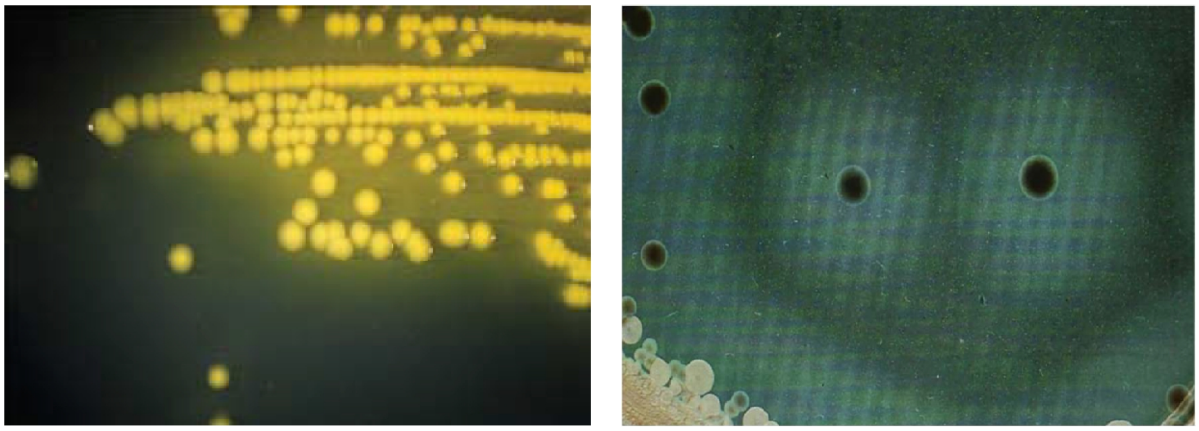
2.6.1.1.2 Inoculation and isolation of suspected V. cholerae

1) An inoculation loop was flamed and after sufficient cooling was used to streak the inoculum smeared on the TCBS and TTGA plates. The loop was flamed in between streaking to ensure dilution and proper isolation of colony forming units.
2) The plates were then incubated at 37°C for 18-24 hours in an aerophilic incubator.

2.6.1.1.3 *V. cholerae* Isolates Detection and Identification

1) After overnight incubation the TCBS and TTGA growth were carefully examined for typical *V. cholerae* colonies.
2) V. cholerae colonies on TCBS agar appear as large (2-4 mm) and yellow because of the fermentation of sucrose. They are characteristically round, smooth, glistening, and slightly flattened.
3) On TTGA media, it appears as small opaque colonies with slightly dark centers. After 24 hours, the centers of the colonies become darker, and eventually the entire colony become “gunmetal” grey in color. In addition to the dark coloration, which is due to the reduction of tellurite, there is also an opaque zone around colonies which resemble a halo. The halo effect is due to the production of the enzyme gelatinase

4) Suspicious colonies for further testing were subcultured on a noninhibitory medium, such as gelatin agar.



A B

Figure 2.20: Vibrio cholerae colonies on TCBS (A) and TTGA (B) plates

2.6.1.1.4 Gram Staining: Gram staining was done in the same process described in section 2.4.1.2.3.

2.6.1.1.5 Kovac's Oxidase Test: Kovac's oxidase test was done in the same process described in section 2.4.2.1.4.

2.6.1.1.6 Anti-Sera Test: *Vibrio* Grouping Antisera were used in qualitative slide agglutination tests for the serological differentiation of *Vibrio cholerae*. Using these antisera, *Vibrio* isolates may be classified into serotypes O1 Ogawa, O1 Inaba, and O139. The following antisera were used for *Vibrio.*

Table 2.11: List of antisera used for identification of *Vibrio cholerae*
Polyvalent (serovar Inaba and Ogawa)
Serovar Inaba
Serovar Ogawa
O139Bengal

The Antisera tests were done in the same process as described in sections 2.5.6.1 and 2.5.6.2

2.6.1.1.7 RBC Hemolysis Test: The classical and El Tor biotypes were differentiated by the ability of the El Tor group to lyse erythrocytes.

1) Fresh chicken red blood cells were used for this assay. A 2.5% suspension of washed and packed (by centrifugation) cells was made in normal saline.

2) A large loopful of the red blood cell suspension was placed on a glass slide.

3) A small portion of the growth from a nonselective agar slant was added to the red cells with a needle or loop, and was mixed well.

4) In a positive test, agglutination of the red cells occurred within 30-60 seconds.

5) Hemagglutinating (El Tor) and nonhemagglutinating (classical) control strains were used with each new suspension of red cells.

2.6.2 Identification and Characterization of Enterotoxigenic *Escherichia coli*

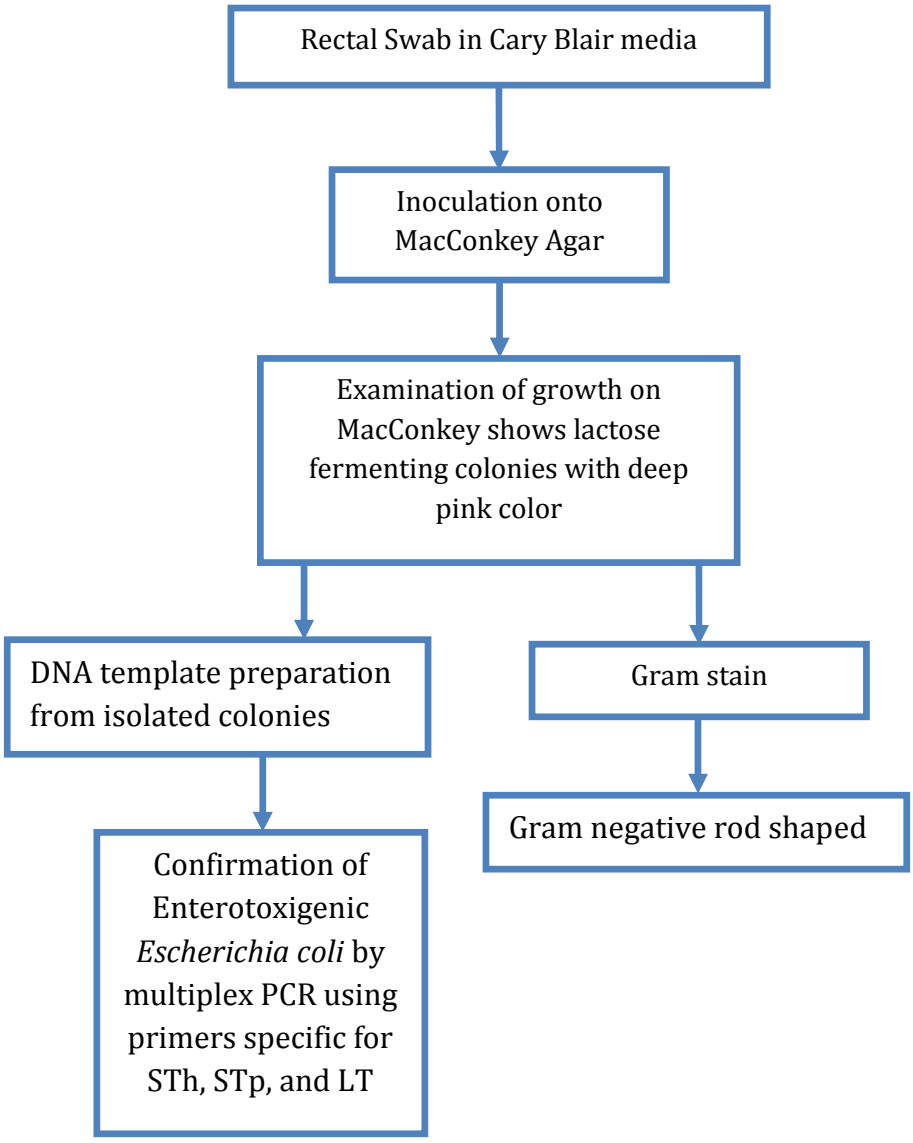


Figure 2.21: Flowchart for Identification and Characterization of Enterotoxigenic *Escherichia coli*.

2.6.2.1 Method

2.6.2.1.1 Inoculation and Isolation of Suspected Enterotoxigenic *E. coli*

1) Within 4-6 hours after collection, the rectal swab was taken from Cary Blair media and used to make smears on MacConkey agar plates.

2) An inoculation loop was flamed and after sufficient cooling was used to streak the inoculum smeared on the MacConkey agar plates. The loop was flamed in between streaking to ensure dilution and proper isolation of colony forming units.

3) The plates were then incubated at 37°C for 18-24 hours in an aerophilic incubator.

2.6.2.1.2 *E. coli* Isolates Detection and Identification

1) After overnight incubation, the MacConkey growth was carefully examined for typical *E. coli* colonies.

2) *E. coli* colonies on MacConkey agar appear as lactose fermenting, deep pink colored and non-mucoid.

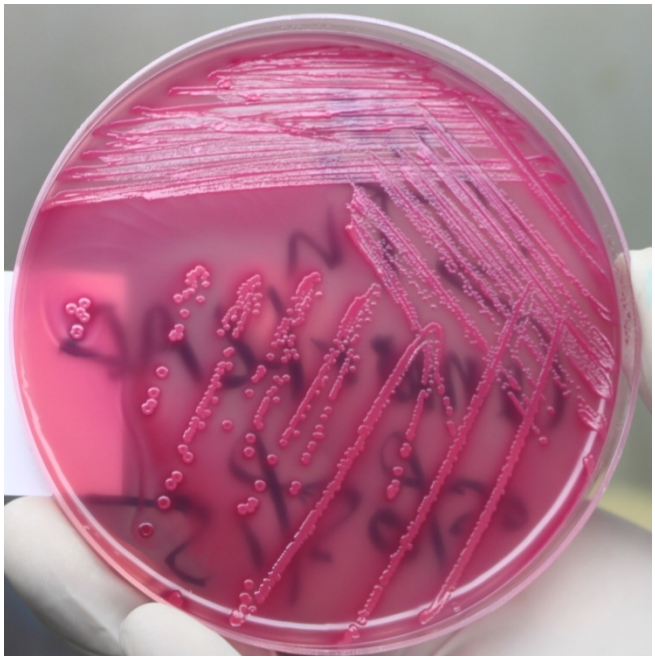


Figure 2.22: ETEC colonies on MacConkey Agar plate

2.6.2.1.3 Gram Staining: Gram staining was done in the same process described in section 2.4.1.2.3.

2.6.2.1.4 Detection of ETEC Toxin Genes Using Multiplex PCR

ETEC strains express one or both of two different enterotoxins, the heat-stable toxins (STh and STp) and a heat-labile toxin (LT). For fast and reliable detection of ETEC, multiplex polymerase chain reaction to amplify the ETEC toxin genes LT and ST was performed in which both LT and ST gene specific primers were used together in one master mix preparation.

2.6.2.1.4.1 Template Preparation

1) Six separate lactose fermenting colonies from each clinical specimen were tested to detect ETEC.
2) Using a sterile loop one loopful of bacteria was taken from a MacConkey Agar plate and suspended in an eppendorf tube containing 100 µl of Phoshate Buffered Saline (PBS)
3) The suspension was heated at 100°C in a water bath for 10 minutes.
4) The tube was immediately transferred to ice and maintained there for a minute.
5) The suspension was centrifuged at 12000 rpm for 10 minutes.
6) The supernatant was then separated and used as the PCR template.

2.6.2.1.4.2 Reference Strains: Different strains of *E. coli* that harbor virulence gene(s) of enterotoxins(s) (LT and/or ST) and strain negative for both of the genes were used as positive and negative control respectively in the multiplex PCR.

Table 2.12: Reference ETEC strains used for the detection of LT and/or ST gene	
Strains	Toxins
E. coli ST 64111	STh+
E. coli 286C2	LT+
E. coli 195	STp+
E. coli VM 75688	LT+, STh+
E. coli E34420C	ST-, LT-

2.6.2.1.4.3 ST and LT gene specific primers: The following ST and LT gene specific primers were used for detection of ETEC by multiplex PCR.

Table 2.13: ST and LT gene specific primers		
Primer	Sequence	Size
LT(Forward)	5'-ACG GCG TTA CTA TCC TCT C-3'	19 nt
LT(Reverse)	5'- TGG TCT CGG TCA GAT ATG TG-3'	20 nt
STp (Forward)	5'-TCT TTC CCC TCT TTT AGT CAG-3'	21 nt
STp (Reverse)	5'-ACA GGC AGG ATT ACA ACA AAG-3'	21 nt
STh (Forward)	5'-TAC AAG CAG GAT TAC AAC AC-3'	20 nt
STh (Reverse)	5'-AGT GGT CCT GAA AGC ATG-3'	18 nt
**nt stands for nucleotide.*		

2.6.2.1.4.4 Preparation of the PCR master mix

Master mix was prepared by mixing all the components of PCR except the component against which the optimization strategy was intended. The PCR component in question was added and the total volume was made 23.5 µL by adding varying amounts of sterilized ultra-pure water. Taq DNA polymerase was added just before starting the reaction.

Table 2.14: PCR master mix used for ETEC multiplex PCR

PCR Reagents		Optimized Quantity (µL)
PCR buffer, with $MgCl_2$ (10X)		2.5
25mM MgCl2		0.5
dNTPs (2.5 mM each)		4.0
Sterile Deionized Water		10.85
Primers	Primer LT mixture (4 pm/µl)	2.0
	Primer STp mixture (4 pm/µl)	2.0
	Primer STh mixture (4 pm/ µl)	2.0
Taq DNA Polymerase (5 U/µl)		0.15
Total		23.5

1.5 µl of template was added in each tube.

2.6.2.1.4.5 Thermal cycling profile used in PCR

The thermal cycling profiles that were programmed for Polymerase Chain Reaction (PCR) for 39 cycles are given below:

Table 2.15: Thermal cycling profile used to amplify ETEC toxin genes

Steps of Thermal Cycling	Temperature	Time	No. of Cycles
Initial denaturation	95°C	5 minutes	1 (First)
Denaturation	94°C	30 seconds	39
Annealing	54°C	30 seconds	39
Elongation	72°C	30 seconds	39
Final Elongation	72°C	5 minutes	1 (last)
Final hold up temp.	4°C	Infinite period	-

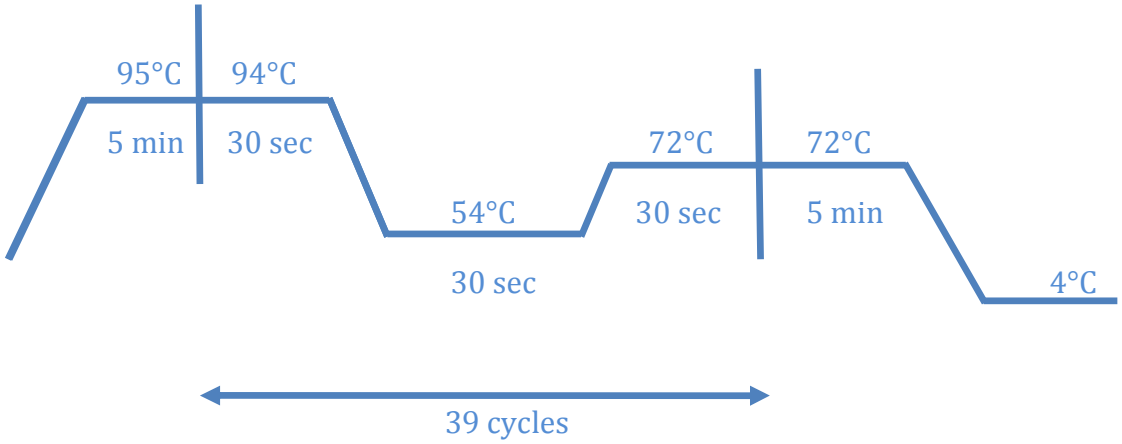


Figure 2.23: Thermal cycling profile used to amplify ETEC toxin genes

Table 2.16: Expected band size of the ETEC PCR products.	
LT	250 bp
STp	163 bp
STh	100 bp

2.6.2.1.4.6 Agarose Gel Electrophoresis and Visualization of PCR Products

Amplified PCR products were analyzed by agarose gel electrophoresis on 2% agarose gel. The gel was prepared by adding 2g Ultra pure agarose in 100 ml 1×TBE buffer (Invitogen, ultra pure) and melted at medium high temperature in a microwave oven for 3-4 minutes. 4 µL of Gel red was added to the gel, mixed well, and poured on a gel casting. 10-15 minutes were allowed to solidify the gel.

For the gel electrophoresis, 4µL of loading dye was mixed with 26 µL of PCR product and loaded into the wells. The PCR products were then separated at 150 volt for 30 minutes. The bands were observed on a Gel documentation system (BIORAD) under UV transillumination.

2.6.3 Identification and Characterization of *Salmonella and Shigella* spp.

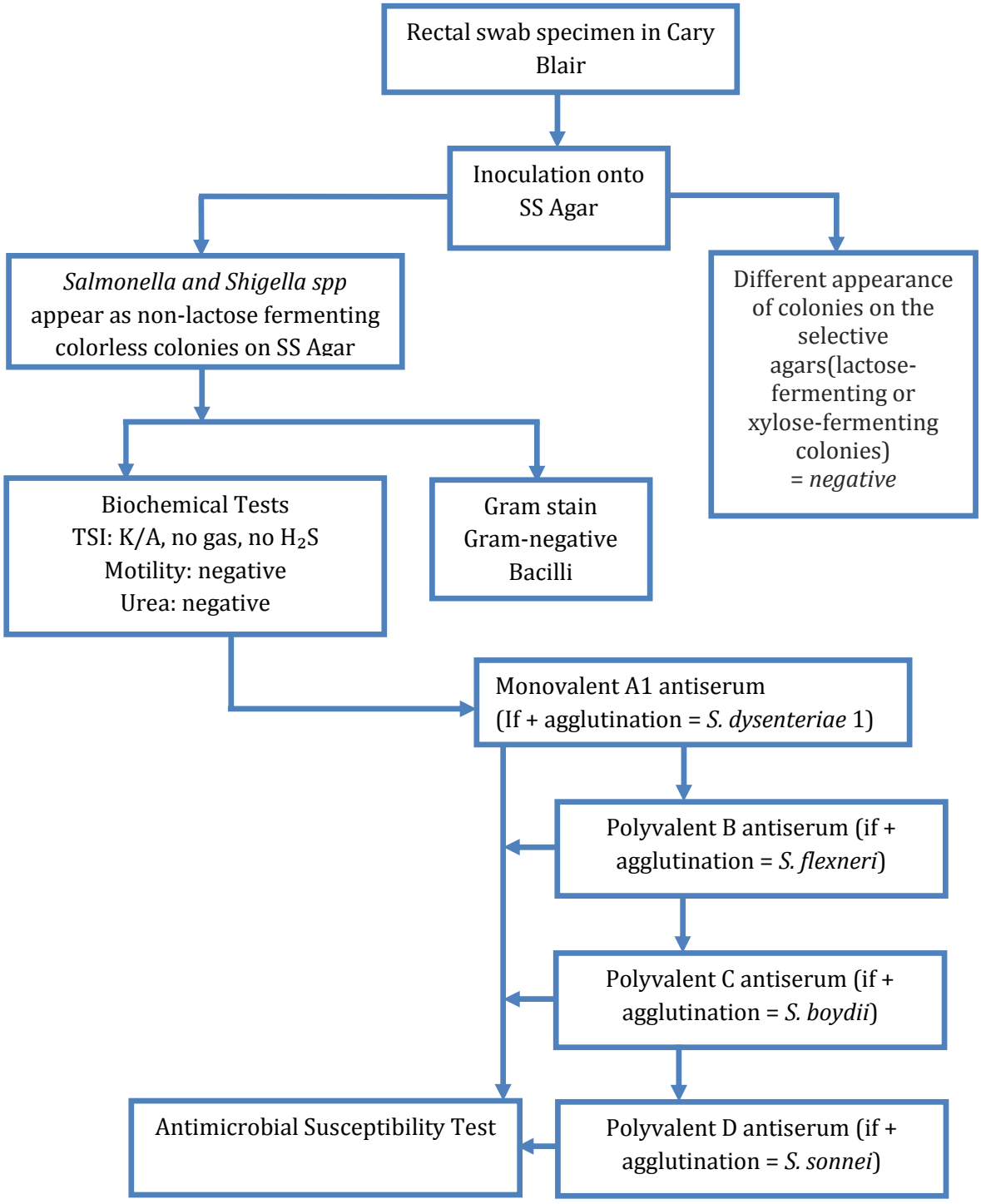


Figure 2.24: Flowchart for Identification and Characterization of *Salmonella Shigella* spp

2.6.3.1 Method

2.6.3.1.1 Inoculation and isolation of suspected *Salmonella Shigella* spp

1) Within 4-6 hours after collection, the rectal swab was taken from Cary Blair media and an area approximately 2.5 cm (1 inch) in diameter was seeded on Salmonella-Shigella Agar plates.

2) An inoculation loop was flamed and after sufficient cooling was used to streak the inoculum smeared on the Salmonella-Shigella Agar plates. The loop was flamed in between streaking to ensure dilution and proper isolation of colony forming units.

3) After streaking, the plates were covered and placed upside down in the incubator to avoid excessive condensation. The plates were incubated for 18–24 hours at 37°C in an aerophilic incubator.

2.6.3.1.2 *Salmonella-Shigella spp* Detection and Identification

1) After overnight incubation, the SS Agar growth was carefully examined for typical *Salmonella-Shigella spp* colonies.

2) *Salmonella spp.* and *Shigella spp*. are non-lactose fermenters and form colorless colonies on Salmonella Shigella Agar. H_2S positive *Salmonella spp*. produces black-center colonies.

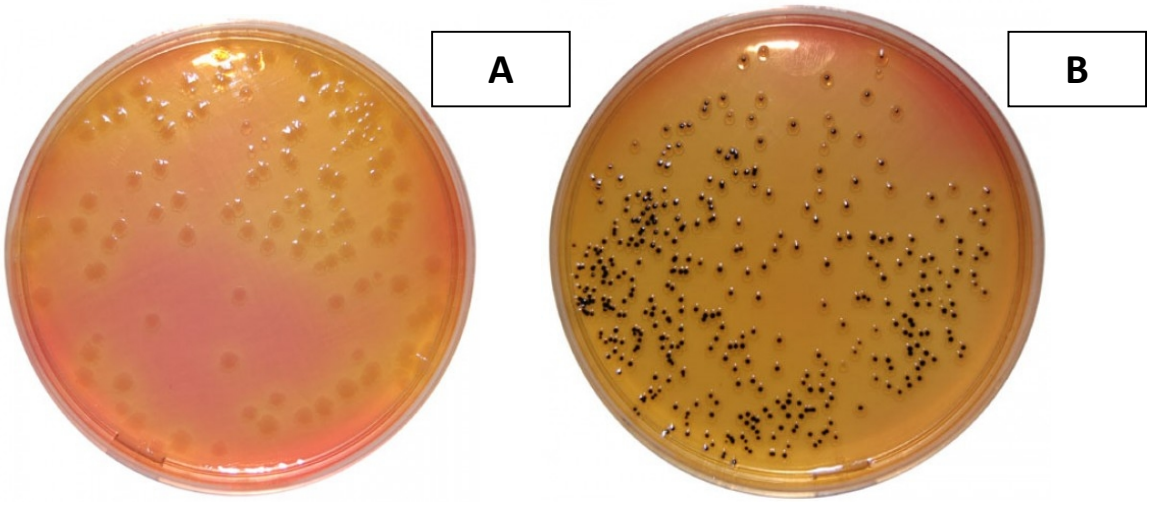


Figure 2.25: Colonies of *Shigella sonnei* (A) and *Salmonella typhimurium* (B) on SS agar plates

2.6.3.1.3 Gram Staining: Gram staining was done in the same process described in section 2.4.1.2.3.

2.6.3.1.4 Biochemical Tests

2.6.3.1.4.1 Triple Sugar Iron Agar: The Triple Sugar Iron (TSI) test was done in the same way as described in section 2.4.3.1.4.1

2.6.3.1.4.2 Motility Indole Urea:: The Motility Indole Urea (MIU) test was done in the same way as described in section 2.4.3.1.4.2

2.6.3.1.4.3 Typical Reactions of *Salmonella* and *Shigella* spp

Table 2.17: Biochemical test references for identification of *Salmonella* and *Shigella* spp.

Organism	Slant	Butt	H_2S	Motility	Indole	Urea
Shigella spp.	R	Y	-ve	-ve	-ve	-ve
Salmonella spp.	R	YG	+ve	+ve	-ve	-ve

R= Red Y= Yellow YG= Gas formed

2.6.3.1.5 Anti-Sera Test: Because *S. dysenteriae* 1 is the most common agent of epidemic dysentery (followed by *S. flexneri* and *S. sonnei*), isolates that reacted typically in the screening biochemical tests were screened first with monovalent A1 antiserum, then with polyvalent B antiserum, polyvalent C antiserum and finally in polyvalent D antiserum. When a positive agglutination reaction was obtained in one of the antisera, the *Shigella* subgroup was identified.

Table 2.18: List of antisera used for identification of *Shigella* spp.	
Subgroup A (*S. dysenteriae*)	Polyvalent A
	Polyvalent A1
Subgroup B (*S. flexneri*)	Polyvalent B
Subgroup C (*S. boydii*)	Polyvalent C
	Polyvalent C1
	Polyvalent C2
	Polyvalent C3
Subgroup D (*S. sonnei*)	Polyvalent D

The Antisera tests were done in the same process as described in sections 2.5.6.1 and 2.5.6.2

2.6.3.1.6 Antimicrobial Susceptibility Test: : The anti-microbial sensitivity testing was done in the same method but using Mueller-Hinton Agar plate as described in section 2.4.1.2.7. The following antibiotics with their concentration per disc were used for the sensitivity testing:

Table 2.19: List of Antibiotics for the AST of *Shigella* spp.	
Antibiotic	Concentration per Disc
Ampicillin (AMP)	10 µg
Cotrimoxazole (COT)	15 µg
Tetracyclin (T)	30 µg
Ciprofloxacin (CIP)	5 µg
Ceftriaxone (CRO)	30 µg
Azithromycin(AZI)	15 µg

Results

Chapter 3

3.1 Acute Respiratory Infection

3.1.1 Study Subjects

In this study, a total of 100 nasal swab specimens from <5 children suspected with acute respiratory infection seeking care in Shaheed Suhrawardy Medical College Hospital and Dhaka Medical College Hospital, Dhaka Bangladesh between September 2014- May 2015 were collected. Each of the specimens was tested for *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Haemophilus influenzae*. The antimicrobial sensitivity patterns of the isolated organisms were also determined. Apart from these, socio-demographic and clinical characteristics of study participants were also analyzed for all specimens.

3.1.2 Prevalence of Respiratory pathogens in the specimens

Table 3.1: Summary of the isolated respiratory pathogens

No. Of Patients with ARI, n	Total No. of Culture Positive Patients, n (% of positive patients)	Name of the Isolated Organisms	No. Isolated Organism		Total, n (% of isolated Organism)
			Male	Female	
n=100	n=30 (30%)	*Streptococcus pneumoniae*	11	4	n=15 (50%)
		Streptococcus species	3	3	n=6 (20%)
		Klebsiella pneumoniae	6	2	n=8 (26.7%)
		Enterobacter agglomerans	0	1	n=1 (3.3%)
		Haemophilus influenzae	0	3	n=3 (10%)

Of the 100 nasal swab specimens that were analyzed, 30% (30 of 100) came out as culture positive. Of the 30 culture positive samples, 63.3% (19 of 30) were from male patients, and 36.7% (11 of 30) were from female patients. Among the culture positive patients, 50% (15 of 30) was positive for *Streptococcus pneumoniae,* 20% (6 of 30) was positive for other *Streptococcus* spp, 26.7% (8 of 30) was positive for *Klebsiella pneumoniae,* 3.3% (1 of 30) was positive for *Enterobacter agglomerans,* and 10% (3 of 30) was positive for *Haemophilus influenzae.*

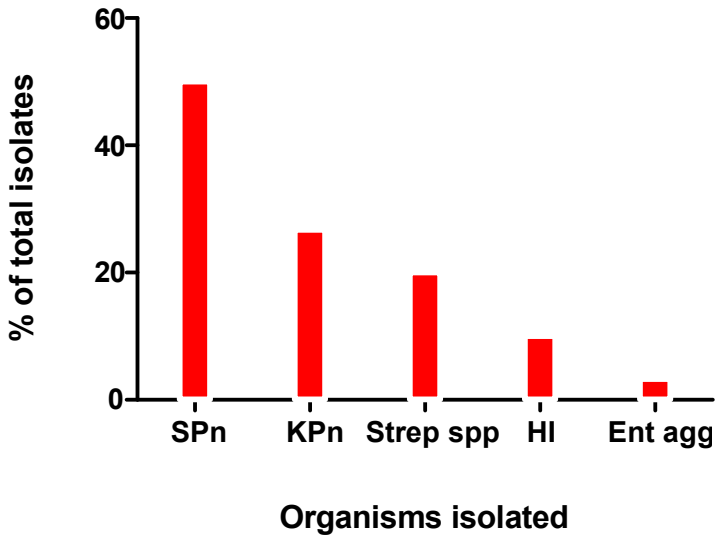


Figure 3.1: Distribution of isolated respiratory pathogens in culture positive nasal swab specimens from Shaheed Suhrawardy Medical College Hospital and Dhaka Medical College Hospital

3.1.3 Antimicrobial Susceptibility Pattern

3.1.3.1 Antimicrobial Susceptibility Pattern of *Streptococcus pneumoniae* Isolates

The susceptibility of the *Streptococcus pneumoniae* isolates to antibiotics was tested according to the Clinical Laboratory Standard Institute (CLSI) Guideline. *Streptococcus pneumoniae* was the most prevalent respiratory pathogen with a susceptibility of 100% to Ampicillin, 46.6% to Erythromycin, 40% to Azithromycin, 6.7% to Trimethoprim+ Sulphamethoxazole, 93.3% to Ceftriaxone, 80% to Cefixime, 93.3% to Levoxine, and 100% to Penicillin.

Table 3.2: Antimicrobial susceptibility pattern of *Streptococcus pneumoniae* isolates

Bacteria	Total no.	Antibiotics	AMP	E	AZI	SXT	CRO	CFM	LEV	P
S. pneumoniae	15	% sensitive	100	46.6	40	6.7	93.3	80	93.3	100

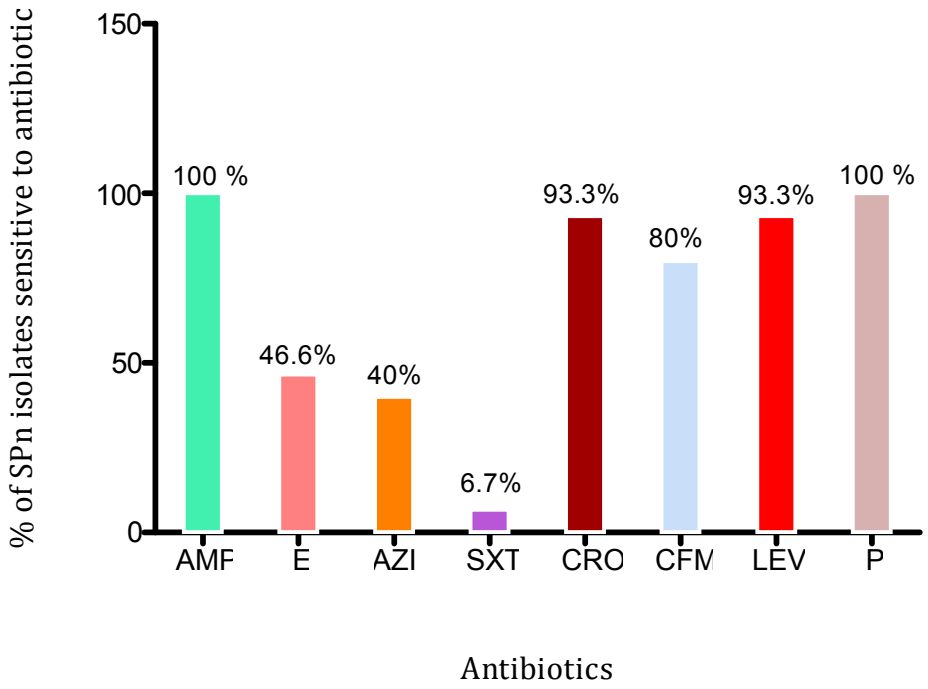


Figure 3.2: Susceptibility pattern of *S. pneumoniae* isolates by percentage to different antibiotics

3.1.3.2 Antimicrobial Susceptibility Pattern of *Streptococcus* spp Isolates

The susceptibility of the *Streptococcus spp.* isolates to antibiotics was tested according to the Clinical Laboratory Standard Institute (CLSI) Guideline. *Streptococcus* species were found to have a prevalence of 20% with a susceptibility of 83.3% to Ampicillin, 66.6% to Cefixime, 0% to Gentamicin, 100% to Ceftriaxone, 66.6% to Erythromycin, and 83.3% to Penicillin.

Table 3.3: Antimicrobial susceptibility pattern of *Streptococcus* spp. isolates

Bacteria	Total no. of isolates	Antibiotics	AMP	CFM	GN	CRO	E	P
Streptococcus spp.	06	% sensitive	83.3	66.6	0	100	66.6	83.3

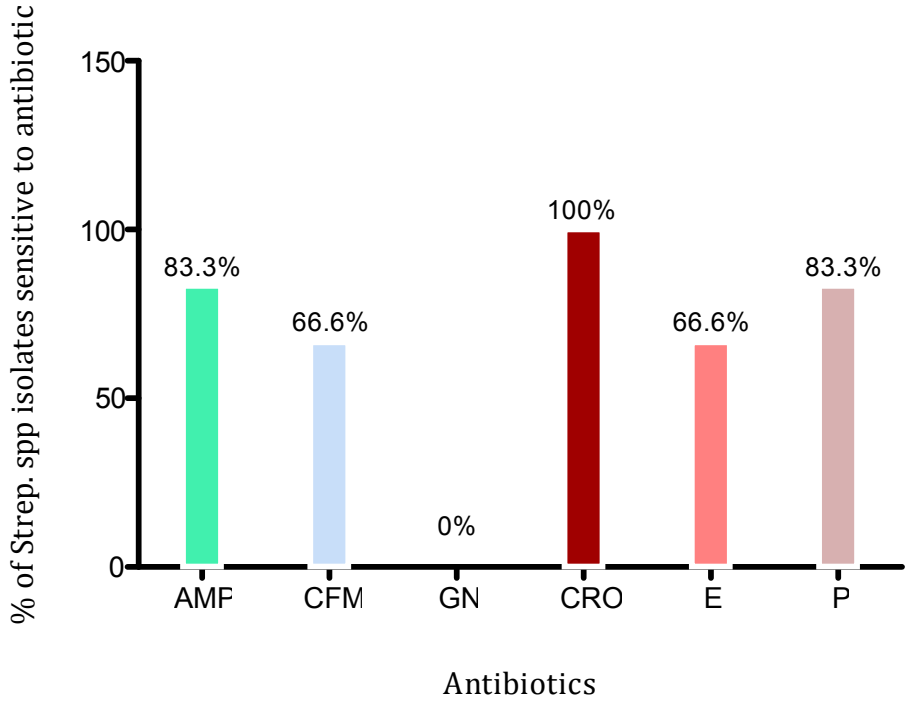


Figure 3.3: Susceptibility pattern of *Streptococcus* spp. isolates by percentage to different antibiotics

3.1.3.3 Antimicrobial Susceptibility Pattern of *Klebsiella pneumoniae* Isolates

The susceptibility of the *Klebsiella pneumoniae* isolates to antibiotics was tested according to the Clinical Laboratory Standard Institute (CLSI) Guideline. *Klebsiella pneumoniae* isolates were found to have a prevalence of 26.7% with a susceptibility of 0% to Azithromycin, 62.5% to Cefixime, 75% to Ciprofloxacin, 87.5% to Gentamicin, 62.5% to Ceftriaxone, 100% to Impenem, 100% to Meropenem, and 62.5% to Tobramycin.

Table 3.4: Antimicrobial susceptibility pattern of *Klebsiella pneumoniae* isolates

Bacteria	Total no.	Antibiotics	AZI	CFM	CIP	GN	CRO	IMP	MEM	TOB
K. pneumoniae	08	% sensitive	0	62.5	75	87.5	62.5	100	100	62.5

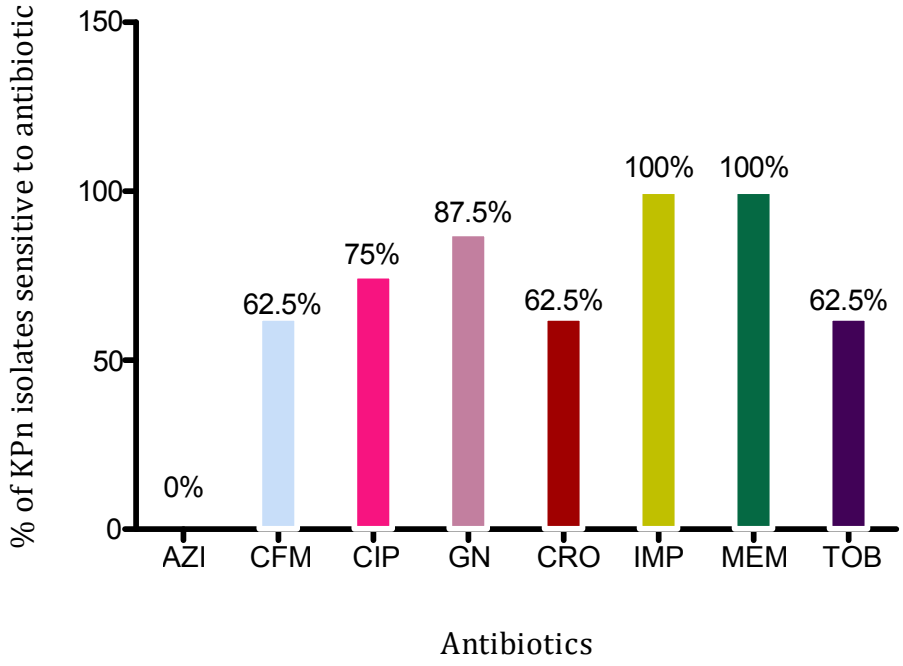


Figure 3.4: Susceptibility pattern of *Klebsiella pneumoniae* isolates by percentage to different antibiotics

3.1.3.4 Antimicrobial Susceptibility Pattern of *Enterobacter agglomerans* Isolate

From the 30 culture positive respiratory samples, one isolate of *Enterobacter agglomerans* was obtained, thus, giving it a prevalence of 3.3%. The susceptibility of the *Enterobacter agglomerans* isolates to antibiotics was tested according to the Clinical Laboratory Standard Institute (CLSI) Guideline.

Table 3.5: Antimicrobial susceptibility pattern of *Enterobacter agglomerans* isolate

Bacteria	Total no.	Antibiotics	AZI	CFM	CIP	CN	CRO	IMP	MEM	TOB
E.agglomerans	01	sensitivity/ Resistance	R	S	S	S	S	S	S	S

In the table above, R indicates resistant and S indicates sensitive.

3.1.3.5 Antimicrobial Susceptibility Pattern of *Haemophilus influenzae* Isolates

The susceptibility of the *Haemophilus influenzae* isolates to antibiotics was tested according to the Clinical Laboratory Standard Institute (CLSI) Guideline. *Haemophilus influenzae* isolates were found to have a prevalence of 10% with a susceptibility of 66.6% to Amoxicillin+ Clavulanic acid, 66.6% to Ampicillin, 100% to Azithromycin, 33.3% to Chloramphenicol, 66.6% to Ceftriaxone, and 33.3% to Trimethoprim + sulphamethoxazole.

Table 3.6: Antimicrobial susceptibility pattern of *Haemophilus influenzae* isolates

Bacteria	Total no. of isolates	Antibiotics	AMC	AMP	AZI	C	CRO	SXT
Haemophilus influenzae.	03	% sensitive	66.6	66.6	100	33.3	66.6	33.3

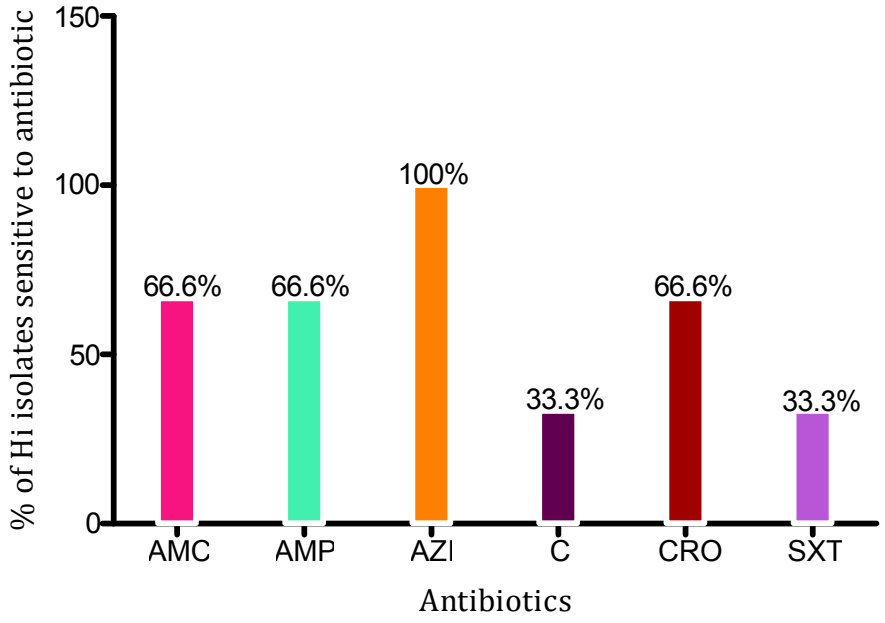


Figure 3.5: Susceptibility pattern of *Haemophilus influenzae* isolates by percentage to different antibiotics

3.1.4 Multidrug Resistant Strains of Respiratory Pathogens

3.1.4.1 Klebsiella pneumoniae

One isolate of *Klebsiella pneumoniae* obtained from a male patient was discovered to be resistant towards most of the first line antibiotics excluding Impenem (IMP) and Meropenem (MEM), and all of the second line antibiotics. The susceptibility of this *Klebsiella pneumoniae* isolate to antibiotics was tested according to the Clinical Laboratory Standard Institute (CLSI) Guideline.

Table 3.7: Antimicrobial susceptibility pattern of *Klebsiella pneumoniae* isolate												
First Line antibiotics								Second line antibiotics				
AZI	CFM	CIP	CN	Cro	IMP	MEM	TOB	AK	NET	TZP	CAR	PB
R	R	R	R	R	S	S	R	R	R	R	R	R

In the table above, R indicates resistant and S indicates sensitive.

3.1.5 Proportion of Isolates Susceptible to a Number of Antibiotics

The proportions of the isolates showing multi-drug resistance are given in Table 3.8. Only one isolate (6.66%) of *Streptococcus pneumoniae* was sensitive to all the antibiotics tested. Some 6.66% (1 of 15) of *S. pneumoniae* isolates were sensitive to 1 - 3 different antibiotics, and 93.3% (14 of 15) were susceptible to more than 3 different antibiotics. For *Streptococcus spp*., 16.7% (1 of 6) of the isolates were sensitive to 1 - 3 different antibiotics, and 83.3% (5 of 6) were susceptible to more than 3 different antibiotics. For *Klebsiella pneumoniae*, 25% (2 of 8) of the isolates were sensitive to 1 - 3 different antibiotics, and 75% (6 of 8) were susceptible to more than 3 different antibiotics. For *Enterobacter agglomerans*, none of the isolates was sensitive to 1 - 3 different antibiotics, and 100% (1 of 1) were susceptible to more than 3 different antibiotics. For *Haemophilus influenzae*, 33.3% (1 of 3) of the isolates were sensitive to 1 - 3 different antibiotics, and 66.7% (2 of 3) were susceptible to more than 3 different antibiotics.

Table 3.8: Proportion of isolates susceptible to a number of antibiotics

Bacteria	Susceptibility to a number of antibiotics			
	N	ALL	≤3	≥4
Streptococcus pneumoniae	15	1 (6.66%)	1 (6.66%)	14 (93.3%)
Streptococcus species	6	0 (0.0%)	1 (16.7%)	5 (83.3%)
Klebsiella pneumoniae	8	0 (0.0%)	2 (25%)	6 (75%)
Enterobacter agglomerans	1	0 (0.0%)	0 (0.0%)	1 (100%)
Haemophilus influenzae	3	0 (0.0%)	1 (33.3%)	2 (66.7%)
Total	33	1	5	28

3.1.6 Co-infection by Respiratory Pathogens

Of the 30 culture positive samples, three cases of bacterial co-infection were discovered. All the three samples were collected from Shaheed Suhrawardy Medical College Hospital. Two of the patients with co-infection were female and one was male. The nasal swab specimen from the male patient was culture positive for *Streptococcus pneumoniae* and *Klebsiella pneumoniae*. The remaining two specimens from the female patients were positive for *Streptococcus pneumoniae* and *Haemophilus influenzae*.

3.2 Enteric Fever

3.2.1 Study subjects

Under-five children who had had fever with ≥39°C temperature for more than three days were considered eligible for this study. A total of 47 participants were enrolled in the study.

3.2.2 Baseline Data of the Participants

The number of male patients and female patients were 29 (61.7%) and 18 (38.29%) respectively. The median age of the patients was 4 years, with a range of 0.6 to 5 years. The median of temperature was 37.75 °C. The duration of fever was 4 days at presentation. 44 of 47 (93%) patients had been treated with antibiotics before blood specimen was collected from them.

Table 3.9: Baseline data of the participants

Feature	Value
Median age in Year (25^{th}, 75^{th} percentile)	4 (1.5, 5)
No. of males (%)	28 (59.6)
No. of females (%)	19 (40.4)
Median Temperature °C (25^{th}, 75^{th} percentile)	37.75°C
Duration of fever in days at presentation (25^{th}, 75^{th} percentile)	4 (3, 5)
No. of patients administered with antibiotic prior to blood collection (%)	44 (93%)

3.2.3 Blood Culture and Antibiotic Susceptibility Test Results

Among 47 patients tested, 4 samples were culture positive. *Salmonella* Typhi was isolated from only one patient (2.1%). The patient positive for *Salmonella* Typhi was female. The remaining three culture-positive samples (6.38%) yielded Coagulase negative *Staphylococcus aureus* (CoNS). The *Salmonella* Typhi isolate was obtained from a patient who had had no antibiotic treatment prior to blood collection for the study. The susceptibility of this *Salmonella* Typhi isolate to antibiotics was tested according to the Clinical Laboratory Standard Institute (CLSI) Guideline. The antibiotic susceptibility pattern of the *S*. Typhi isolate has been tabulated below.

Table 3.10: Antimicrobial susceptibility pattern of *Salmonella* Typhi isolate

Bacteria	Total no.	Antibiotics	AMP	AZI	CFM	C	CIP	COT	CRO	NA
Salmonella Typhi	01	Sensitivity/ Resistance	S	I	S	S	S	S	S	R

In the table above, R indicates resistant, I indicates intermediate, and S indicates sensitive.

Among the 3 patients from whom CoNS was isolated, 2 (66.7%) were male and 1 (33.3%) was female. The 3 CoNS isolates were also tested for their susceptibility to antibiotics using the Clinical Laboratory Standard Institute (CLSI) Guideline. The 3 CNS isolates were found to have a susceptibility of 66.7% to Ciprofloxacin, 100% to Gentamicin, 66.7% to Cotrimoxazole, 100% to Linezolid, and 100% to Vancomycin.

Table 3.11: Antimicrobial susceptibility pattern of CoNS isolates

Bacteria	Total no.	Antibiotics	CIP	CN	COT	LZD	VAN
Coagulase-negative *Staphylococcus aureus*	03	% Sensitive	66.7	100	66.7	100	100

3.2.4 TPTest Results

The 47 blood specimens that were tested by culture were also tested for the presence of *Salmonella* Typhi and Paratyphi specific IgA immunoglobulins using ELISA, which would indicate presence of an active immune response against *Salmonella* Typhi and Paratyphi infection in the host's system. The number of samples that were positive for TPTest having a level of Typhi specific IgA >10 ELISA Units was 23 (49%). All patients with confirmed *S.* Typhi bacteremia were positive in the TPTest. Among 46 suspected of having enteric fever but blood culture negative, 22 patients were positive by the TPTest. One TPTest positive patient was bacteremic for CoNS.

Table 3.12: TPTest results		
No. of total suspected patients		47
No. of TPTest positive patients (%)		23 (49%)
No. of both Blood culture and TPTest positive patients (%)		2 (4.3%)
No. of isolated organism by blood culture	*Salmonella* Typhi	1
	Salmonella Paratyphi	0

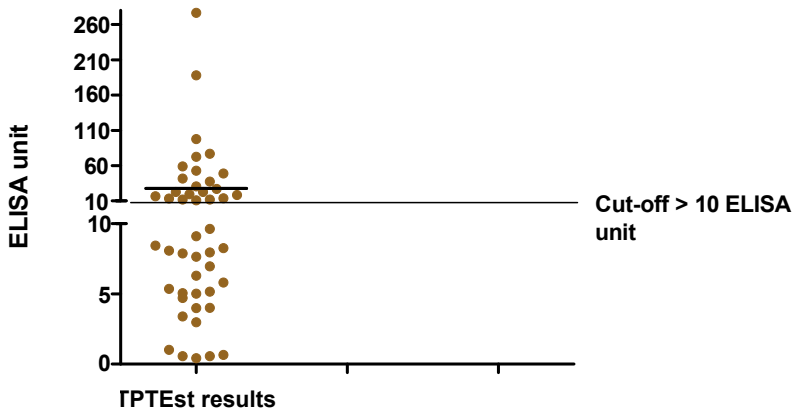


Figure 3.6: Distribution of the antibody titers from TPTest results

3.3 Diarrhea

3.3.1 Study Subjects

In this study, a total of 17 specimens from diarrheal patients under five years of age attending Shaheed Suhrawardy Medical College Hospital, Dhaka, during September 2014 to May 2015 were tested. All specimens were screened for enterotoxigenic *E. coli* (ETEC); *Vibrio cholerae* (*V. cholerae*), and *Salmonella-Shigella* spp. Socio-demographic and clinical characteristics of study participants were analyzed for all specimens.

3.3.2 Prevalence of Diarrheal pathogens in the Specimens

Table 3.13 Prevalence of diarrheal pathogens in the specimens

No. of Diarrheal Patients	No. of Culture Positive Patients, n (%)	Name of the Isolated Organisms		No. Isolated Organism		Total (% of isolated organism)
				Male	Female	
n=17	n=5 (29.4%)	Enterotoxigenic *E.coli*	STp	0	1	n=3 (60%)
			STh	2	1	
		Vibrio cholerae		1	0	n= 1 (20%)
		Shigella dysenteriae		1	0	n=1 (20%)

Of the 17 stool specimens that were analyzed, 29% (5 of 17) came out as culture positive. Of the 5 culture positive samples, 60% (3 of 5) were from male patients, and 40% (2 of 5) were from female patients. Among the culture positive patients, 60% (3 of 5) was positive for Enterotoxigenic *E.coli,* 20% (1 of 5) was positive for *Vibrio cholerae*, and 20% (1 of 5) was positive for *Shigella dysenteriae***.**

3.3.2 Enterotoxigenic *Escherichia coli* (ETEC)

3.3.2.1 Prevalence of ETEC

About 17.6% (3 of 17) of all specimens were found positive for ETEC. Of the 3 patients who were positive for ETEC infection, 2 were female and 1 was male.

3.3.2.2 Molecular Toxin Profiling of ETEC Isolates

E. coli isolates from the stool specimens were tested for toxin production. Heat labile enterotoxin (LT) and heat stable enterotoxin (ST) gene specific primers were used for PCR detection of the toxin genes. Multiplex PCR was used for simultaneous detection of the gene *eltB* (LT) and the ST variants *st1* (STp) and *estA* (STh) more specifically. Of the 3 *E.coli* isolates 33.3% (1 of 3) was positive for the heat stable enterotoxin STp, 100% (3 of 3) was positive for heat stable enterotoxin STh, and 33.3% (1 of 3) was positive for both STp and STh. None was positive for LT.

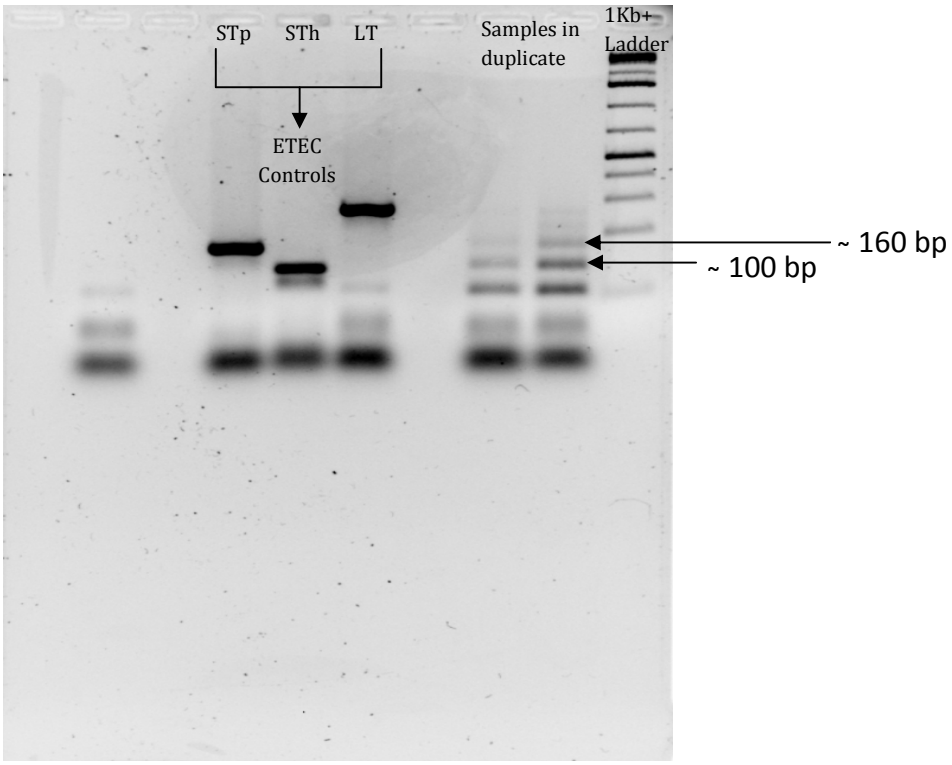


Figure 3.7: Gel electrophoresis of ETEC PCR product

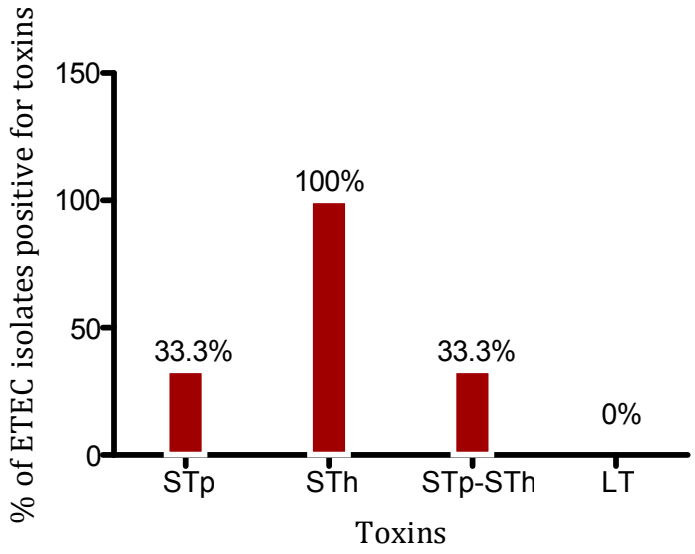


Figure 3.8: Toxin profile of ETEC isolates by percentage of total isolates

3.3.3 *Vibrio cholerae*

3.3.3.1 Prevalence of *Vibrio Cholerae*

About 5.8% (1 of 17) of all specimens were found positive for *Vibrio cholerae.* The isolate was obtained from a male patient.

3.3.3.2 Serotype and Biotype of the Isolate

The serotype of the isolate was determined to be Ogawa, and the biotype was determined to be El Tor

3.3.4 *Salmonella* and *Shigella* spp

3.3.4.1 Prevalence of *Salmonella* and *Shigella* spp

From the 17 specimens that were tested, 1 yielded an isolate of *Shigella dysenteriae* polyvalent A1. No *Salmonella* isolates were found. The prevalence was 5.8%. The isolate was obtained from a male patient.

3.3.4.2 Antimicrobial Susceptibility Pattern of the *Shigella dysenteriae* Isolate

The susceptibility of the *Shigella dysenteriae* isolate to antibiotics was tested according to the Clinical Laboratory Standard Institute (CLSI) Guideline. The antibiotic susceptibility pattern of the *Shigella dysenteriae* isolate has been tabulated below.

Table 3.14: Antimicrobial susceptibility pattern of *Shigella dysenteriae* isolate

Bacteria	Total no.	Antibiotics	AMP	AZI	CIP	COT	CRO	T
Shigella dysenteriae	01	Sensitivity/Resistance	R	S	S	S	S	S

In the table above, R indicates resistant, and S indicates sensitive.

Discussion

Chapter 4

4.1 Acute Respiratory Infection:

Despite the progress that Bangladesh has made in fulfilling the millennium development goal 4 of reducing under-five mortality rate by two-third by 2015, acute respiratory infections (ARI) remain the leading cause of death in this age group. Report by (Ahmad, Lopez and Inoue, 2000); and (Baqui, et al. 2001) suggest that Pneumonia is the leading cause for 90% of all childhood and infant death in Bangladesh. Regardless of this rather striking statistics, information about the true burden of acute respiratory infection on under-five children in Bangladesh is quite limited. Traditionally respiratory tract infections have been diagnosed and treated through clinical and radiological findings. However, in the recent decades, over-usage and over-exposure of antibiotics have led to an emergence of increased antibiotic resistance amongst major pathogens. This emerging antibiotic resistance has rendered traditionally used antibiotics ineffective for many respiratory tract pathogens, hence compromising their treatment. These circumstances call for definitive bacteriological diagnosis and testing susceptibility of isolated pathogens to antimicrobial agents for an effective management of ARI, and reduction of under-five mortality and morbidity by ARI. Therefore, this study aimed at filling in information on the actual burden of major bacterial respiratory pathogens and their antimicrobial sensitivity pattern among under-five children in two hospital based urban settings in Dhaka, Bangladesh.

A total of 100 patients were included in the study. Of the samples analyzed, some 30 bacteria of various species were isolated, giving a prevalence rate of 30%, which is similar to what was reported in a similar study in India. A community-based prospective study investigating incidence, pattern, and severity of Acute Respiratory Infections among infants and toddlers of a peri-urban area of Delhi reported prevalence of ARI to be 34.3% (Walke et al., 2014). The aetiology of the respiratory infection in culture negative cases can be attributed to viral pathogens. The culture positive cases consisted of 63.3% from male patients and 36.7% from females. Samples obtained from male subjects yielded more bacteria than those obtained from females. The bacteria isolated from the samples included *Streptococcus pneumoniae* (50%), *Streptococcus spp* (20%),

Klebsiella pneumoniae (26.7%), *Haemophilus influenzae* (10%), and *Enterobacter agglomerans* (3.3%). These isolates clearly represented clinically significant pathogens, and are similar to the results reported in Nigeria (Olugbue, and Onuoha, 2011) and also falls within the range of frequencies reported in other countries such as Iran (Imani, Rouhi, and Ganji, 2007).

All the isolates exhibited variable susceptibility to the antimicrobials tested. The sensitivity tests also indicated that the isolates were resistant to one or more antibiotic. The most effective antimicrobials for *Streptococcus pneumoniae* were Ampicillin (100%), Penicillin (100%), Ceftriaxone (93.3%), Levoxin (93.3%), Cefixime (80%), Erythromycin (46.6%), and Azithromycin (40%), while the least effective was Trimethosporine+ Sulphamethoxazole (6.7%). The *Streptococcus* spp. isolates were most susceptible to Ceftriaxone (100%), Ampicillin (83.3%), Penicillin (83.3%), Cefixime (66.6%), and Erythromycin (66.6%), but were resistant to Gentamicin. *Klebsiella pneumoniae* isolates were found to have maximum susceptibility to Impenem (100%), Meropenem (100%), Gentamicin (87.5%), Ciprofloxacin (75%), Cefixime (62.5%), Ceftriaxone (62.5%), and Tobramycin (62.5%), and complete resistance to Azithromycin (0%). The single *Enterobacter agglomerans* isolate when tested for the same array of antibiotics as *K. pneumoniae* isolates was susceptible to all the antibiotics except Azithromycin. The *Haemophilus influenzae* isolates had the highest susceptibility to Azithromycin (100%), Amoxicillin+ clavulanic acid (66.6%), Ampicillin (66.6%), Ceftriaxone (66.6%) and the least susceptibility to Chloramphenicol (33.3%), and Trimethosporine+ Sulphamethoxazole (33.3%).

The sensitivity patterns of respiratory pathogens to antimicrobials obtained from this study did not concede with the findings reported in other similar studies outside Bangladesh including, Nigeria (Olugbue, and Onuoha, 2011), Pakistan (Jafari et al., 2009). The findings of this study suggest that some of the antibiotics that are commonly used to treat acute respiratory infections are still effective. The results show that antibiotic resistance in this region is relatively low compared with other countries

During this study, one isolate of *Klebsiella pneumoniae* was found which was resistant to most of the first line and all of the second line antibiotics it was tested for. It was found sensitive only for the antibiotics Impenem and Meropenem. Emergence of such multi-drug resistant strains is a matter of concern. Several different mechanisms exist that impart antimicrobial resistance in bacterial pathogens causing acute respiratory infection. These include presence of lactamases and the ability to exchange resistance markers like plasmids and transposons (Aydemir et al., 2006; Jonaidi et al., 2009). Redundant usage and overexposure of antibiotics to bacterial pathogens have been known to contribute significantly in development of antibiotic resistance in bacteria (Mincey and Parkulo, 2001). However, there have been arguments that the emergence of resistance to a particular antibiotic doesn't always represent the antibiotic consumption in a given region. Rather, in addition to selection pressure created by overconsumption of antibiotics, horizontal transfer of gene also acts as a significant cause for development of resistance in pathogenic bacteria (Brown et al., 2005).

This study provides valuable information about the antimicrobial susceptibility pattern of common ARI pathogens in under-five children in Dhaka, which is unique and is custom suited for the pathogens prevalent in the local population. This information can help guide medical professionals regarding use of appropriate antibiotics in management of ARI in under-five children. However, a larger study where more children can be enrolled would be important for assessing the prevalence more accurately.

4.2 Enteric Fever:

A fever surveillance study conducted in an urban area in Kamalapur, Dhaka identified children under-five years of age to be at least 8.9 times more prone towards developing bacteremic enteric fever compared to any other age groups (Brooks et al. 2005). However, no comprehensive data on the burden of enteric fever of under-five children in Bangladesh currently exists. A major reason for this void of information is the absence of

an optimal assay for diagnosing acute enteric fever in patients and determining its burden within a region. Use of microbiological culture and nucleic acid level detection has their limitations due to the very low count of organism in peripheral blood, antibiotic consumption prior to collection of blood for culture, and serodiagnostic approaches in endemic zones are often plagued by the very probable likelihood of prior exposure to the pathogens. Sheikh et al. 2009, and Khanam et al. 2013 reported development of a novel immunodiagnostic assay for early diagnosis of enteric fever, the TPTest (Typhoid Paratyphoid Test). This test utilizes the detection of *Salmonella* Typhi and Paratyphi specific mucosal immune response in form of IgA immunoglobulins to diagnose an acute infection. Immediately after an infection, activated mucosal lymphocytes migrate temporarily to the peripheral blood circulation from where they can be separated and cultured in-vitro to harvest their culture supernatant. This supernatant also termed as antibody in lymphocyte supernatant (ALS) is then used for analyzing IgA immunoglobulins against membrane preparation (MP) of *Salmonella* Typhi and Paratyphi by ELISA within 24-48 hours. The assay has a sensitivity of 100% with a specificity of 78-97%, and requires a small volume of blood. Using this novel immunodiagnostic assay and the conventional blood culture method the enteric fever part of this study was conducted to determine the burden of enteric fever in under-five children in two hospitals in Dhaka.

A total of 47 patients clinically suspected of typhoid fever were enrolled into the study. Of the 47 samples tested, only 4 were culture positive, and among them only one was positive for *Salmonella* Typhi, giving a prevalence of 2.1%. The remaining three were culture positive for Coagulase negative *Staphylococcus aureus.* On the other hand, TPTest results of the 47 clinical suspects showed 23 positive results, giving a prevalence of 49%. The only sample that was culture positive for *Salmonella* Typhi was also positive for TPTest. Such massive difference in the blood culture and TPTest results can be attributed to the administration of antibiotics in the clinical suspects participating in the study as part of their treatment regimen prior to collection of blood specimens for culture. The clinical evaluation data of the study participants reveal that 93% (44 of 47)

of the participants had been treated with antibiotics prior to blood collection, and that in 84% (37 of 44) of the cases the preferred antibiotic used was Ceftriaxone, which has been shown to be effective against the only *Salmonella* Typhi isolate obtained from this study. The only patient who was positive for *Salmonella* Typhi in blood culture had had no exposure to antibiotics prior to blood collection. So, while blood culture is dependent upon the presence of organism in peripheral blood and is sensitive to antibiotic consumption, TPTest targets an entirely different marker of *Salmonella* Typhi and Paratyphi infection, the host's immune response. The CoNS isolates were probably resulted from improper sterilization of skin surface before intravenous blood collection, as CoNS are part of the normal flora of human skin and risk factors for their infection include presence of foreign devices like IV needles.

The findings of this study thus lend support to findings reported by (Sheikh et al. 2009); and (Khanam et al. 2013, Khanam et al. 2015). The TPTest being an immunological detection method of *Salmonella enterica* Serovar Typhi and Paratyphi infection holds promises in becoming the basis of an improved diagnostic assay for typhoid, while its high specificity and sensitivity also makes it a prime candidate for using in surveillance studies. However, the isolation of a single organism from the study due to prior consumption of antibiotics prevents us from determining a statistically significant antimicrobial susceptibility pattern of the prevalent pathogens of enteric fever in under-five children.

4.3 Diarrhea:

For the diarrheal part, a total of 17 diarrheal patients were inducted in the study. Rectal specimens from all the participants were tested for ETEC, *Vibrio cholerae*, and *Salmonella and Shigella* spp. Of the 17 patients tested, 5 (29.4%) were found culture positive for diarrheagenic pathogens. Enterotoxigenic *Escherichia coli* (ETEC) was found to be the most prevalent with 3 isolates separated, giving it a prevalence of 60%. Molecular analysis of toxin genes of the ETEC isolates revealed one to be positive for

both heat stable enterotoxin STp and STh, and remaining two to be positive for STh only. Only one isolate of V. cholerae and Shigella dysenteriae was found.

4.4 Limitations of the Study:

This study has a number of drawbacks. This study does not explore the viral aetiology of acute respiratory infection which is a major contributor of respiratory infections in under-five children. The antibiotic consumption by majority of the enteric fever suspected patients likely affected their likelihood of blood culture positivity as well as the host–pathogen interactions that could have occurred during infection including antibody responses. The very small sample size for the diarrheal part of the study most likely affects the statistical significance of the results. The site selection for diarrheal sample collection was probably not appropriate.

4.5 Future Work Plan:

Further works in the foreseeable future regarding this project will include:

1) Investigating the viral aetiological agents of acute respiratory infection in under-five children using real-time PCR.

2) Investigating the molecular biological basis of multi-drug resistance in the multi-drug resistant *Klebsiella pneumoniae* strain discovered.

3) Carrying out a more comprehensive analysis with involvement of more hospitals and a larger enrollment of patients.

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Chapter 5

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Appendix A

Laboratory Apparatus

1. Eppendorf tubes and micropipette tips were taken from Eppendorf® and Sigma, and were sterilized by autoclaving at 121°C for 20 minutes.

2. Petri dishes used in the experiments were provided by either Sterilin or Gibco. Screw capped tubes and other glass wares were taken from Pyrex® Labware, USA.

3. Plastic tubes and pipettes were of Falcon®; both were the brands of Becton, Dickinson and Company. 96-well ELISA plates were obtained from Nunc™, Sweden.

4. Micropipettes were from Thermo Labsystems.

5. Mini scale centrifugations were carried out in a Sorvall® *pico* microfuge and large-scale centrifugation were carried out in a Sorvall® Legend™ X*RT* super speed centrifuge. ELISA reading was taken using Biotek EON reader.

6. Heparin-coated sterile vacutainer tubes (Becton Dickinson, Ruther-ford, NJ)

7. Multi-channel dispenser (Lab System, USA)

Appendix B

Chemicals/Reagents:

1. Acetic acid, Sigma-758-12-3

5. FBS (Foetal Bovine Serum Albumin), Gibco BRL- 16140-071

6. Ficoll, Pharmacia LKB Biotechnology AB Uppsala, Sweden.

8. Rabbit anti-human IgA-HRP, Southern Biotechnology Associates, Inc. 2050-05

9. H2O2 (Hydrogen Peroxide), Fisher Scientific, H-325

10. Na-acetate, Sigma-127-09-3

11. NaHCO3 (Sodium bi-carbonate), Fisher Scientific- S233-500

12. NBT (Nitro blue tetrazolium), BioRad, 170-6532

13. NaCl (Sodiun Chloride) *Fischer Scientific, Pittsburgh, PA, USA)*

APPENDIX C

Buffers and Substrate Solutions

1. Preparation of phosphate buffered saline (PBS) (Vacutainer System; Becton Dickinson, Rutherford, NJ) (pH 7.2)

NaCl *(*0.136mM *Fischer Scientific, Pittsburgh, PA, USA*) ----------80.00 g

Na2HPO4----------27.50 g

KH2PO4 ----------2.75 g

KCL (*Fischer Scientific, Pittsburgh, PA, USA*) ----------2.00 g

Deionized water ----------1000.0 mL

The concentrated solution (10xPBS) was diluted ten times and was used as working solution.

2. Preparation of 0.05% PBS-Tween (1000ml)

Tween ----------0.5 mL

PBS ----------1000 mL

3. Preparation of 1% BSA in PBS (500 ml)

Phosphate Buffer Saline (PBS) ----------500 mL

Bovine Serum Albumin (BSA) ----------5 g

5. Preparation of 0.1% BSA in PBS-Tween (500 ml)

Phosphate Buffer Saline (PBS) ----------500 mL

Bovine Serum Albumin (BSA) ----------0.5 g

Tween ----------250 µL

6. Preparation of Sodium citrate buffer (pH 4.5) (1000ml)

Tri-natrium citrate ($Na_3C_6H_5O_4.2H_2O$) ----------29.4 g

H_2O (deionized) ----------1000.0 mL

7. Preparation of Orthophenylene diamine - H2O2 substrate (10 ml)

OPD----------10.0 mg

0.1 M sodium citrate (pH 4.5) ----------10.0 mL

30% H_2O_2----------4.0 µL

14. Preparation of trypan blue (100ml)

NaCl ----------0.81 g

KH_2PO_4 ----------0.06 g

Tryphan blue ----------0.4 g

Deionized water ----------100.0 mL

APPENDIX D

Media

1. Preparation of RPMI complete medium (220 ml)

RPMI 1640 (1X) ----------200 mL

Fetal Bovine Serum (FBS- 10%) ----------20 mL

Pen/Strp (Penicillin-Streptomycin- 1%) ----------2 mL

Pen-10,000 IU/mL, Strep-10,000 ug/mL

Na-Pyruvate 100 mM (1%) ----------2 mL

L- Glutamine 200 mM (1%) ----------2 mL

2. Preparation of TTGA media (100 ml)

Tryptone----------1 g

Sodium chloride----------1 g

Sodium choleate----------0.5 g

Sodium carbonate anhydrous ----------0.1 g

Sodium hydroxide----------200 µl

Gelatin----------3 g

Agar ----------1.5 g

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

After autoclaving, addition of 1 ml Potassium tellurite

3. Preparation of TCBS media (100 ml)

TCBS powder----------8.8 g

Deionized water

4. Preparation of SS agar media (100 ml)

SS agar powder----------6.302 g

Deionized water

5. Preparation of MacConkey agar media (100 ml)

MacConkey agar powder----------5 g

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

6. Preparation of Mueller Hinton Broth (100 ml)

Mueller Hinton powder----------2.1 g

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

7. Preparation of Mueller Hinton agar (100 ml)

Mueller Hinton powder----------1.5 g

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

8. Preparation of Gelatin agar (1000 ml)

Peptone----------4 g

Yeast extract----------1 g

Gelatin powder----------15 g

Sodium chloride----------10 g

Agar----------15 g

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

9. Alkaline Peptone water (1000 ml)

Peptone----------10 g

Sodium chloride----------10 g

Sodium choleate----------5 g

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

10. Preparation of STGG media (100 ml)

Skim milk----------2 g

Tryptic Soy Broth----------3 g

Glucose----------0.5 g

Glycerol----------10 ml

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

11. Preparation of Cary-Blair media (1000 ml)

Cary-Blair (dehydrated) ----------12.6 g

1% calcium chloride----------9 ml

Autoclaved at 121°C for 15 min 1 atmospheric pressure

12. Preparation of Blood Agar media (1000 ml)

Blood agar powder----------40 g

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

After the media comes to 50°C, defibrinated sheep blood is added

13. Preparation of Chocolate Agar media (1000 ml)

Blood agar base----------40 g

Hemoglobin----------10 g

Autoclaved separately at 121°C and 15 lbs for 15 min

Polyvitex (BioMerieux) ----------10 ml

14. Preparation of Tryptic Soy Broth (1000 ml)

TSB powder----------30 g

Deionized water

Autoclaved at 121°C and 15 lbs for 15 min

15. Preparation of Blood culture media

TSB----------3%

Sodium polyanethole sulfonate ----------0.025%

Autoclaved at 121°C and 15 lbs for 15 min

16. Preparation of SS agar media (1000 ml)

SS agar powder----------60 g

Heated with frequent agitation and boiled for completely dissolving

No autoclaving required.

Appendix E

Reference strains for ETEC PCR

Strains	Toxin types
E. coli ST 64111	STh+
E. coli 286C2	LT+
E. coli 195	STp
E. coli VM 75688	LT+, STh+
E. coli E34420C	ST-, LT-

Appendix F

Softwares

1. MS word
2. Microsoft Excel
3. Endnote 1
4. Eon 1
5. Graph Pad Prism 5.0