ISOLATION AND IDENTIFICATION OF *SALMONELLA* AND *SHIGELLA* SPECIES COLLECTED FROM HUMAN STOOL AT DINAJPUR GENERAL HOSPITAL, BANGLADESH.

A THESIS

BY

ABDIWAHAB AHMED

REGISTRATION NUMBER: 1905305

SEMESTER: JANUARY-JUNE, 2020

SESSION: 2019

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

JUNE, 2020

DEDICATED

TO MY

BELOVED WIFE

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The Author

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ABSTRACT

Salmonellosis and Shigellosis bear the potential threat for human health. Outbreaks of Salmonellosis and Shigellosis remain serious health problems worldwide. Therefore, the present study was conducted to isolate and identify *Salmonella* and *Shigella* species from stool samples of human patients. A total of 33 samples were collected from the Dinajpur General Hospital in Bangladesh and brought to the laboratory of Microbiology, Faculty of Veterinary and animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur. This study was performed by morphological, cultural, biochemical test, serological test. In addition, antibiotic sensitivity was evaluated by disc diffusion method during the period from January 2020 to June 2020.

The present study was obtained that the percentage of positive cases of *Salmonella* was 52.7% while the percentage of positive cases of *Shigella* was 28.6%. The study revealed that the prevalence of *Salmonella* and *Shigella* isolated from human stool samples related to male and female was (57.1% and 44.4%) respectively. The current study indicated that the male was higher prevalence than the female. Out of 17 patients 6 were positive and others were negative according to their age, the age of these participants were ranged 5-15 years found (28.5%) (n=2) and (14.2%) (n=1) and 16-45 years of age obtained of result (30%) (n=3) and (0.0%) (n=0) was found positive in *Salmonella* and *Shigella* respectively. Also the overall prevalence related to age was (42.7% and 30%) of *Salmonella* and *Shigella* respectively. *Salmonella* species isolated, out of 33 patients were susceptible to Gentamicin, Cefixime and Chloramphenicol antibiotics. The remaining patients displayed resistant to Cefoxitin, Erythromycin, Azithromycin, Amoxicillin, Tetracycline and Ampicillin while intermediate to Levofloxacin. On the other hand, *Shigella* species were sensitive to Gentamicin, Azithromycin and Chloramphenicol. The isolates were resistant to Cefoxitin, Erythromycin, Levofloxacin, Amoxicillin, Tetracycline and Ampicillin while intermediate to Cefixime. The present study showed that the prevalence of *Salmonella typhi*, *Salmonella paratyphi A* and *Salmonella paratyphi B* using qualitative slide agglutination reaction results of Widal test was 39.4%, 12.1% and 6.1%, respectively.

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LIST OF ABBREVIATION AND SYMBOLS

%	Percentage
/	Per
_	Negative
+	Positive
<	Less than
>	Greater than
≥	Greater than or equal
µg	Microgram
^{0}C	Degree Celsius
A	Acid
AAP	American Academy of Pediatrics
AIDS	Acquired Immune Deficiency Syndrome
BAM	Bacteriological Analytical Manual.
BGA	Brilliant green agar
BSAC	British Sub-Aqua Club
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
e.g.	Example
EI	Extra Intestinal
et al.	Associated
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration

LIST OF ABBREVIATION AND SYMBOLS (Contd.)

G	Gas
gm	Grams
$H_2 O_2$	Hydrogen Peroxide
$H_2 S$	Hydrogen Sulfide
HE	Hektoen enteric
HIV	Human immunodeficiency virus
HSTU	Hajee Mohammad Danesh Science and Technology University
K	Alkaline
KOH	Potassium Hydroxide
LPS	Lipopolysaccharide
MCA	MacConkey Agar
Min	minute
MIU	Motility Indole Urease
ml	Milliliter
MOH	Ministry of Health
MR	Methyl Red
N	Number
NA	Nutrient Agar
No.	Number
NTS	non-typhiodal Salmonella
pH	Power of Hydrogen
PHEM	Public Health Emergency Management

LIST OF ABBREVIATION AND SYMBOLS (Contd.)

PVT-Ltd	Private Limited
spp.	Species
Sq	Square
SS	Salmonella Shigella
SSA	Salmonella-Shigella agar
Stx	Shiga toxin
TSI	Triple Sugar Iron
UK	United Kingdom
US	United State
VP	Voges Proskauer
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

CHAPTER 1

INTRODUCTION

Salmonella and *Shigella* species are one of the most important pathogens in the family *Enterobacteriaceae*. *Salmonella* and *Shigella* organisms are responsible for causing an acute and chronic infection of the intestine which is known as Salmonellosis and shigellosis respectively (Galan *et al.*, 1992). Salmonellosis and shigellosis are global human health problems, especially in developing countries such as Bangladesh, where substandard hygiene and unsafe water supplies prevail which is aggravated by multidrug resistance determined the prevalence and antimicrobial susceptibility patterns of *Salmonella* and *Shigella* isolates among diarrheic patients, which helps in disease management by showing the disease burden and allowing for selection of appropriate antibiotics for empiric treatment in rural communities of resource limited countries such as Bangladesh as well, *Salmonella* and *Shigella* species cause a significant amount of morbidity in and mortality in rural communities (Dekker and Frank, 2015).

According to the latest nomenclature, which reflects recent advances in taxonomy (Grimont and Weill, 2007), the genus *Salmonella* consists of only two major species: *S. enteric* and *S. bongori*. *Serotypes typhi* (*S. typhi*), *S. paratyphi* are highly adapted to human. *S. typhi* and *S. paratyphi* have humans as their main reservoir and enteric fever (typhoid and paratyphoid fever) as their most important clinical manifestation. Enteric fever continues to be an important cause of morbidity and mortality in developing countries. Although, primarily intestinal bacteria, salmonellae are wide spread in the environment and commonly found in farm effluents, human sewage and in any material subject to faecal contamination. Typhoid fever is a life threatening illness caused by the bacterium *Salmonella typhi* and was observed by Eberth (1880) in the mesenteric nodes and spleen of fatal cases of typhoid fever. Food borne diseases are common in developing countries including Bangladesh due to the prevailing poor food handling and sanitation practices, inadequate food safety laws, weak regulatory systems, lack of financial resources to invest in safer equipment and lack of education for food handlers (Gonzalez-Escobedo, 2013).

Globally, food borne diseases remain a major public health problem. The problem is severe in developing countries due to difficulties in securing optimal hygienic food handling practices. Data reported that an estimated 70% of cases of diarrheal diseases are

associated with the consumption of foods contaminated with pathogenic microorganisms. Among these microorganisms *Salmonella* and *Shigella* are the major ones. Moreover, Congress of the International Association of Microbiologists *Shigella* Commission that *Shigella* be adopted as the generic name and that species subgroups be designated A (*Shigella dysenteriae*), B (*S. flexneri*), C (*S. boydii*) and D (*S. sonnei*) (Girma, 2015).

Acute gastroenteritis is one of the leading causes of illness and death in infants, children, immuno-compromised and aged individuals throughout the world, especially in developing countries. Asia, Africa and Latin America, had an estimated 2.5 million deaths each year in children less than 5 years of age. Among the enteric pathogens *Salmonella* and *Shigella* species are of particular concern as causes of enteric fevers, food poisoning and gastroenteritis. Although more prevalent in developing countries, shigellosis is a worldwide problem with *Shigella sonnei* in predominating in Europe and US and *Shigella flexneri* more prevalent in Asian and African countries (Mengistu *et al.*, 2014).

Salmonellosis and shigellosis public health sector is a growing concern day by day throughout the world. In Bangladesh, there is limited information among *Salmonella* and *Shigella* isolated from stool and there is lack of sufficient and gap on studies emphasizing isolation and characterization of *Salmonella* and *Shigella* considering human stool in Bangladesh. This type of work is rare in Bangladesh and therefore it will improve our understanding about the exposure of various population groups to this microorganism and their response against it. Therefore, present study was conducted to isolate and identify Salmonella and Shigella from human patients as well as antibiotic sensitivity test was performed. Our study will help to choose better antibiotic for clinically diagnosed patients.

Research Objectives:

- To isolate and identify *Salmonella* and *Shigella* species from human stool samples at Dinajpur General Hospital.
- To estimate the prevalence of *Salmonella* and *Shigella* species from human stool collected different patients at Dinajpur General Hospital.
- To determine the antimicrobial sensitivity of isolated pathogen.

CHAPTER 2

LITERATURE REVIEW

2.1. Isolation of *Salmonella*

Muthoni (2016) detailed that bacterium, *Salmonella typhi,* is known to cause typhoid fever, a life threatening illness. Annually, typhoid fever is known to record cases estimated to be between 13-16 million, which result in 216,000 deaths. The most affected are children and young adults between the ages of 5 and 19 years. As of 2010, these cases caused about 190,000 deaths, a rise from 137,000 recorded in 1990. In Kenya, typhoid incidence rate of 39/100,000 have been reported but these figures may be underestimates because facilities capable of performing the blood culture tests essential for diagnosis are absent from many regions. A study carried out by Breiman *et al.* (2012) found out that typhoid incidence rate is higher in urban slums than in rural areas. The rates of typhoid fever among children aged 2 to 4 years were 15 times higher in Kibera (urban slum) than in Lwak (rural setting) for the same age group. This can be linked to overcrowding and suboptimal access to safe water, sanitation and proper hygiene. In Bangladesh, multidrug *Salmonella typhi* isolates from adults and school age children associated with sporadic outbreaks in resource poor settings especially in slum areas have been reported.

Barrett *et al.* (2013) reported *salmonella* make up a large genus of gram-negative bacilli within the family *Enterobacteriaceae* and it constitute a genus of more than 2300 serotypes that are highly adapted for growth in both humans and animals and that cause a wide spectrum of disease. The growth of *S. typhi* and *S. paratyphi* is restricted to human hosts, in whom these organisms cause enteric (typhoid) fever. The remainder of *Salmonella* serotypes, referred to as non-typhoidal *Salmonella*, can colonize the gastrointestinal tracts of a broad range of animals, including mammals, reptiles, birds, and insects. More than 200 of these serotypes are pathogenic to humans, in whom they often cause gastroenteritis and can also be associated with localized infections and/or bacteremia. Furthermore, members of the genus *Salmonella* are ubiquitous pathogens found in humans and livestock, wild animals, reptiles, birds, insects and can multiply under various environmental conditions outside the living hosts *Salmonella*e are gram-negative, non-spore forming, and facultative anaerobic bacilli and 2 to 3 by 0.4 to 0.6 μm in size.

Lunguya *et al.* (2012) investigated that epidemiologic class of Salmonella is primarily based totally at the host preferences. The first organization consists of host-constrained serotypes that infect most effective human beings along with S. Typhi. The 2nd organization consists of host-tailored serotypes that are related to one host species however can motive sickness in different hosts serotypes along with S. Pullorum in avian. The 1/3 organization consists of the closing serotypes. Typically, Salmonella Enteritidis, Salmonella Typhimurium and Salmonella Heidelberg are the 3 maximum common serotypes recovered from human beings every year. The genus includes species: the primary is S. Enterica that's divided into six subspecies S. Enterica subsp. Enterica, S. Enterica subsp. Salamae, S. Enterica subsp. Arizonae, S. Enterica subsp. Diarizonae, S. Enterica subsp. Houtenae and S. Enterica subsp. Indica; and the second one is S. Bongori(previously referred to as S. Enterica subsp. Bongori) Salmonella enterica subspecies I is particularly remoted from warm-blooded animals and bills for extra than 99% of scientific isolates while closing subspecies and S. Bongori are particularly remoted from cold-blooded animals and account for much less than 1% of scientific isolates.

Pui *et al.* (2011) Studied that *Salmonella* bacterium was first described by Theobald Smith (1859-1934) and then in 1885, two American veterinarians, Salmon and Smith isolated the bacterium causing hog cholera from infected pigs. The name *Salmonella* was subsequently adopted in honor of Dr. Salmon. Over the decades following the pioneering work of Salmon and Smith, many other *Salmonella* were isolated from both animals and humans. The antigenic classification or serotyping of *Salmonella* used today is a result of years of study of antibody interactions with bacterial surface antigens by Kauffman and White in the 1920s to 1940s (Kauffmann, 1950). According to this Kauffmann-White scheme, each *Salmonella* serotype is recognized by its possession of a particular lipopolysaccharide (LPS) or O antigen and a flagellar or H antigen. This led to the description of more than 2500 serotypes at present.

Shrivastava *et al.* (2011) reported that as according to the WHO Global Salm-Surv, during 2000-2002, *S.* Enteritidis was by far the most common serotype reported from humans globally. In 2002, it accounted for 65% of all isolates, followed by *S.* Typhimurium at (12%) and *S.* Newport at (4%). Among non- human isolates, S. Typhimurium was the most commonly reported serotype in all the three years, accounting for (17%) of isolates in 2002 followed by *S.* Heidelberg (11%) and S.

Enteritidis (9%). *Salmonella* Enteritidis, *S. Typhimurium* and *S. Typhi* were ranked among the fifteen most common human serotypes in all regions of the world throughout the three-year study period. *Salmonella* Agona, *S.* Infantis, *S.* Montevideo, *S.* Saintpaul, *S.* Hadar, *S.* Mbandaka, *S.* Newport, *S.* Thompson, *S.* Heidelberg and *S.* Virchow were also widespread. In Africa in 2002, *S. Enteritidis* and *S. Typhimurium* were each reported from approximately one fourth of isolates from humans.

Anjum *et al*. (2011) stated that the *Salmonella* is one of the leading causes of bacterial foodborne disease in industrialized as well as developing countries even though the incidence seems to vary between The wide variations in the national prevalence of Salmonellosis likely arise from limited scope of studies and lack of coordinated epidemiological surveillance systems, under-reporting of cases and the presence of other diseases considered being of high priority. *Salmonella* is a leading bacterial cause of acute gastroenteritis and constitutes a huge health burden in both developing and developed countries. Symptoms of acute infection (e.g., diarrhea, fever, abdominal pain, nausea, and vomiting) are easy to identify and treat using antibiotics or through natural clearance of the organism. However, *Salmonella* infection can exist in host cells persistently causing patients to chronically carry the pathogen resulting in further spread of the bacteria.

Gilman *et al*. (2011) reported that Salmonellosis is the most common foodborne disease in both developing and developed countries, although incidence rates vary according to the country (Stevens *et al.*, 2006). The fecal wastes from infected animals and humans are important sources of bacterial contamination of the environment and the food chain. Members of *Salmonella enterica* subspecies *enterica* are widely distributed in the environment and in the intestinal tracts of animals (Anjum *et al.*, 2011). People can become infected following a failure of personal hygiene after contact with infected animals and or other infected people. Environmental contamination, especially untreated water is also important. Most human infections are acquired through consumption of contaminated food of animal origin.

Kramer *et al.* (2011) observed that *Salmonella* infection appears to be one of the most common examples of an enteric disease that is transmitted from animals to humans. The transmission occurs both through food products, such as meat, dairy products, and eggs, and by direct contact between animals and humans through the fecal-oral route. Stool

cultures remain positive for four to five weeks after infection. Morbidity and mortality associated with salmonellosis are highest among the elderly, infants, and immune compromised individuals, including those with hemoglobinopathies and those infected with HIV or with pathogens that cause blockade of the reticuloendothelial system (e.g., patients with bartonellosis, malaria, schistosomiasis, or histoplasmosis).

Abera *et al*. (2010) studied that *Salmonella* infection were susceptible to both human and animals while some of this infections causes disease; the majority probably leads to subclinical cases resulting in a healthy carrier state with intermitent excretion of the *Salmonella* in stool. Whether a human develop disease following ingestion of *Salmonella* depend on dose of organism, the species of *Salmonella* and up on the specific and non-specific immunological factors. Species such as *S. typhimurium* and *S. enteritidis* usually causes gastrointeritis (food poisoning). The majority of food poisoning outbreaks caused by *Salmonella* follow the consumption of food directly or indirectly associated with infection in animals. The chain of transmission is often from contaminated animal food staffs to animal and then from contaminated animal carcasses to man. Salmonellosis is most commonly caused by *S. enterica* subsp. *typhimurium* or *S. enterica* subsp. *enteritidis*. Secondly, *S. enterica* subsp. *typhi* and *S. enterica* subsp. *paratyphi* are the causes of typhoid fever or paratyphoid fever, respectively. *Salmonella* can replicate both inside the vacuoles of host cells and in the external environment. *Salmonella* are the second most common pathogens isolated from humans with gastroenteric disease in developed countries.

Okonko *et al*. (2010) reported that Salmonellosis in the human host is generally associated with *Salmonella enteric* subspecies *enteric* and acute infections can present in one of four ways: enteric fever, gastro-enteritis, bacteremia, and extra intestinal (EI) focal infection. As with other infectious diseases the course and outcome of the infection are dependent upon a variety of factors including inoculating dose, immune status of the host and genetic background of both host and infecting organism. Broadly speaking the *Salmonella enteric* from human infections can be subdivided in to two groups: the enteric fever (typhoidal) group and non-typhoidal *Salmonella* (NTS), which typically cause gastroenteritis but can cause invasive disease under certain conditions. *Salmonella* infections in humans can range from a self-limited gastroenteritis usually associated with non-typhoidal *Salmonella* (NTS) to typhoidal fever with complications such as a fatal intestinal perforation. Non-typhoidal *Salmonella* is one of the principal causes of food

poisoning worldwide with an estimated annual incidence of 1.3 billion cases and 3 million deaths each year. Outbreaks of salmonellosis have been reported for decades, but within the past 25 years the disease has increased in incidence in many continents. The disease appears to be most prevalent in areas of human as well as animals.

Ley *et al.* (2010) mentioned that typhoid fever is an acute systemic febrile illness caused by the bacterium *Salmonella enterica* serovar Typhi. *Salmonella enterica* serovars Paratyphi A, B, and C cause the clinically similar condition, paratyphoid fever Typhoid and paratyphoid fevers are collectively known as enteric fevers. While both diseases share clinical features, paratyphoid fever tends to have a more benign course of illness. Enteric fever is an endemic disease in the tropics and sub-tropics primarily affecting children and young adults whereas in high-income countries it is mainly a disease of returning travelers. The disease is most commonly acquired by ingestion of water and food contaminated with feces or urine of carriers. Human beings are the only reservoir host for enteric fever. The illness due to enteric fever may be mild or severe but sometimes fatal. Enteric fever commonly presents with nonspecific clinical features such as fever, flu-like symptoms with chills, a dull frontal head-ache, malaise, anorexia, poorly localized abdominal dis-comfort, a dry cough and myalgia, nausea, vomiting, constipation, and diarrhea which are indistinguishable from other causes of fever such as malaria.

Omuse *et al*. (2010) estimated that the global burden of typhoid and paratyphoid fever indicated that in 2000, there were 22 million new cases of typhoid fever, 210,000 typhoid fever-related deaths, and 5.4 million cases of paratyphoid fever. Developing nations share the highest burden due to rapid population growth, increased urbanization and limited safe water and health system. The incidence of this neglected illness in some parts of South Asia is as high 1600 per 100,000 populations. The case-fatality rate is higher in children under 5 years than school aged children and adolescents. It is highest in Sub-Saharan Africa and North Africa/Middle East regions. In Ethiopia, enteric fever is among diseases claiming the life of many people particularly children. A study made in Ethiopia Swedish hospital in Addis Ababa (1984–1995) reported intestinal perforation in 27 patients (25%) of which 10 (37%) children died. In untreated cases, the case fatality ranges be-tween 10 and 30%, however, with appropriate and timely antimicrobial treatment it falls to 1–4%.

Boyen *et al*. (2008) stated that bacterium, *Salmonella typhi* (*Salmonella* enteric serotype *typhi*), causes typhoid fever. *Salmonella typhi* is an obligate, motile, gram-negative, rod-shaped enteric bacillus and belongs to *Enterobacteriaceae* family. The intestines of humans are known to be its principal habitat. The major route of transmissions of enteric fever (typhoid fever) is either through feco-oral route or urine-oral route. When the bacteria are ingested through food or water that is contaminated (containing at least 104 bacteria), it finds its way into the small intestine after 1-2 weeks' period of incubation which varies. After they attach themselves to the intestinal epithelium where they penetrate lamina propria and sub mucosa, *S. typhi* are engulfed by monocytes

Ponce *et al*. (2008) studied that typhoid fever is one of the infectious human diseases. Outbreaks of typhoid fever caused by *Salmonella typhi* remains a serious health problem worldwide. There are a number of tests available presently, from molecular to immunological and biochemical to microbiological. However, misdiagnosis is usually experienced since most health care facilities use only Widal test without confirmation of results with a second test method. This study aimed at evaluating the performance of Widal test and stool culture in the laboratory diagnosis of typhoid fever using blood culture as gold standard. Typhoid fever is a systemic prolonged febrile illness caused by certain *Salmonella* serotypes including *Salmonella typhi*, *S. paratyphi* A, *S. paratyphi* B and *S. paratyphi* C. Human beings are the only reservoir host for typhoid fever, and the disease is transmitted by faecally contaminated water and food in endemic areas especially by carriers handling food. The World Health Organization (WHO) estimates about 21 million cases of typhoid fever with >600,000 deaths annually. The cases are more likely to be seen in India, South and Central America, and Africa i.e. in areas with rapid population growth, increased urbanization, and limited safe water, infrastructure, and health systems.

Beyene *et al*. (2008) described that the typhoid fever and paratyphi are prevalent in areas that are characterized by overcrowding and at the same time have poor access to proper sanitation. Estimates by the WHO depict South East Africa, South Central Asia and Southern Africa as having high incidences of *Salmonella typhi* infection i.e. more than 100 cases per 100,000 persons per year. Those with medium incidences include the Caribbean, Latin America among some other regions of Asia as well as Africa (10-100 cases per 100,000 persons per year). The factual incidences of typhoid fever are however, difficult to estimate especially in developing countries due to what Hook

(2005) terms as lack of rapid inexpensive diagnostic tools, poor disease reporting systems, laboratory testing infrequency and the often common confusion between the clinical presentation of the disease and other common febrile illness.

Rajapati *et al.* (2008) investigated that the accurate diagnosis of typhoid fever at an early stage is important not only for diagnosis of etiological agent, but also to identify individuals that may serve as a potential carrier, who may be responsible for acute typhoid fever outbreaks. Options for the diagnosis of typhoid fever are clinical signs and symptoms, serological markers, bacterial culture, antigen detection and DNA amplification. Blood, bone marrow and stool culture are the most reliable diagnostic methods but they are expensive techniques and some bacterial culture facilities are often unavailable. Typhoid fever is a major health problem in developing countries and its diagnosis on clinical ground is difficult. Diagnosis in developing countries including Bangladesh is mostly done by Widal test. In many countries including Bangladesh, the Widal test is the most widely used test in typhoid fever diagnosis because it is relatively cheaper, easy to perform and requires minimal training and equipment.

Prajapati *et al.* (2008) identified that the earlier findings in the present study indicated that stool culture has a high sensitivity and specificity. This finding support an earlier study that established that stool culture picked highest typhoid fever cases. *Salmonella typhi* is an enteric microorganism and its principle habitant is in the intestine of humans, so it is easily detectable in stool even in cases of early ingestion of bacteria. Stool culture is an important adjunct for diagnosis; it may be positive even when blood culture is negative and it is also important for the monitoring of carriage of *S. typhi* after apparent clinical cure, which is a risk factor for people involved. Improvement of stool culture sensitivity can be done by culturing stool in triplicate. Additionally, increasing the quantity of stool used for culture has been shown to increase the sensitivity with culturing two grams of stool rather than the standard one gram increasing isolation by 10%.

Torpdahl *et al*. (2007) described that blood culture is a microbiological culture of blood. It is employed to detect infections that are spreading through the blood stream (such as bacteremia and septicemia amongst others). Blood cultures are inoculated in suitable differential and selective media and incubated at 37oC for 3 days. Results are reported as positive (cultures with bacteria present thus indicating the patient is bacteremic) or

negative blood culture. If the culture is positive, biochemical tests are carried out to confirm the presence of bacteria which are as follows; Gram reaction, lactose fermentation, motility test, citrate utilization test and glucose fermentation test.

Galanis *et al.* (2006) examined that blood culture is the standard diagnostic method for *Salmonella typhi and paratyphi* infection. Blood is normally a sterile environment, so detection of bacteria in the blood is an indication of systemic infection. Culture of blood is the most sensitive method for detection of bacteremia However, even though the blood culture has the highest sensitivity in detecting *S. typhi* infection, it is not widely applicable in most of the rural health care facilities because these health centers lack trained laboratory personnel, scientific know how and technical expertise necessary to perform a blood culture. Due to lack of financial resources, setting up and maintaining a microbiology laboratory with a blood culture facility is a big challenge. The inability to maintain stable and reliable electricity supply in rural health facilities poses a great challenge to performing a blood culture. Access to blood cultures is limited to the well-equipped private hospitals located in urban centers. The inability to perform effective diagnosis has led to poor health outcomes, indiscriminate antibiotic use and increasing drug resistant. It has also made targeted cost effective vaccination strategies impossible to implement. Some of the limitations of blood culture include venipuncture which is associated with pain, therefore creating fear to patients especially children. The size of veins varies in different individuals; therefore, locating one is sometimes difficult. Risk factors associated with giving a blood sample include blood accumulating under the skin (hematoma), excessive bleeding especially those that are haemophilic, then fainting and infections.

Renuka *et al.* (2005) studied that results in the previous study found out that Widal test has low sensitivity and specificity. The case of many false positive in Widal test in the present study could be because individuals who had *S. typhi* infection in the past may have developed *Salmonella typhi* antibodies during an unrelated or closely related infection (anamnestic response) thereby giving false positive results. Patients who had received vaccines against *Salmonella* gave false positive reactions since antibodies to 'O' and 'H' antigens were detectable in serum. Other causes of drawback are the procedural aspects when conducting the tests and variability in the reagents as well, since varying antigens in the different test kits available perform differently. Positive Widal agglutination tests have also been reported in association with dysgammaglobulinaemia

of chronic active hepatitis and other autoimmune diseases. In addition, the infecting strains of *S. typhi* are poorly immunogenic. There is therefore, big conflicting evidence as to the relative importance of somatic and flagella agglutinin tests for the diagnosis of typhoid fever. These findings concur with the study carried out other researchers which found out that Widal test had registered high cases of false positives. The case of false positive in the present study are possibly because the 'O' and 'H' agglutination usually appear around 8-12 days of infection, hence the probability of them not detected in case of early diagnosis. Immunosuppressed patients lack antibody responses. Infection of a site such as the synovial cavity which is not part of the reticuloendothelial system or early treatment with ampicillin or chloramphenicol can have a profound inhibitory effect on agglutinin production.

Jain et al. (2005) survey that the previous study, a single sample test was used, as is the case in hospitals since outpatients rarely return for medical follow up. This could be a drawback to interpreting the results because of high background rates of circulating antibodies to *S. typhi.* Due to polyvalent nature of Widal antigens, there is a high possibility of cross-reactivity with bacterial and non-bacterial infections. Since some diseases such as Malaria, ulcerative colitis, non-typhoidal *Salmonellae*, rheumatoid arthritis and nephrotic syndrome may show similar symptoms and produce high 'O' antibody test, they should always be evaluated as differential diagnosis. The gold standard for typhoid fever and paratyphoid diagnosis is currently the blood culture and when evaluating diagnostic tests for typhoid fever, blood culture is universally used as the reference standard. Where culture is conducted, bone marrow culture is not common due to invasiveness and technical difficulty of the procedure (Gilman *et al*., 2011). The potential of using molecular detection of *S. typhi and S.paratyphi* from blood has been demonstrated by a number of studies. However, the true utility of Widal test as a typhoid fever diagnostic tool is still being debated.

2.2. Isolation of *Shigella*

Arena *et al.* (2015) studied that *Shigella* are gram-negative, non-motile, facultative anaerobic, non-sporulating, rod shaped bacteria that cause the disease shigellosis, which is also known as bacillary dysentery. Organisms of the genus *Shigella* belong to the *Enterobacteriaceae* family. There are four subgroups that have been historically treated as separate species, although recent genetic analysis indicates that they are members of

the same species. These include subgroup A (*Shigella dysenteriae*), subgroup B (*S. flexneri*), subgroup C (*S. boydii*), and subgroup D (*S. sonnei*). Bacteria are considered highly infectious, as infection can result from ingestion of 10-200 organisms. This group of bacteria were first described by and named after Japanese scientist Kiyoshi Shiga in 1898, after he isolated what he called *Bacillus dysenteriae* (now known to be *Shigella dysenteriae* serotype from a patients' stool during a dysentery epidemic in Japan in 1897. *Shigella* infection is a major public health problem in developing countries with poor sanitation. Humans are the natural reservoir for this organism. Endogenous *Shigella* species are not present in any natural food products, but a wide variety of foods may be contaminated. Transmission of the bacteria occurs by the fecal–oral route. *Shigella* species have a very low infective dose, as low as 10 to 100 organisms. Once ingested these survive gastric acidity and invade the colonic mucosa, resulting in mucosal abscesses and ulceration. Destruction of the epithelial layer causes symptoms like watery diarrhea, severe abdominal pain and cramping, eventuating in the bloody mucoid stool characteristic of bacillary dysentery. If needed treatment is not provided, then shigellosis patients may develop secondary complications such as septicaemia and haemolytic uremic syndrome.

Sureshbabu *et al.* (2014) Shigellosis is endemic in most developing countries and the most important cause of bloody diarrhea worldwide. Most shigellosis cases are sporadic. Globally, *Shigella* is estimated to cause at least 80 million cases of bloody diarrhea and 700,000 deaths annually. Ninety-nine percent of infections caused by *Shigella* occur in developing countries and the majority of cases and deaths occur among children less than five years of age. Outbreaks of shigellosis have been associated with food, water, men who have sex with men (MSM) and conditions of crowding and/or where personal hygiene is poor such as child care centers and institutionalized populations. Transmission is fecal-oral including direct person-to- person contact. It is most likely to occur in children and those who fail to clean hands thoroughly, including under fingernails after defecation. Transmission occasionally occurs with sexual contact. Two features of the disease facilitate person-to- person transmission: the infective dose is low (as few as 100 viable organisms) so minor hygiene omissions allow fecal-oral spread, and many persons have only a mild illness, so they remain in contact with and can transmit the infection to others. Transmission may be indirect through ingestion of contaminated food or water and less commonly through contaminated inanimate objects. Flies may serve as vectors

for transmission of shigellosis, particularly in settings where disposal of human feces is inadequate

Sureshbabu *et al.* (2014) Shigellosis is the most common cause of outbreak of bloody diarrhea worldwide with secondary infection rates as high as 40% in the household and case fatality rate of 15-20%. Outbreaks may result in large scale mortality as occurred in an outbreak of *Shigella dysenteriae* type 1(Sd1) which caused very high fatality of 20,000 in one month among Rwandan refugees in Zaire in 1994. *Shigellae* has very low infectious dose; ingestion of as few as 10- 200 viable bacteria resulting in clinical disease after an incubation period of 12-96 hours. Patients may, however, present only with acute watery diarrhea without visible blood or mucus, and without the other symptoms described above, especially at the beginning of their illness. The bacteria can be transmitted by ingestion of contaminated food or water, or through person-to- person contact. Flies can breed in infected feces and then contaminate food; vegetables can become contaminated if they are harvested from a field irrigated with untreated sewage. *Shigellae* bacteria are present in the stools of infected persons while they are sick and for 1-2 weeks after resolution of symptoms. A majority of *Shigella* infections are transmitted via the fecal-oral route (2- 5). Globally the annual number of *Shigella* cases is estimated to be 164.7 million, of which 163.2 million were in developing countries (with 1.1 million deaths) and 1.5 million in industrialized countries.

Jarząb *et al*. (2013) observed that *Shigella dysenteriae* is also referred to as Group A. It has 13 serotypes *S. dysenteriae* serotype 1 is known as Shiga's bacillus. It is indole negative and is the only member of the family that is always catalase negative. It is the agent of epidemic shigellosis and is responsible for large-scale outbreaks in Central Africa, Southeast Asia, and the Indian subcontinent. In endemic areas it is isolated from up to 30% diarrheal patients. *Shigella dysenteriae* serotype 1 is unique among *Shigella* species because, it produces a potent toxin known as the Shiga toxin (Stx). Stx is a bipartite molecule composed of a single enzymatic A subunit and a pentamer of receptor-binding B subunits. The toxin binds to a glycolipid receptor found in target cells, globotriaosylceramide. It is then endocytosed preferentially by the clathrin-coated pathway. The Shigellosis or bacillary dysentery is a leading cause of morbidity and mortality worldwide. The use of appropriate antimicrobial therapy for shigellosis may reduce symptom severity and illness duration, and may also prevent potentially lethal complications and further disease transmission.

Dewan *et al.* (2013) detailed that Shigellosis is one of the major diarrheal diseases in Bangladesh and several other developing countries and is caused by any one of the four species or groups of *Shigella*, namely. Dysenteriae, *S. flexneri, S. boydii,* and *S. sonnei.* Each serogroup contains multiple serotypes, and at least 47 serotypes are currently recognized based on their biochemical and/or lipopolysaccharide characteristics. Shigellosis occurs as an endemic disease in Bangladesh, with the predominant species being *S. flexneri*. Shigellosis is transmitted via the fecal-oral route through ingestion of contaminated food or water or by person to person contact. *Shigella* is one of the major diarrheal diseases in Bangladesh, especially among children, as well as in several other developing countries is shigellosis. The symptoms of shigellosis can range from mild diarrhea to severe dysentery with the passage of frequent bloody, mucoid stools, fever and abdominal cramps. The population of Bangladesh, which consists of different groups due to their different lifestyle, food habit, health condition, may all be exposed to this pathogen, *Shigella flexneri*, at one time or other. Human blood serum from these various groups has the ability of exhibiting complement activity against this organism. Serum is the first body defense met by pathogen when it breaks through and enters the skin or epithelial lining of the organs. It contains complement, which can exhibit bactericidal activity through the classical or alternative pathway

Jarząb *et al.* (2013) described that *Shigella flexneri* is named after Flexner (1900), who described the first of the mannitol fermenting shigellae from Phillipines. The species is also referred to as Group B and has 6 serotypes. It causes shigellosis or bacillary dysentery. *S. flexneri* is widespread in developing countries and is responsible for the worldwide endemic form of this disease. Among the four species of *Shigella, S. flexneri* is the most prevalent species in Bangladesh. This human-specific pathogen is transmitted via the fecal–oral route. It targets the large intestine, resulting in acute inflammation, tissue edema, and erosion of the colonic epithelium. Moreover, *Shigella boydii* is named after Boyd, who first described these strains from India (1931). The species is also referred to as Group C and has 18 serotypes. In comparison with other *Shigella* serogroups this species has been less often reported worldwide. *S.boydii* is typically related with people who have travelled to endemic areas but it is relatively rare in developed countries. Isolation rate of this species is less than 1–2% of the total *Shigella* isolates, except in the Indian subcontinent. Therefore, Shigellosis is an important public health problem and outbreaks have been reported both in developed and developing

countries. The major modes of transmission are contaminated food as well as contaminated fresh produce, contaminated water and person-to-person contact.

Das *et al*. (2013) studied that *Shigella sonnei* is named after Sonne, who first described these strains from Denmark (1915). The species is also referred to as Group D and has 1 serotype. *Shigella sonnei* normally causes mild self-limiting infection. In recent years it has become the most usual *Shigella* species in the developed world. The spread of *S.sonnei* generally occurs in institutional or crowded settings, such as day-care centers and prisons and in military field settings. Shigellosis is the third leading bacterial gastrointestinal disease in the United States, with 25,000 cases reported in 1998 and 18,000 cases reported in 1999. Approximately 900 cases of *S.sonnei* infection are reported annually in the United Kingdom, and 15 cases of *S.sonnei* infection were reported to the National Disease Surveillance Centre in Ireland in 2001. In most of the patients infected with *S.sonnei*, watery diarrhea occurs as a prodrome, or as the only clinical manifestation. However, some patients with *Shigella* infection- especially those with *S.sonnei*-never progress to the dysenteric phase, whereas others may develop dysentery without a prodrome.

Saeed *et al*. (2012) considered that the distribution of *S. dysenteriae* according to age of the patients. No infection was recorded in children samples below 1 year of age in this study which may be due to the immunity conferred by breast feeding. Breast feeding is associated with a substantial decrease of the risk of sever *Shigella* infection or might be due to the boiled drinking water usually given to infant. The highest percent (49%) were at 1-4 years of age, because of low health consciousness and poor personal hygiene at this age. This result was also noted in India, which is an endemic area. The lowest percent in elder patient may be due to the acquired immunity gained from repeated exposure to the disease, whether clinical or asymptomatic infection. This result was dissimilar with when found the percentage of the disease was 16.3% in ‹1 year of age. Found the highest incidence of clinical Shigellosis is usually observed among toddlers, pre-school children. Shigellosis can affect people of any age, including teens. It's especially common in children ages 2 to 3 years old because they're often not toilet trained yet or they don't wash their hands after using the bathroom. That means that *Shigella* bacteria can spread easily within families, schools, child-care centers, nursing homes, and other institutions.

Breiman *et al*. (2012) examined that the diarrheal of diseases claim the lives of at least five million children per year in developing countries and shigellosis or bacillary dysentery is responsible for approximately 10% of these deaths. *Shigella flexneri* is responsible for the worldwide endemic form of this disease. According to symptoms Shigellosis is a severe form of bloody diarrhea which is endemic in developing countries. The symptoms occur within 24-48 hours of ingestion of the etiologic agent and may persist in untreated adults for up to 7 days. The organism may be cultivated from stools for 30 days or longer. The best way to prevent shigellosis is following proper hand washing and hygiene practices. Foodborne transmission can be reduced by ensuring proper hygiene and sanitation during harvesting, production, distribution and preparation of food. Infected food workers should be discouraged from working while ill. Waterborne outbreaks of shigellosis can be prevented by making sure that safe drinking water is present. Drinking water should be protected and kept well away from toilet facilities as *Shigella* can survive in tap or sterilized water for as long as 4-6 weeks. Additionally, the transmission of shigellosis in community settings, such as daycare centers, can be prevented by providing children and child care staff with fully stocked and operational hand washing stations. The children should be supervised and assisted during hand washing. Other security measures include elimination of water play areas and exclusion of child care workers handling diapers from preparing food in child care centers or in restaurants.

Atyabi *et al.* (2012) reported that the prevalence of *Salmonella* and *Shigella* are increasing gradually worldwide. It is estimated that more than 6 million cases of Salmonellosis and Shigellosis occur worldwide annually. Salmonellosis and Shigellosis occur sporadically or in limited outbreaks but is most likely under-reported. *salmonella* and *Shigella* are more prevalent in India and other Asian countries, Eastern Europe and South America and Africa.

Rahman *et al.* (2011) described that Bangladesh, located in South Asia, has a population that is mostly insolvent; thus, it is probable that *salmonella* and *Shigella* prevalence is usually high, yet little is known about the distribution of this disease in Bangladesh. *Salmonella* and *Shigella* are around the year problem which sometimes takes epidemic proportions in this country. Monsoon months have the highest disease occurrences followed by the pre-monsoon and post-monsoon season the reasons behind such occurrences are unsafe water supply, defective sewage system and unhygienic food

handling practice. In Bangladesh has reported that half of cases are resistant to commonly used antibiotics leading to salmonellosis and Shigellosis case fatality rates as high as 30% (WHO) indicated.

Bugla-Ploskonska *et al.* (2009) studied that in Bangladesh; a population-based study suggested that children and young adults had the highest age-specific rates of salmonellosis and Shigellosis. Highest prevalence has been reported to occur in children 5–15 years of age; however, in regions where the disease is highly endemic, children <5 years of age may have among the highest infection rates. Another study in Dhaka reported that Prevalence of salmonellosis and Shigellosis is high among the patients of school going age group (66.67%), habituated with unsafe drinking water (58.33%) and junk foods (72.92%).

Erdman *et al.* (2008) reported that effective antimicrobial therapy reduces the rate of morbidity and mortality. Early antibiotics therapy has transformed a previously life threatening illness of several weeks' duration. Without therapy, the illness may last for 3–4 weeks and case-fatality rates may exceed 10%. With appropriate treatment, clinical symptoms subside within a few days, fever recedes within 5 days, and mortality is reduced to approximately 1% (Perilla *et al.,* 2002). Antibiotics such as ampicillin, chloramphenicol, trimethoprim–sulfamethaxazole, nalidixic acid, azithromycin, ciprofloxacin, cefixime, ceftriaxone, ofloxacin, gatifloxacin, imipenem, meropenem etc. are choice of drugs for treating both typhoid and paratyphoid fever. But resistance to drugs is such a never ending process through which organisms can evade themselves from the effect of such antibiotic. Besides because of the ready availability of over-the-counter antibiotics and subsequent resistance to these drugs in areas of endemicity, enteric fever is becoming harder to treat. Depending on the evaluation of resistant organisms, antibiotics are suggested for successful treatment of typhoid and paratyphoid. Chloramphenicol, ampicillin, and trimethoprim-sulphamethoxazole became prevalent in some Asian countries during the late 1980s and early 1990s and resistant *S. Typhi* and *S. Paratyphi* emerged as a significant therapeutic problem. In most strain the resistant organisms developed by plasmids (Jacoby *et al.,* 1980). Ciprofloxacin and ofloxacin have for some years been the drugs of choice for enteric fever, but resistance to these drugs has become very common in South Asia and has sporadically been reported in Sub-Saharan Africa. Nalidixic acid sensitivity has to be carried out in all enteric fever organisms isolated from South Asia, and if resistance is noted ciprofloxacin should not

be used. Injectable third-generation cephalosporins are often the empiric drug of choice when the possibility of fluoroquinolone non susceptibility is high. Azithromycin is increasingly used to treat typhoid fever. On the other hand, such typhoid serovers often become susceptible to particular antibiotics after a certain period of time. For typhoid and paratyphoid although same antibiotics are used but susceptibility to particular antibiotic of these bacteria may vary. Thus both *S.Typhi* and *S.Paratyphi* have a varying degree of susceptibility to different antibiotics. Such susceptibility may change due to selective pressure; plasmid transfer or resistant gene transfer after sometimes or at the time of treatment.

Ranjbar *et al.* (2008) investigated that the treatment of shigellosis can be done by using oral rehydration therapy or appropriate antibiotics. The use of oral rehydration therapy is an effective and safe measure for the treatment of acute diarrhea. However, if the patient is vomiting or is in shock from severe dehydration, then intravenous fluid replacement is required until initial fluid and electrolyte loss are corrected. Shigellosis is generally a self-limited disease and does not need antibiotic therapy, except in patients with severe colitis, who are benefitted by it. *Shigella* species demonstrate increased resistance to both ampicillin and trimethoprim-sulfamethoxazole (TMP-SMX), as shown by a recent US surveillance data, which is why cefixime and ceftriaxone is recommended as alternative antibiotics in treatment of infections, caused by the bacteria. Azithromycin can be used for the treatment of *Shigella* infections in pediatric patients, as suggested by AAP (American Academy of Pediatrics). Fluoroquinolones are at present contraindicated in children due to potential safety concerns, but they are recommended as a potential alternative therapy by the AAP for the treatment of *Shigella* infections in pediatric patients.

Bergelson *et al.* (2008) stated that rationale for antibiotic treatment with effective antibiotic therapy, clinical improvement occurs within 48 hours, resulting in a decreased risk of serious complications and death, shorter duration of symptoms, the elimination of *Shigella* from the stool and subsequently decreased transmission of infection.3 In fact, one of the primary arguments for treatment of *Shigella* infection is due to its public health effect by diminishing transmission through decreasing the duration of faecal carriage (from approximately 4 weeks to 3 days) with effective treatment. The 2005 guidelines recommend ciprofloxacin as first-line treatment and noted that pivmecillinam (amdinocillin pivoxil) and ceftriaxone were ‘the only antimicrobials that are usually

effective for treatment of multi-resistant strains of *Shigella* in all age groups', yet their usage is limited by their high cost and formulation (four times daily dosing for pivmecillinam, and parenteral administration for ceftriaxone). Pivmecillinam and ceftriaxone were therefore only listed for usage when local strains of *Shigella* are known to be resistant to ciprofloxacin. Azithromycin was included as a second-line therapy for adult patients; this was (most likely) not recommended for children in these guidelines due to limited evidence at that time in regards to its efficacy.

Von *et al*. (2006) reported that a third (high-quality) systematic review11 assessing *in vitro* resistance patterns of all *Shigella* spp. to ciprofloxacin alone found increasing resistance in the Asia-Africa regions (analyzed together), from 0.6% in 1998-2000 (95% CI 0.2 to 1.3%) to 29.1% (95% CI 0.9 to 74.8%) in 2007-2009 – a 49-fold increase over 12 years. This increase in resistance was significantly above the (very minimal) increase documented in the Europe-America region, which only reached 0.6% (95% CI 0.2 to 1.2%) by 2007-2009. Of note, this review also found higher resistance in children, with respective rates (globally) of 7.5% (95% CI 4.3 to 11.5%) in paediatric patients versus 3.6% (95% CI 2.2 to 5.3%) in adults. The same authors conducted a review of *in vitro* resistance of all *Shigella* spp. to third-generation cephalosporins (ceftriaxone, cefotaxime and ceftazidime) between 1999-2012,21 finding markedly increased resistance in the Asia-Africa region (with ceftriaxone resistance to *Shigella* spp. reaching 14.2% (95% CI 3.9 to 29.4%) by 2012). Both studies, however, exhibited a lack of data pertaining to patient outcomes. The authors concluded that ceftriaxone and cefotaxime may not be appropriate for treating shigellosis in Asia-Africa. Assessing the Asia region independently, a 2006 multi-center study (of 2,927 *Shigella* isolates in children and adults) across Bangladesh, China, Pakistan, Indonesia, Thailand and Vietnam documented ciprofloxacin-resistant *S. flexneri* isolates in China (18/305, 6%), Pakistan (8/242, 3%), and Vietnam (5/282, 2%).

Marcus *et al.* (2002) examined that bloody diarrhea occurs widespread throughout Bangladesh and is associated with outbreaks. Shigelloses infections are not usually fully reported to the health facilities, with less than 1% typically reported. The MOH/PHEM guideline sets the threshold for epidemic detection and action as a cluster of acute bloody diarrhea cases in the same settlement in one week. Therefore, detection of a cluster of more than 30 cases of shigellosis in 3 days (with total of 104 patients in 2 weeks) in the campus was declared an outbreak. Infections may be acquired from eating contaminated

food, although contaminated food usually looks and smells normal. Food may become contaminated by infected food handlers who don't wash their hands with soap after using the bathroom. Vegetables can become contaminated if they are harvested from a field with sewage. Shigelloses infections can then be acquired by drinking, swimming in or playing with the contaminated water. Comprehensive surveillance data on laboratory confirmed cases of Shigellosis is limited in Ethiopia. However, bloody diarrhea can be used as an indicator of shigellosis cases. From the year 2006 to 2008 a mean of 75,531 outpatient cases of bloody diarrhea were reported per year (approximately 98/100,000 persons per year); the mean number of admission was 1901 patients and 72 deaths per year by the surveillance system of the government Ethiopia.

Wilson *et al.* (2001) isolation that *Shigella* usually by culture from stool specimen. If the patient cannot pass a stool, a sample should be collected with a sterile rectal swab and placed in transport media. Fresh stool samples should reach the laboratory within two hours as *Shigella* species are fragile organisms. If this is not possible, specimens should be placed in transport medium, refrigerated immediately and processed within 72 hours. Infection is usually associated with the presence of pus cells in the stool. Contact your direct-service laboratory if multiple tests for other organisms are required. Antimicrobial susceptibility testing is available upon request. Serotyping is performed on all isolates, through samples and isolates submitted to Cadham Provincial Laboratory.

CHAPTER 3

MATERIALS AND METHODS

This research work was conducted during the period from January 2020 to June 2020 at laboratory of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur. The details outline of the materials and methods are given below:

3.1 Study area and period

The study area included patients from Dinajpur General Hospital in Bangladesh. The research work was conducted during the period from January 2020 to June 2020.

3.2 Study population

We collected sample from patients that were clinically diagnosed as *Salmonella* and *Shigella* infection who presented with fever of 38^0 C and above that had lasted for at least three days, had bowel disturbances, headache, malaise, anorexia etcetera who were seeking medical attention at the hospital. For control group samples were collected from patients with other laboratory-confirmed illness such as malaria, amoebic dysentery, diabetes, AIDS, cholera, parasitic worm infestation, infectious hepatitis, etc. Individuals selected for the study were identified by special code numbers.

3.3 Research design

The study was involved obtaining blood and stool samples from patients suspected of having Salmonellosis and Shigellosis. Blood samples were centrifuged to obtain serum that was subjected to Widal test. Stool samples were inoculated into suitable agar media for growth of *Salmonella* and *Shigella* bacteria.

3.3.1 Inclusion criteria

The inclusion criteria for enrolment in the current study included patients who presented with fever at Dinajpur General Hospital, aged five years or more and willing to participate the study.

3.3.2 Exclusion criteria

The exclusion criteria for the study subjects included patients who did not present with fever at Dinajpur General Hospital, aged less than 5 years and declined to participate in the study.

3.4 Collection of Sample from the Dinajpur General Hospital

Specimens were collected from patients' ≥ 5 years old with symptoms of illness who presented themselves at the study site. They were asked to give verbal consent and answer a brief questionnaire about clinical signs and symptoms, antimicrobial treatment, history of Salmonellosis and Shigellosis.

A total of 33 samples were collected from the Dinajpur General Hospital in Bangladesh. The samples were collected under aseptic condition with the help of clean new containers and immediately transferred into the microbiology laboratory, HSTU. The bacteriological tests were undertaken within 6 hours after collection to avoid the growth or death of microorganisms in the sample.

3.4.1 Blood collection and stool collection

Samples of 5ml of blood were obtained from the study population upon routine venipuncture for Widal test. Blood samples were allowed to clot and the clot was removed by centrifuging at 1,000-2,000 revolution per minute for 10 minutes in a refrigerated centrifuge and the supernatant obtained was serum. Serum samples were stored at -20^0C until analyzed. Freshly passed feces were collected from patients visited at Dinajpur General Hospital, Bangladesh. Samples were collected aseptically into a sterile wide mouthed which placed on ice-box and brought to the bacteriology laboratory, Department of Microbiology, HSTU, Dinajpur with necessary precautions for bacteriological examination. Each sample container was labelled with the patient code number, date and time.

3.4.2 Serological test to confirm *S. Typhi* and *S. Paratyphi*

The serological study was performed by slide agglutination test. The dry and clean glass slide was marked into several parts with a way pencil. A drop of normal saline was placed on each block. An isolated colony of *Salmonella* species was touched with a sterile loop and suspended in normal saline on the slide. Commercially available

Salmonella antisera (Murex diagnostic reagents, UK) were added to the suspension. After back and forth movement of the slide, agglutination was observed two minutes later against diffuse high, Stereotyping was done using specific antiserum (Nuzhat, 2016).

3.5. Laboratory preparation

All items of glassware including test tubes, pipettes, cylinder, flasks, conical flasks, glass plate, slides and agglutination test tubes soaked in a household dishwashing detergent solution for overnight, contaminated glassware were disinfected in 2% sodium hypochloride solution prior to cleaning. The glassware was then cleaned by brushing, washed thoroughly and finally sterilized by autoclaving for 15 minutes at 121℃ under 15 lbs pressure per square inch. Autoclaved items were dried in a hot air oven at 50℃.Disposable plastic ware (micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50℃ for future use.

3.5.1 Appliances and Instruments

The different kinds of glass wares and appliances used during the course of the experiment:

- Aluminum foil roll
- Autoclave
- Bacteriological incubator
- Biosafety cabinet type ii
- Clean free grease slide
- Compound microscope
- Conical flask,
- Cover slips
- Durham’s tube
- Electronic compact balance
- Filter paper
- Hof air oven
- Ice-box
- Inoculating loop (Straight and coiled)
- Jar, Beaker, Cylinder

- ❖ Marker pen
- ❖ Micropipette Pipette
- ❖ Petri dishes
- ❖ Phase contrast microscope
- ❖ Plastic syringe (5 ml)
- ❖ Refrigerator
- ❖ Sealed poly bags
- ❖ Spirit lamp and
- ❖ Sterile cotton
- ❖ Stool collection containers
- ❖ Stop Watch
- ❖ Test tube rack
- ❖ Water bath, Detergent power

3.5.2 Chemicals, solutions and reagents

The chemicals, solutions and reagents used for performing the different bacteriological tests:

- ✓ Acetone alcohol
- ✓ Alpha-naphthol
- ✓ Crystal violet
- ✓ Distilled water
- ✓ Glycerin
- ✓ Gram's iodine
- ✓ Immersion oil
- ✓ Kovac's reagent
- ✓ Methyl-Red solution
- ✓ Phenol red solution
- ✓ Phosphate buffered saline solution
- ✓ Potassium hydroxide solution
- ✓ Safranin and other common laboratory reagents and chemicals.

3.6 Bacteriological media for culture

3.6.1 Liquid media

Nutrient broth the medium was prepared by adding 13 g of nutrient broth powder to one litter of distilled water and well mixed. The pH was adjusted to 7.4. The mixture was distributed in 5 m1 volumes into clean bottles, and then sterilized by autoclaving at 121 °C (15 lb/inch2) for 15 minutes. This medium was prepared by dissolving 10 g of peptone water and 5g sodium chloride in 1littre of distilled water. The mixture was distributed in 5 ml volumes into clean bottles, and sterilized by autoclaving at 121°C (15lb/inch2) for 15 minutes.

3.6.2 Solid media

3.6.2.1 Nutrient agar

Twenty-eight grams of nutrient agar powder (Hi-media, India) was suspended in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petridish and allowed to solidify. After solidification of the medium in the Petri dishes, these were incubated at 37°C for overnight to check their sterility and used for culture characterization (Carter, 1979).

3.6.2.2 MacConkey agar

51.50 grams of dehydrated Bacto-MacConkey agar (Difco) was suspended in 1000 ml of cold distilled water taken in a conical flask and was heated up to boiling to dissolve the medium completely. After sterilization by autoclaving, the medium was poured sterile glass Petri dishes. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the Petri dishes partially removed. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.6.2.3 Salmonella Shigella Agar

Salmonella Shigella (SS) agar is the selective medium for the isolation of *Salmonella* and *Shigella*. 63.0 grams SS agar powder was dissolved in 1000 ml of distilled water. It was mixed well until a homogeneous suspension is obtained. It was heated with frequent

agitation and boiled for one minute. It did not sterilize by autoclaved. It was cooled to 45ºC and 50°C and distributed in Petri plates and allow the medium to solidify partially uncovered (HIMEDIA and Leifson *et al.,* 1935).

3.6.2.4 Xylose Lysine Deoxycholate (XLD Agar)

Xylose-Lysine Deoxycholate Agar (XLD Agar) is a selective medium recommended for the isolation, identification and enumeration of *Salmonella Typhi* and other *Salmonella* species from clinical and nonclinical samples in accordance with FDA BAM 1998. It was suspended 56.93 grams of XLD agar powder in 1000 ml distilled water. Then it was heated with frequent agitation until the medium boiled. It was transferred immediately to a water bath at 50°C. After cooled, it was poured into sterile Petri plates. (HIMEDIA).

3.6.2.5 Brilliant Green Agar

Brilliant Green Agar is a selective and differential medium for the isolation of *Salmonella* species other than *S. Typhi* and *S. paratyphi* from clinical specimens. It was suspended 58.09 grams of powder in 1000 ml purified /distilled water. It was heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then cooled to 45-50°C. finally it was mixed well and poured into sterile Petri plates (Kristensen *et al.,* 1925).

3.6.2.6 Hektoen Enteric (HE) Agar

Hektoen enteric agar is a selective as well as differential media for the isolation and differentiation of enteric pathogens from clinical specimens. It was suspended 75 g of the medium in one liter of purified water. Then it was heated with frequent agitation and boiled for one minute to completely dissolve the medium. Then cooled to 45-50°C. Finally, it was mixed well and poured into sterile Petri plates.

3.6.2.7 Mueller Hinton Agar

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. 38 grams of Mueller Hinton agar powder was suspended in 1000 ml of distilled water. It was mixed well. It was heated agitating frequently and boiled for about one minute. It was dispensed and sterilized in autoclave at 116 - 121°C (15 lbs. sp) for 15 minutes. It was cooled to 45° or 50°C (Carter, 1979).

3.6.2.8 Motility Indole Urease medium (MIU)

18 grams of MIU agar (Difco) was suspended in 950 ml of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. Ninety-five ml was dispensed into flasks and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Then was Cooled to about 50-55°C and aseptically add 5ml was added of sterile 40% basal medium. After mixing were dispensed into sterile test tubes. Allow to cool in an upright position. The sterility of the medium was judged and used for cultural characterization or stored at 4°C in refrigerator for future use (Carter, 1979).

3.7 Reagents preparation

3.7.1 Methyl Red-VogesProskauer broth

A quantity of 3.4 grams of Bacto MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube and then the test tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and used for biochemical characterization or stored at 4°C in refrigerator for future use (Cheesbrough, 1984).

3.7.2 Methyl Red solution

The indicator methyl red (MR) solution was prepared by dissolving 0.1 gm of Bacto methyl red (Difco) in 300 ml of 95% alcohol and diluting this to 500 ml with the addition of 200 ml of distilled water.

3.7.3 Voges-Proskauer solution

Alpha-naphthol solution was prepared by dissolving 5 grams of 1-naphthol in 100 ml of 95% ethyl alcohol.

3.7.4 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of potassium hydroxide crystals in 100 ml of cold distilled water.

3.7.5 Indole reagent

This solution was prepared by mixing 25 ml of concentrated Hydrochloric acid in 75 ml of a methyl alcohol and to this mixture 5 grams of paradimethyl-aminohenzyldehide

crystals were added. This was then kept in a flask equipped with rubber cork for future use (Merchant and Packer, 1967).

3.8 Experimental Layout

The present study was conducted to isolate and identify *Salmonella* and *Shigella* species on human stool and blood samples collected from various patients visited at Dinajpur General Hospital, Bangladesh. Isolation of bacteria from the stool samples were processed on Brilliant Green Agar (BGA), Xylose-Lysine Deoxycholate Agar (XLD Agar), MacConkey agar (MAC) Nutrient agar (NA) *Salmonella Shigella* agar (SSA). Identification of bacteria was performed by morphological staining, cultural characteristics, biochemical test, serological test and antibiotic sensitivity pattern.

Experimental layout

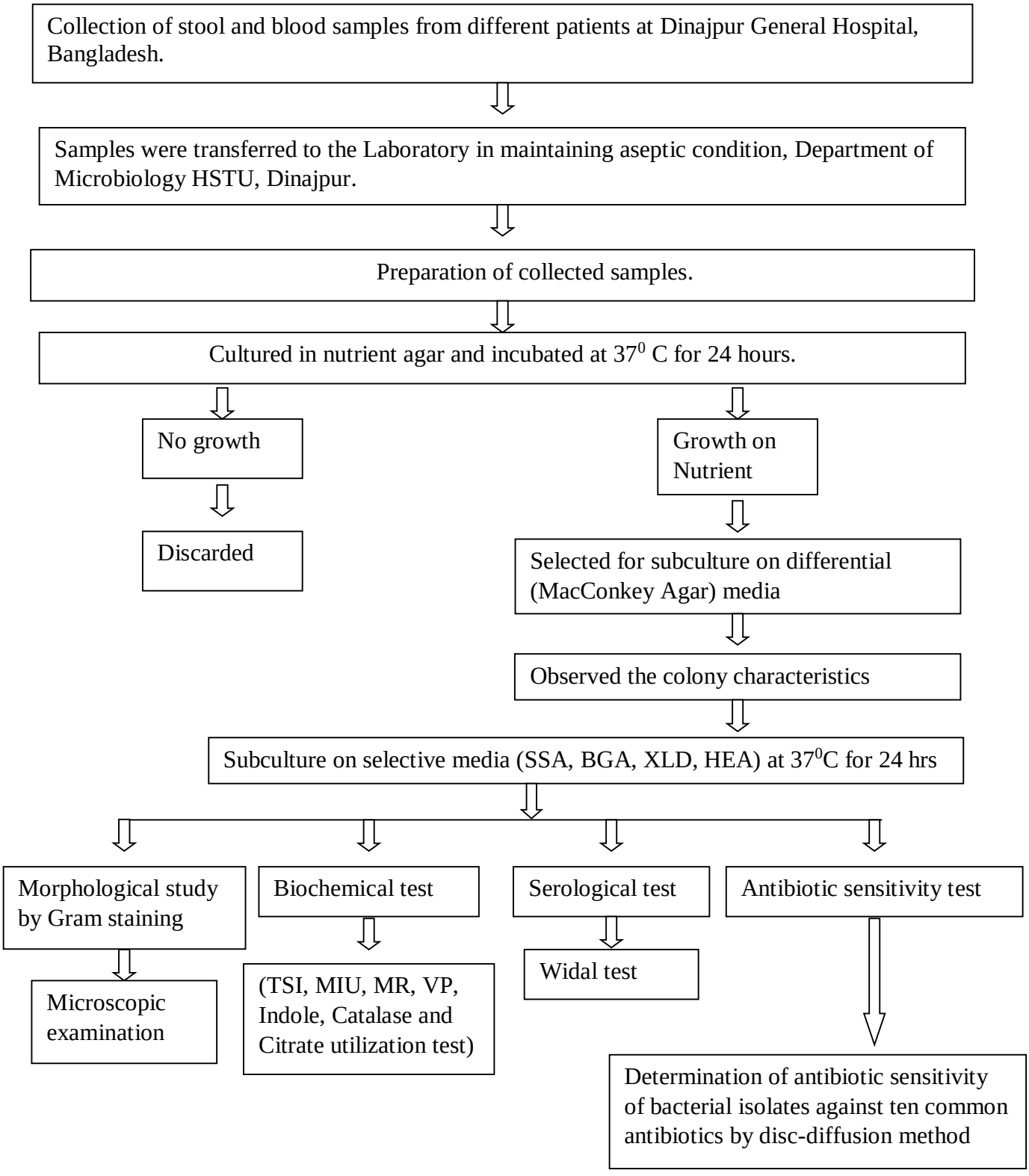

Figure 1: The schematic illustration of the experimental layout.

3.9 Ethical considerations

Ethical clearance was obtained from research supervisor and chairman of the department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur. Permission was also obtained from the Dinajpur General Hospital administration. Samples were collected after informed consent was obtained from each volunteer and guardian.

3.9.1 Sample preparation and laboratory Analysis

Most of the selected samples were diarrheal sample; other samples were mixed with 100 ml of normal saline by mechanical stirring. Then it was inoculated into Nutrient agar and incubated at 37^0C for 24 hours, after 24 hours it was streaked into MacConkey agar medium by inoculating loop.

3.10. Isolation and identification of pathogens

The entire samples were selected for bacteriological culture.

3.10.1 Culture in ordinary media

Samples were inoculated separately into ordinary media like nutrient agar, nutrient broth and were incubated at 37°C for overnight. The colonies on primary cultures were 5 of 1.0 ml 5 of 10 ml 5 of 0.1 ml repeatedly sub-cultured by streak plate method (Cheesbrough, 1984) until the pure culture with homogenous colonies were obtained.

3.10.2 Isolation of bacteria in pure culture

For isolation of bacteria in pure culture, the mixed culture was inoculated into nutrient agar media by streak plate technique to obtain isolated colonies as per:

Step-1: Inoculum was picked up with a sterile loop and spread on an area of the medium in the Petridish.

Step-2: The loop was sterilized by being heated as red hot in a flame.

Step-3: The inoculum was spread over the reminder of the plate by drawing the cooled parallel line. This method was repeated as many times as necessary to obtain a culture containing only one type of colony and usually at least two more times to ensure purity.

3.11 Examination of plates

3.11.1 Gross colony study

Morphological characteristics (shape, size, surface, texture, edge, elevation and color) developed after 24 hours of incubation were carefully studied as described by Merchant and Picket (1967) and recorded.

3.11.2 Gram's staining technique

The most widely used staining procedure in the microbiology is the gram stain, discovered by the Danish scientist and physician Hans Christian Joachim Gram in 1884, gram staining is a differential staining technique that differentiates bacteria into two groups gram positive and gram negative. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. gram negative bacteria are decolorized by the alcohol losing the color of the primary stain purple color positive bacteria are not decolorized by alcohol and will remain as purple after decolorization step. A counter stain is used to impart a pink color to the decolorized gram negative organisms.

3.11.3 Morphological characteristics of bacteria by gram's staining method

The gram staining was followed to study the morphological and staining characterization of bacteria and provide information about the presumptive bacterial identification as per recommendation of (Cowan and Steel 1979).

Procedure:

1. A loopful sterile distilled water was placed in the center of a clean sterile slide.
2. A small colony was picked up with a bacteriological loop and was mixed with distilled water of a slide.
3. The colony was made to thin smear on a slide.
4. The smears were fixed by air driving.
5. 0.5% of crystal violet solution was then applied on the for two minutes
6. Then washout with clean water.
7. Grams iodine was then added to act as mordant for one minute.
8. Then washed out with clean water.
9. Acetone alcohol was then added to decolorize for 1-2 seconds.

10. Washed out with clean water.
11. Safranin was as counter a stain and allowed for one minute.
12. Washed out with water.
13. Then the slide was blotted with blot paper and was allowed to dry. The slide was examined under microscope with high power objective (100X) using with immersion oil.

Grams staining observation:

Gram Positive: Dark purple.

Gram Negative: Pale to dark red, Cocci: Round shape, Bacilli: Rod shape.

3.12 Culture into differential media

3.12.1 Mac-Conkey agar

Samples were sub-culture on Mac-conkey agar media and incubated at 37°C for overnight. After that lactose non-fermented bacteria (pale color colony) were selected.

3.12.2 Culture on selective media

3.12.2.1 Salmonella -Shigella agar

Non-lactose fermenter samples were taken and sub-culture on SS agar media and incubated at 37°C for overnight, which after inoculation, raised, black centered, smooth round colony was present.

3.12.2.2 Xylose Lysine Deoxycholate (XLD Agar)

Materials from nutrient agar were inoculated into Xylose Lysine Deoxycholate (XLD Agar) containing plates and incubated at 37°C for overnight, which after inoculation, red or pinkish to reddish colonies with black centers were present.

3.12.2.3 Brilliant Green Agar

After non-lactose fermenters were observed on the primary plate cultures were inoculated into Brilliant Green Agar containing plates and incubated at 37°C for overnight, which after inoculation, Smooth, white colonies with pinkish background was present.

3.12.2.4 Hektoen Enteric (HE) Agar

HE Agar is examined for typical colonial morphology after incubation. Colonies of *Salmonella* and *Shigella* spp. are green to bluish-green in color. *Salmonella* spp. that produces H_2S appears as blue-green colonies with black centers. H_2S producers form black-centered colonies in the presence of ferric ammonium citrate and sodium thiosulfate.

3.13 Identification of isolated bacteria

The purified isolates were identified according to criteria described by Barrow and Feltham (1993). This included staining reaction, cell morphology, cultural characteristics and biochemical characteristics.

3.14 Microscopic examination

Smears were made from each type of colony on primary culture and from purified colonies. Then fixed by heating and stained by Gram staining method as described by Barrow and Feltham (1993). The stained smears were examined microscopically under oil immersion lens for cell morphology, cell arrangement and staining reaction.

3.15 Biochemical methods for identification of isolated bacteria:

All biochemical tests were performed as described by Barrow and Fellham (1993). They included the following tests:

- Catalase test
- Citrate utilization test
- Indole test
- Methyl test
- Motility indole
- Motility, Indole and Urease (MIU) test.
- Triple sugar iron (TSI) Test
- Voges-Proskauer (VP) test

3.15.1 Catalase test

The present of catalase is determined by its ability to break down peroxide into water and oxygen, releasing bubbles of oxygen. This test can be used as an aid to the identification of *Enterobacteriaceae* (Cheesbrough, 1985).

Procedure:

- Use a loop or sterile wooden stick to transfer a small amount of colony growth in the surface of a clean, dry glass slide.
- Place a drop of 3% H_2O_2 in the glass slide.
- Observe for the evolution of oxygen bubbles.

Observations:

Positive- Copious bubbles produced, active bubbling

Negative- No or very few bubbles produced.

3.15.2 Indole test

The test organism was cultured in a medium containing tryptophan. The organisms break down tryptophan and indole are released. It was detected by the action of Kovac's reagent or Ehrlich reagent (formation of red colored compound). This test was important for the identification of Enterobacteria such as *Salmonella, Shigella* etc. by (Cheesbrough, 1985).

Procedure

(a) Tryptophan containing broth was inoculated with bacteria.

(b) The tube was incubated at 37° C for 24 hours.

(c) Added 0.5 ml of the Kovac's reagent after the bacterial growth.

(d) If indole positive within a 30 second a red color ring appeared at the junction of medium in the tube.

(e) Negative: No color development or slightly pink color. The test culture was inoculated into peptone water and incubated at 37°C for 48 hours. One ml of Kovacs

reagent was added to the tube. The appearance of a pink color in the reagent layer within a minute indicated positive reaction.

3.15.3 Methyl Red (MR) test

This test was performed to differentiate Enterobacteriaceae. Some Enterobacteriaceae when cultured in buffered glucose peptone water, ferment glucose to produce sufficient acidity, which gives red color with methyl red indicator (pH range: 4.4-6.2, Color change: red yellow) by (Cheesbrough, 1985).

Procedure

(a) Sterile MR-VP broth was inoculated with the test organism and following incubation at 37°C for 48 or 72 hours.

(b) Few drops of methyl red solution were added.

Observations:

A distinct red color indicated MR positive test

Yellow or orange color indicated a negative result.

3.15.4 Voges-Proskauer (VP) test

The Voges-Proskauer (VP) test is used to determine if an organism produces acetylmethyl carbinol from glucose fermentation. If present, acetylmethyl carbinol is converted to diacetyl in the presence of ∝- naphthol, strong alkali (40% KOH), and atmospheric oxygen. The test organisms were cultured in glucose phosphate peptone water for 72 hours at 37° C. This test was used to assist in the differentiation of *Enterobacteriaceae* (Cheesbrough,1985).

Procedure

1. Prior to inoculation, allow medium to equilibrate to room temperature.
2. Using organisms taken from an 18-24 hours pure culture, lightly inoculate the medium.
3. Incubate aerobically at 37 degrees C. for 24 hours.
4. Following 24 hours of incubation, aliquot 2 ml of the broth to a clean test tube.
5. Re-incubate the remaining broth for an additional 24 hours.

6. Add 6 drops of 5% alpha-naphthol, and mix well to aerate.
7. Add 2 drops of 40% potassium hydroxide, and mix well to aerate.
8. Observe for a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.

Observations:

Positive Reaction***:*** A pink-red or bright red color at the surface

Negative Reaction***:*** A lack of a pink-red color

3.15.5 Citrate utilization test

The test organism was cultured in a medium containing sodium citrate, an ammonium salt and bromothymol blue indicator. The organisms use citrate (the only source of 28nitrogen).

The citrate utilization is followed by alkaline reaction (change of the color form light to blue) and growth in the medium was indicated by appearance of turbidity. This test was performed in the identification of *Enterobacteriaceae* by (Cheesbrough, 1985).

Procedure

1. A loop of bacteria was spread across the surface of the agar.
2. Kept the tubes at 37° C for 24 hours for incubation. Examined the tubes for the result.

Observations:

Positive: - produce blue color,

Negative: - no color

3.15.6 MIU (Motility Indole Urease) test

MIU is a semisolid medium designed for detection in *Enterobacteriaceae* of urease activity, motility, and indole production by Cheesbrough, 1985).

Procedure

1. Inoculate tubes with a pure culture by stabbing the center of the column of medium to greater than half the depth.
2. Incubate tubes for 18-48 hours at 35 ± 2 °C in aerobic atmosphere.
3. Motility was observed by growth extending from the line of inoculums or diffuses turbidity of the medium. Non motile organisms grow only along the line of inoculation.
4. Urease activity was observed by a change of color to red.
5. Indole production is indicated by the formation of a pink to red color after the addition of three or four drops of Kovac's reagent to the surface of the medium.

Observations:

A negative reaction is indicated by the development of a yellow color.

The red color of phenol red in alkaline pH did not interfere because of the acidity of kovac's reagent by (Cheesbrough, 1985).

3.15.7 Triple Sugar Iron (TSI) agar slant

65 grams TSI agar base powder was mixed in 1000 ml of cold distilled water in a flask and mixed thoroughly, then heated to boiling for dissolving the medium completely (necessary calculation was done for required number of test tubes). The medium was then sterilized by autoclaving for 15 minutes at 121°c. Then 20/10 ml of medium was poured into each sterilized test tubes and allowed to cool and to solidify (kept in horizontal position). After solidification test tube were used for biochemical characterization and incubated at 37°c for 24 hours.

Procedure

1. A loop of bacteria was spread across the surface of the agar.
2. A needle of bacteria was inserted (stabbed) into the bottom (butt) of the tube.
3. Kept the tubes at 37°c for 24 hours for incubation. Examined the tubes for the result.

Table 1: Triple sugar Iron (TSI) agar slant

Result (Slant/butt	Symbol	Interpretation
Red/yellow	K/A	Glucose fermentation only
Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red/Red	K/K	No fermentation
Yellow/Yellow with bubbles	A/A, G	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/Yellow with bubbles	K/A, G	Glucose fermentation only; Gas produced
Red/Yellow with bubbles and black precipitation	K/A, GH_2S	Glucose fermentation only; Gas produced H2SProduced
Red/Yellow with black precipitation	K/A,H_2S	Glucose fermentation only; H_2S Produced
Yellow/Yellow with black precipitation	A/A,H_2S	Glucose and lactose and/or sucrose fermentation;H_2S Produced
No Change/ No Change	NC/NC	No fermentation

Note: A=acid production: K= alkaline reaction: G=gas production: H_2S= Hydrogen sulfide production.

3.16 Antibiotic sensitivity tests

The antibiotic susceptibility pattern of all the isolated bacteria from each sample as well as control samples was determined using the disk diffusion according to Bauer-kibry technique (Bauer *et al.,* 1966). It was Labeled the covers of each of the agar plates with name of the test organisms was inoculated.

- Using sterile technique, inoculated all agar plates with their respective test organisms as follow:
- Dipped a sterile cotton swab into a well-mixed saline test culture and removed excess inoculums by pressing the saturated swab against the inner wall of the culture tube.
- Using the swab streaked the entire agar surface horizontally, vertically, and around the outer edge of the plate to ensure a heavy growth over the entire surface.
- Allowed all culture plates to dry for about 5 minutes.
- Distributed the individual antibiotic discs at equal distance with forceps dipped in alcohol and flamed.

- ❖ Gently pressed each disc down with the wooden end of the cotton swab or sterile forceps to ensure that the discs adhered to the surface of the agar.
- ❖ The plates were then inverted and incubated at 37°c for 24 hours.
- ❖ After incubation, the plates were examined and the diameter of the zones of complete inhibition was measured in mm.
- ❖ The zone diameter for individual anti-microbial agents was used to determine susceptible, intermediate, and resistant categories by referring to an interpreting table

Table 2: Antimicrobial agents with their disc-concentration

Serial number	Anti-microbial Agents	Symbol	Disc concentration (µg/disk)
1	Gentamicin	(GEN)	10 µg/disc
2	Levofloxacin	(LE)	5 µg/disc
3	Cefixime	(CFM)	5 µg/disc
4	Cefoxitin	(COT)	25 µg/disc
5	Erythromycin	(E)	15 µg/disc
6	Azithromycin	(AZM)	30 µg/disc
7	Amoxicillin	(AMX)	30 µg/disc
8	Chloramphenicol	(C)	30 µg/disc
9	Tetracycline	(TE)	30 µg/disc
10	Ampicillin	(AMP)	25 µg/disc

Source: Clinical and Laboratory Standards Institute (2013).

3.17 Interpreting results

After 24 hours of inoculation, each plate was examined if plate was satisfactory streaked, and the inoculum was corrected, the resulting zone of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies were apparent, the inoculum was too light and the test must be repeated. The diameters of the zone of complete inhibition were measured, including the diameter of the disc. Zones were measured to the nearest whole millimeter, using calipers or an inches above a black, non-reflecting background and zones are measured in millimeter from the upper

surface of the agar illuminated with reflected light, with the cover removed (EUCAST,2013).

3.18 Stock culture

After completion of characterization of bacterial pathogens, it was necessary to preserve the isolated organisms for longer periods. For this purpose, pure culture of the isolated *Salmonella* and *Shigella* species were stored in 10% glycerin and used as stock culture. Prior to use glycerin must be autoclaved. The equal volume of 10% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at -48°C. The isolated organisms were given code name for convenience.

Figure 2: Stock culture

CHAPTER 4

RESULTS

The present study was conducted to isolate and identify *Salmonella* and *Shigella* species on human stool and blood samples collected from patients visited at Dinajpur General Hospital, Bangladesh. A total of 33 samples of human blood and stool were processed for isolation and identification of *Salmonella* and *Shigella* species through morphological, staining, cultural characteristics, biochemical test, antibiotic sensitivity pattern and serological test. The result presented in different tables and described below under the following heading: -

4.1 Isolation of bacteria

Two genera of bacteria such as *Shigella* and *Salmonella* species were isolated from human stool and blood samples.

4.2 Identification of bacteria

Identification of bacteria was performed by determining staining reactions, cultural characteristics and different biochemical properties.

4.3 Staining characteristics

The staining characteristics of the isolated organisms were determined according to Gram's staining technique. Morphological and staining characteristics of bacteria recorded from human stool samples by Gram's staining are presented in (Table 3).

Table 3: **Morphological and staining properties of the bacterial isolates of human stool sample by Gram's staining technique**

Serial number	Staining Characteristics			Remarks	Plate number
	Shape	Arrangement	Gram's Staining character		
01	Rod in shape	Single or pair	Gram negative	*Salmonella* spp.	6
02	Rod in shape	Single or pair	Gram negative	*Shigella* spp.	8

4.4 Cultural Characteristics

Cultural characteristics of each type of bacteria isolated from human stool were studied for the determination of colony characteristics in different bacteriological media. The staining property of primary culture of the human stool samples indicated the presence of more than one type of bacteria in the same smear. The pure cultures of the organism from each mixed culture were obtained by repeated streak plate method using different sample and selective solid media for study. The cultural characteristics of *Shigella* and *Salmonella* species exhibited on the media are presented in (Table-4).

Table 4: Identification of isolated bacterial pathogens by cultural properties

Serial Number	Suspected case of Bacteria	Name of Media	Cultural Characteristics	Plate number
01	*Salmonella* species	Salmonella-Shigella agar	Opaque, smooth, round with black centered colonies were found.	2
		XLD agar	Red colonies with black centers	3
		Brilliant green agar	Smooth, white colonies with pinkish background	4
		Hektoen agar	Blue-green colonies with black centre	5
02	*Shigella* species	SS agar	Smooth, round pinkish colonies were found.	6

4.5 Biochemical tests

The isolated organisms were confirmed by different biochemical tests.

Table 5: Result of biochemical test for *Salmonella* spp.

Biochemical test	***Salmonella* spp.**		
	Change of the media	**Results**	**Plate number**
Methyl Red(MR) test	Red color	Positive	9
Voges Proskauer (VP) test	No color change	Negative	10
Triple sugar iron (TSI) test	Slunt=Red Butt=yellow	Slunt=Alkaline Butt=Acid, gas (+), H_2S (+)	11
Motility Indole Urease (MIU) test	No turbidity and no changing of color of media	Negative	13
Indole test	No color change	Negative	12
Citrate utilization test	No color change	Negative	14
Catalase test	Bubbles produced	Positive	15

Table 6: Result of biochemical test for *Shigella* spp.

Biochemical test	***Shigella* spp.**		
	Change of the media	**Results**	**Plate number**
Methyl Red(MR) test	Red color	Positive	16
Voges Proskauer (VP) test	No color change	Negative	17
Triple sugar iron (TSI) test	Slunt -Red Butt -yellow	Slunt -Alkaline Butt -Acid, gas (+), H_2S (+)	18
Motility Indole Urease (MIU) test	No turbidity and no changing of color of media	Negative	19
Indole test	No color change	Positive	20
Citrate utilization test	No color change	Negative	21
Catalase test	No bubble production	Negative	22

4.5.1 Result of catalase test

Catalase test of *Salmonella* and *Shigella* species showing bubble formation indicating positive reaction. No bubble formation indicates negative reaction (Plate 15 & 22).

4.6 Maintenance of stock culture

The stock culture was maintained following the procedures of Choudhury *et al. (*1987). During the experiment it was necessary to preserve the isolated organism for long periods. For this purpose, pure culture of the isolated organisms was stored in sterilized 80% glycerin and used stock culture. The equal volume of 80% glycerin and bacterial culture were mixed and sealed with a paraffin wax and stored in freezer for future use.

4.7 Percentage of isolated pathogens from human stool

The percentage of positive cases of *Salmonella* was 52.7% while the percentage of positive cases of *Shigella* was 28.6%.

Table 7: Percentage of isolated pathogens from human stool

Isolated organism	**Number of collected samples**	**Percentage (%)**	**No. of positive cases**	**Percentage (%) of positive cases**
Salmonella spp.	19	57.6	10	52.7
Shigella spp.	14	42.4	4	28.6
Total	**33**	**100**	**14**	**81.3**

4.8 Prevalence of isolated bacteria from human stool according to sex and age (N=33)

A total of 8 patients were examined (37.5%) (n= 3) and (12.5%) (n= 1) found to be a male positive patients *Salmonella* and *Shigella* respectively. Likewise, out of 11 samples from patients obtained that prevalence was (18.2%) (n=2) and (18.2%) (n=2) were found female in *Salmonella* and *Shigella* (Table 8). An overall prevalence revealed that the prevalence of *Salmonella* and *Shigella* isolated from stool samples related to male and female was (50% and 36.4%) respectively (Table 8). The current study indicated that the

male was higher prevalence than the female, which also indicated the most patients collected from the sample were female. Out of 17 patients 6 were positive according to their age. The age of these participants were ranged 5-15 years found (28.5%) (n=2) and (14.2%) (n=1) and 16-45 years of age obtained of result (30%) (n=3) and (0.0%) (n=0) (Table 8) was found positive in *Salmonella* and *Shigella* respectively. Also the overall prevalence related to age was (42.7% and 30%) of *Salmonella* and *Shigella* respectively. (Table 8)

Table 8: Prevalence of isolated bacteria from human stool according to sex and age

Variable	**Number of Samples examined**	***Salmonella* Positive**	***Shigella* Positive**	**Overall Prevalence**
Sex				
Male	8	3(37.5%)	1(12.5%)	4(50%)
Female	11	2(18.2%)	2(18.2%)	4(36.4%)
Age				
5-15	7	2(28.5%)	1(14.2%)	3(42.7%)
16-45	10	3(30%)	0(0.0%)	3(30%)
Total	**33**	**10**	**4**	**14**

4.9 Prevalence of *Salmonella* species in blood sample

Prevalence of *Salmonella typhi*, *Salmonella paratyphi A* and *Salmonella paratyphi B* using Qualitative slide agglutination reaction results of Widal test was 39.4%, 12.1% and 6.1% respectively (Table 9).

Table 9: Percentage of *Salmonella* species in blood sample

Salmonella species	No. of samples tested	No. of *S.typhi* reactive	% of *S.typhi* reactive	No. of *S.paratyphi* A reactive	% of *S.paratyphi A* reactive	No. of *S.paratyphi B* reactive	% of *S.paratyphi B* reactive
Blood sample	33	13	39.4%	4	12.1%	2	6.1%

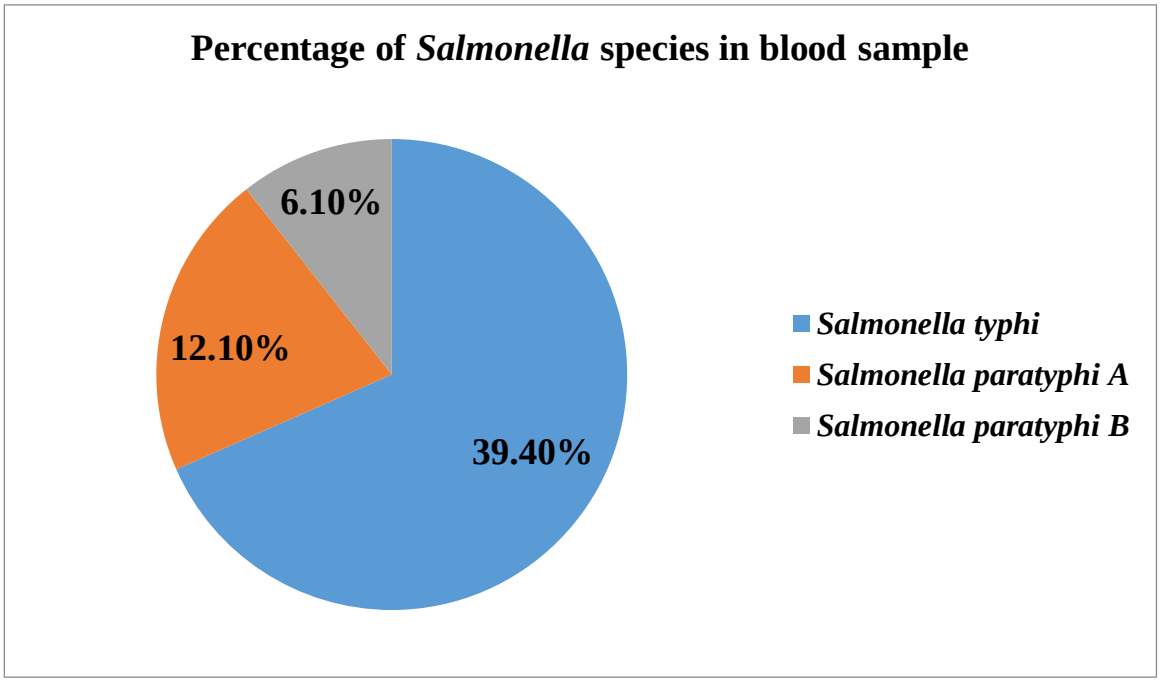

Figure 3: Percentage of *Salmonella* species in blood sample

4.10 Observation of antibiotic sensitivity

Anti-microbial sensitivity testing was performed using Muller Hinton agar (HI-media laboratory PVT ltd) plates as recommended by clinical and laboratory standards institute.

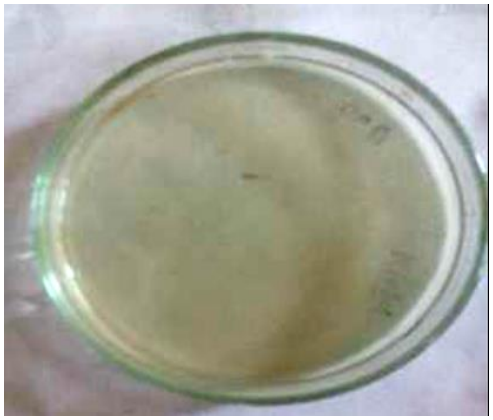

Figure 4: Mueller-Hinton agar

4.10.1 Antibiotic sensitivity of *Salmonella* species

The antibiotic sensitivity test revealed that the isolated *Salmonella* species were sensitive to Gentamicin, Cefixime and Chloramphenicol. The isolates were resistant to Cefoxitin, Erythromycin, Azithromycin, Amoxicillin, Tetracycline and Ampicillin while intermediate to Levofloxacin.

Table 10: Antimicrobial profile of against *Salmonella* species

Organism	Name of the antibiotics	Disc concentration (µg/disk)	Zone of inhibition (mm)	Interpretation
Salmonella species	Gentamicin (GEN)	10 µg/disc	20	S
	Levofloxacin (LE)	5 µg/disc	18	I
	Cefixime (CFM)	5 µg/disc	20	S
	Cefoxitin (COT)	25 µg/disc	0	R
	Erythromycin (E)	15 µg/disc	0	R
	Azithromycin (AZM)	30 µg/disc	12	R
	Amoxicillin (AMX)	30 µg/disc	0	R
	Chloramphenicol (C)	30 µg/disc	22	S
	Tetracycline (TE)	30 µg/disc	0	R
	Ampicillin (AMP)	25 µg/disc	0	R

Note: R=Resistant, S=Sensitive, I=intermediate

4.10.2 Antibiotic sensitivity of *Shigella* species

The antibiotic sensitivity test revealed that the isolated *Shigella* species were sensitive to Gentamicin, Azithromycin and Chloramphenicol. The isolates were resistant to Cefoxitin, Erythromycin, Levofloxacin, Amoxicillin, Tetracycline and Ampicillin while intermediate to Cefixime.

Table 11: Antimicrobial profile of against *Shigella* species

Organism	Name of the antibiotics	Disc concentration (µg/disk)	Zone of inhibition (mm)	Interpretation
Shigella species	Gentamicin (GEN)	10 µg/disc	22	S
	Levofloxacin (LE)	5 µg/disc	10	R
	Cefixime (CFM)	5 µg/disc	18	I
	Cefoxitin (COT)	25 µg/disc	0	R
	Erythromycin (E)	15 µg/disc	10	R
	Azithromycin (AZM)	30 µg/disc	20	S
	Amoxicillin (AMX)	30 µg/disc	0	R
	Chloramphenicol (C)	30 µg/disc	25	S
	Tetracycline (TE)	30 µg/disc	05	R
	Ampicillin (AMP)	25 µg/disc	0	R

Note: R=Resistant, S=Sensitive, I=intermediate

Plate 1: Sample collection

Plate 2: *Salmonella* spp grows on *Salmonella-Shigella* agar produced black colony (left) compared with control petridish (right).

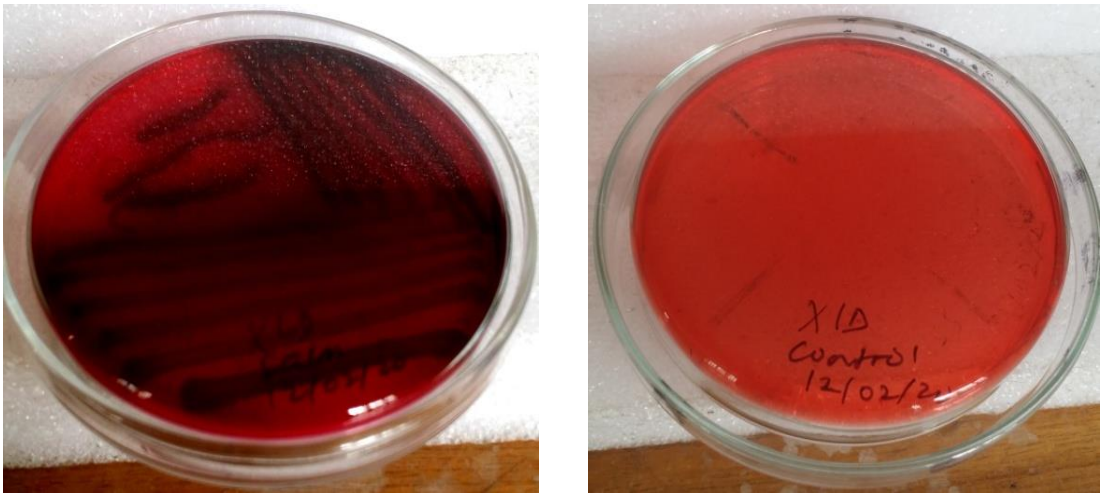

Plate 3: *Salmonella* spp grows on XLD agar produced red colonies with black centers (left) compared with control petridish (right).

Plate 4: *Salmonella* spp grows on Brilliant Green Agar produced white colonies with pinkish background (left) compared with control petridish (right).

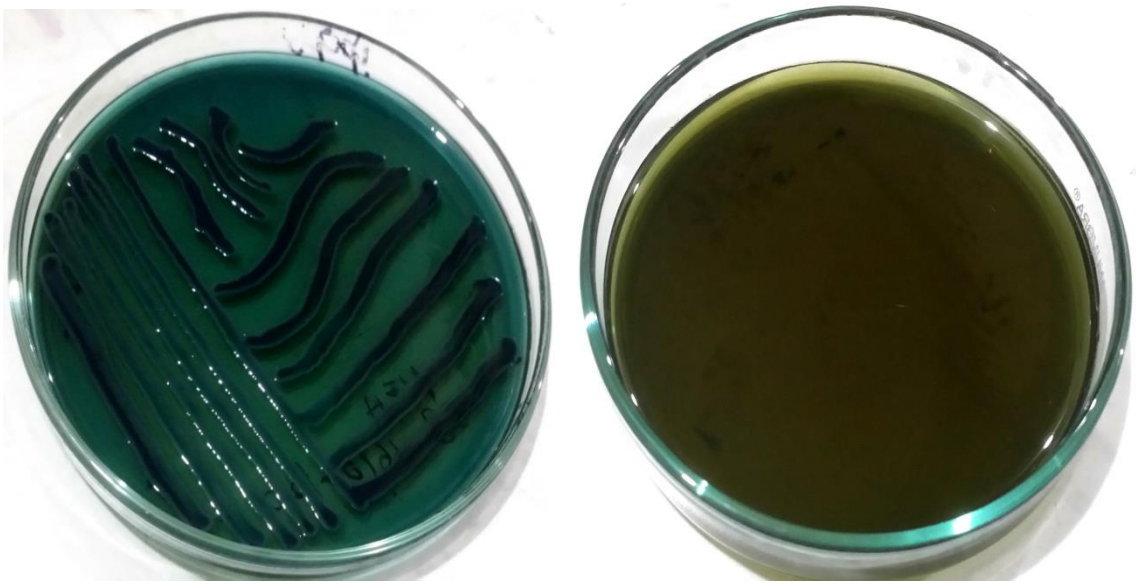

Plate 5: *Salmonella* spp grows on Hektoen Agar produced Blue-green colonies with black centre (left) compared with control petridish (right).

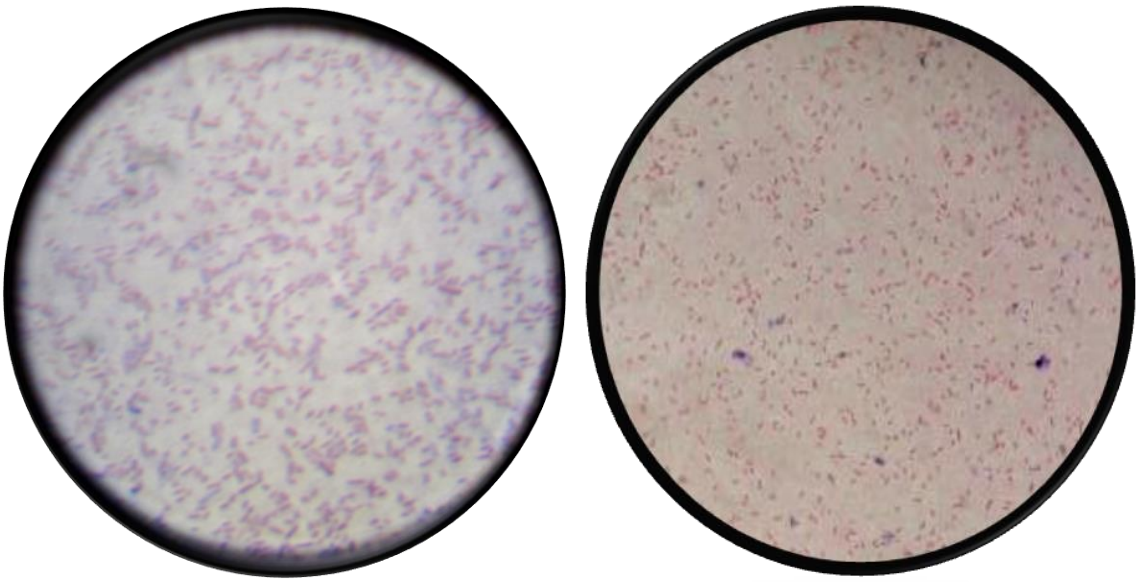

Plate 6: Gram negative *Salmonella* spp, pink colored, very short plump, rods, single or paired seen under microscope

Plate 7: *Shigella* spp grows on *Salmonella Shigella* agar produced pinkish colony (left) compared with control petridish (right)

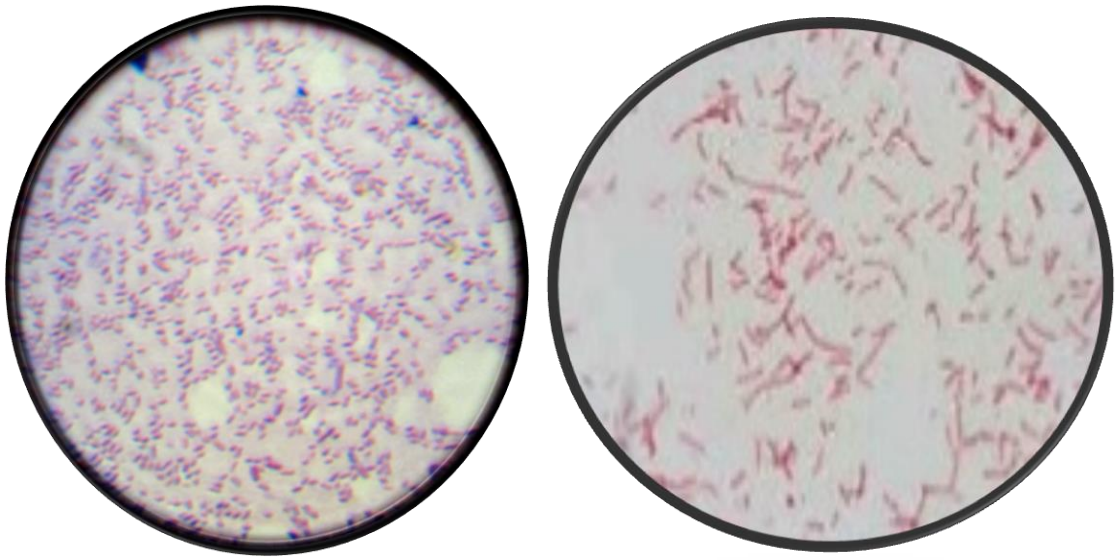

Plate 8: Gram negative *Shigella* species pink colored, very short plump, rods, single or paired seen under microscope

Biochemical test result of *Salmonella*

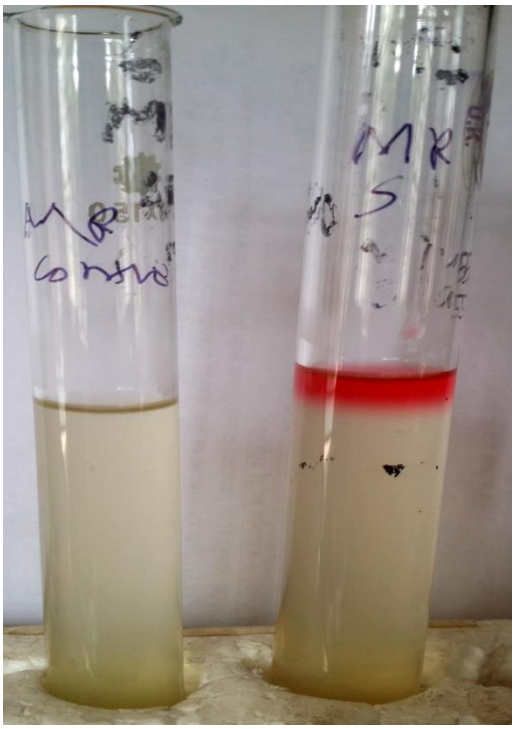

Plate 9: *Salmonella spp* showing positive result (Right) on Methyl Red test with control (Left).

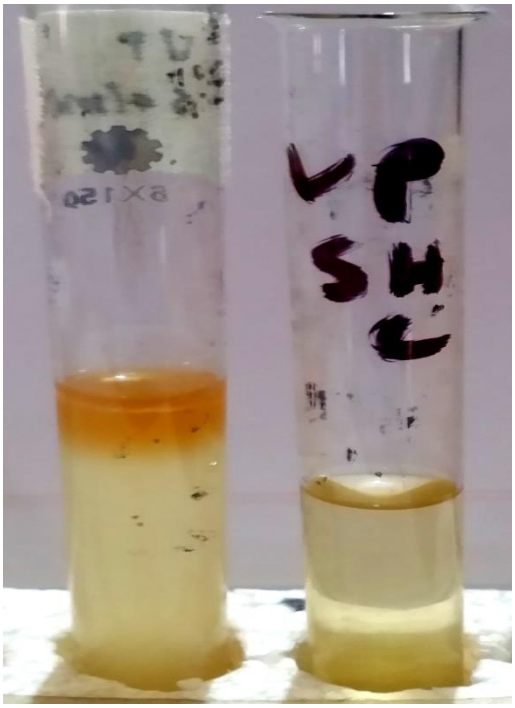

Plate 10: *Salmonella* spp showing negative result (Left) on Voges-Proskauer test with control (Right).

Plate 11: *Salmonella* spp showing positive result (Right) on Triple sugar iron (TSI) test with control (Left).

Plate 12: *Salmonella spp* showing Negative result (Left) on Indole with control (Right)

Plate 13: *Salmonella spp* showing Negative result (Right) on MIU with control (Left)

Plate 14: *Salmonella spp* showing negative result (Right) on Citrate with control (Left)

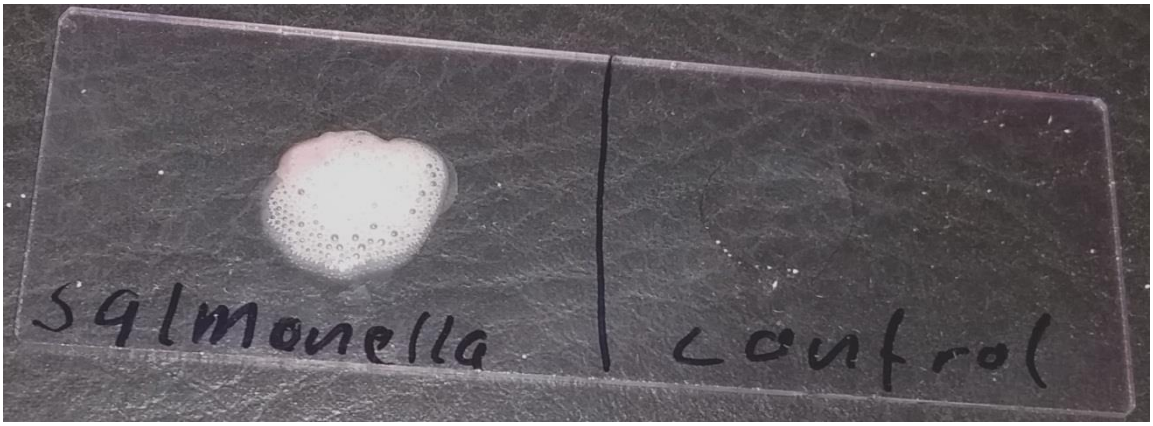

Plate 15: *Salmonella spp* showing positive result (Left) on Catalase with control (Right)

Biochemical test result of *Shigella*

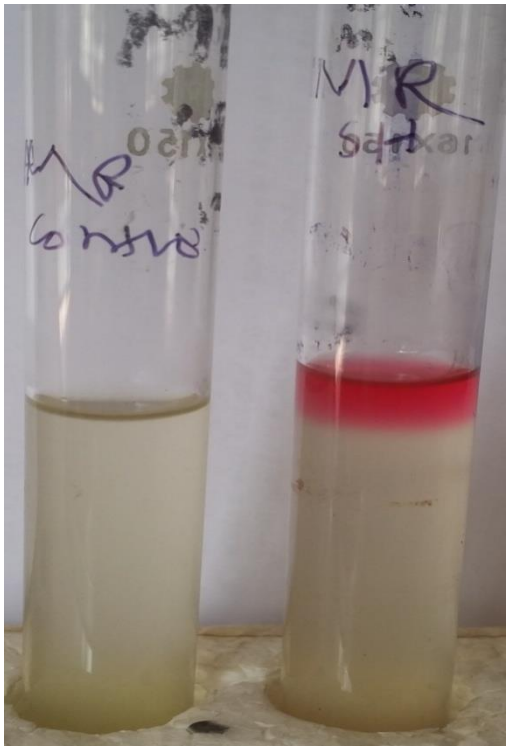

Plate 16: *Shigella* spp showing Positive result (Right) on MR with control (Left)

Plate 17: *Shigella* spp showing Negative result (Left) on VP with control (Right)

Plate 18: *Shigella* spp showing positive result (Left) on TSI with control (Right)

Plate 19: *Shigella* spp showing Negative result (Right) on MIU with control (Left)

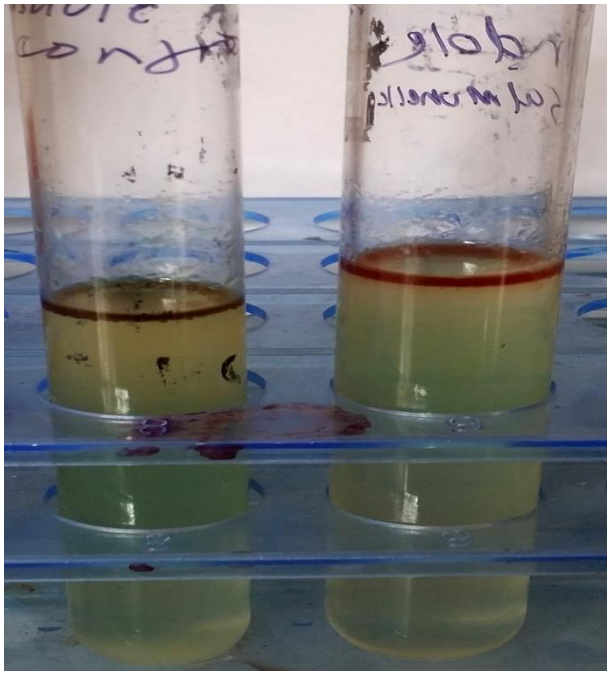

Plate 20: *Shigella* spp showing Positive result (Right) on Indole with control (Left)

Plate 21: *Shigella* spp showing negative result (Right) on Citrate with control (Left)

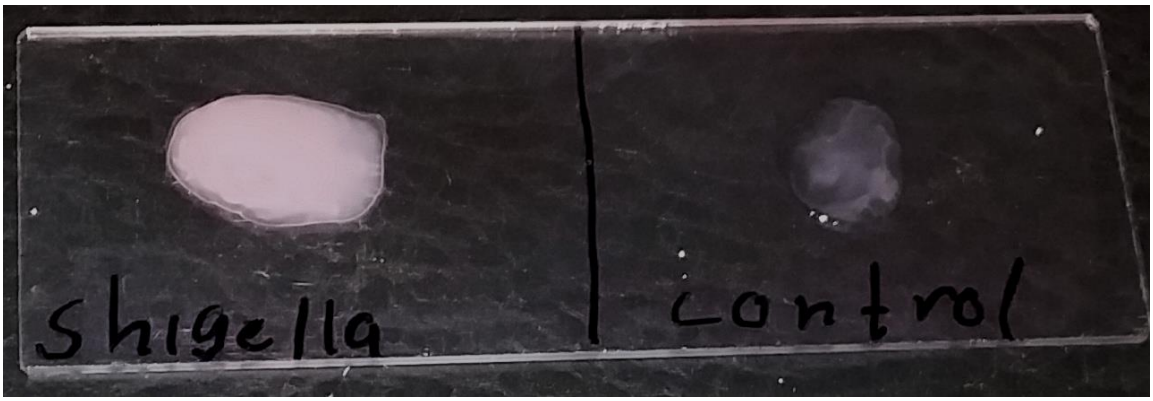

Plate 22: *Shigella* spp showing negative result (Left) on Catalase with control (Right)

Serological test (Widal test)

Figure 5: Widal test reagent

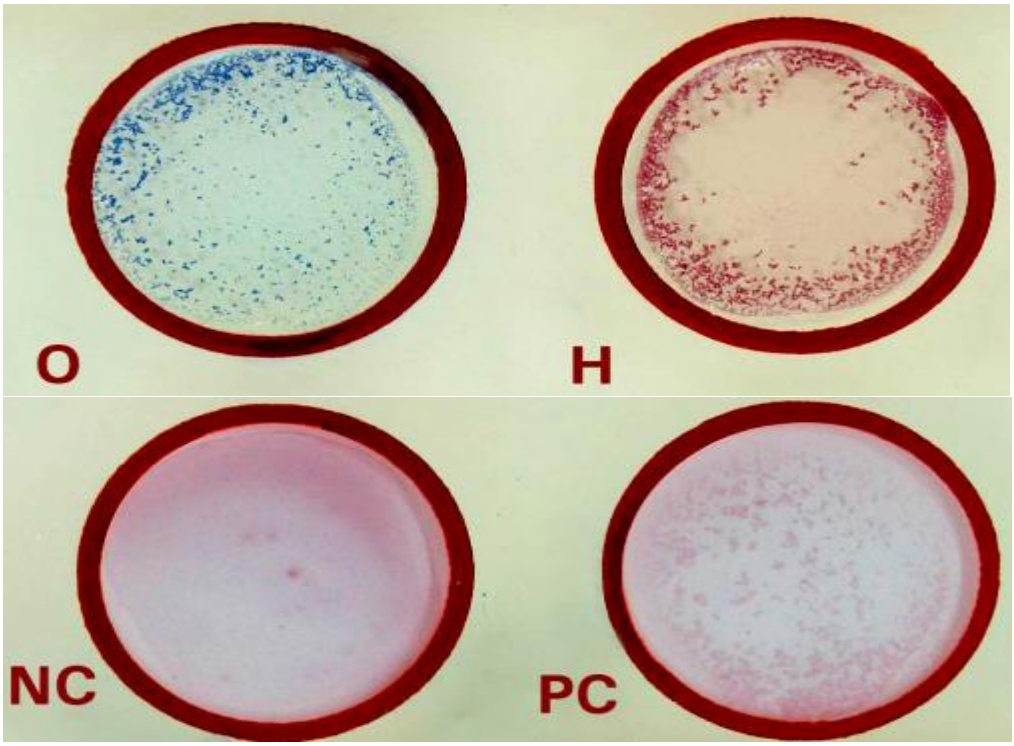

Plate 23: *Salmonella typhi* showing O and H positive with negative and positive controls

Result of antibiotic test of *Salmonella* species

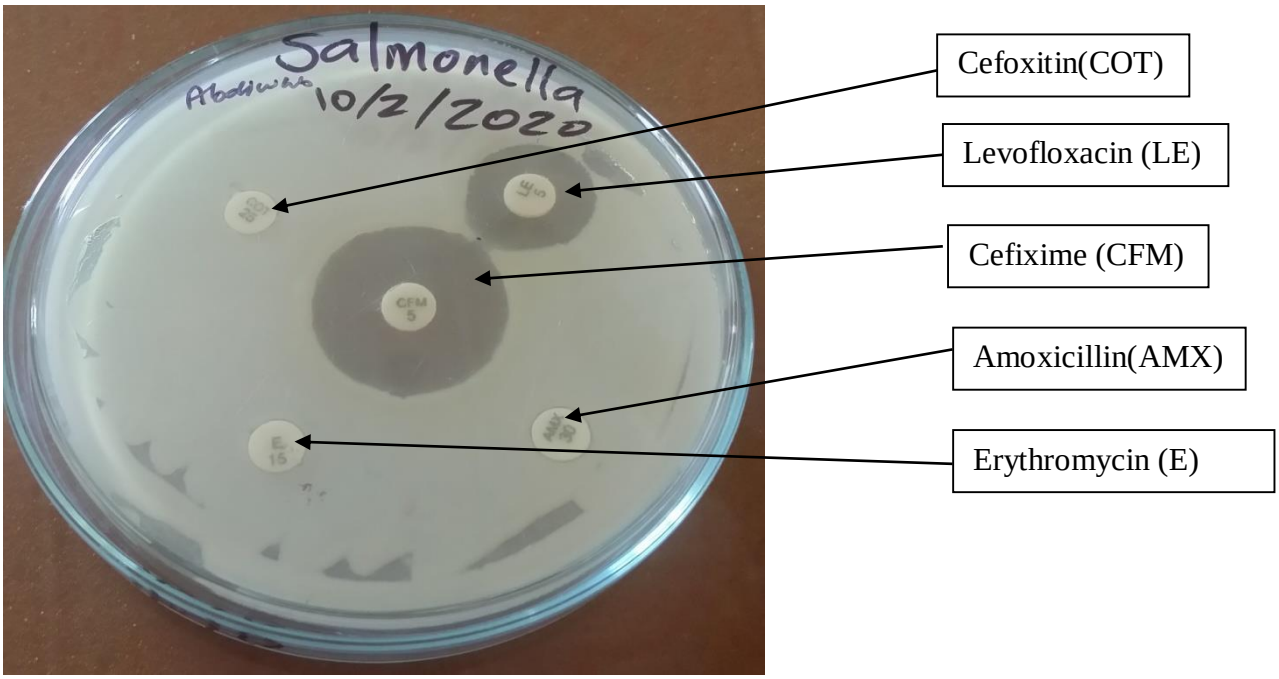

Plate 24: Antibiotic sensitivity test result of *Salmonella* on Muller-Hinton agar

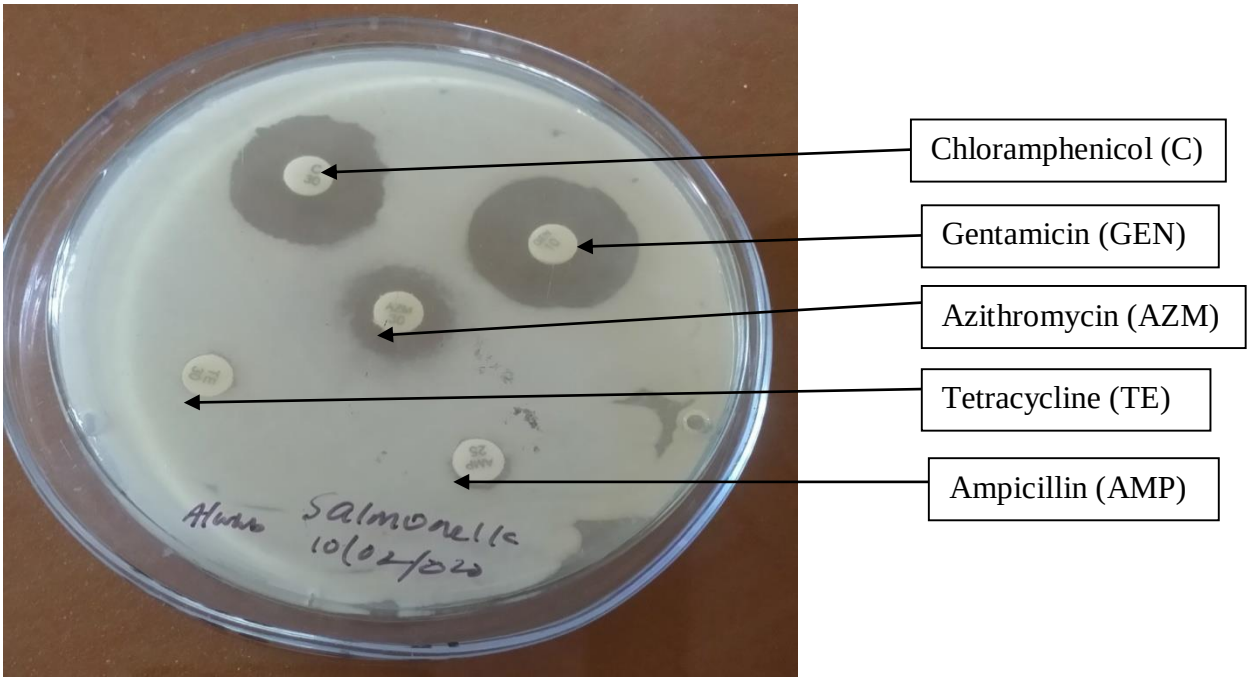

Plate 25: Antibiotic sensitivity test result of *Salmonella* on Muller-Hinton agar

Result of antibiotic test of *Shigella* species

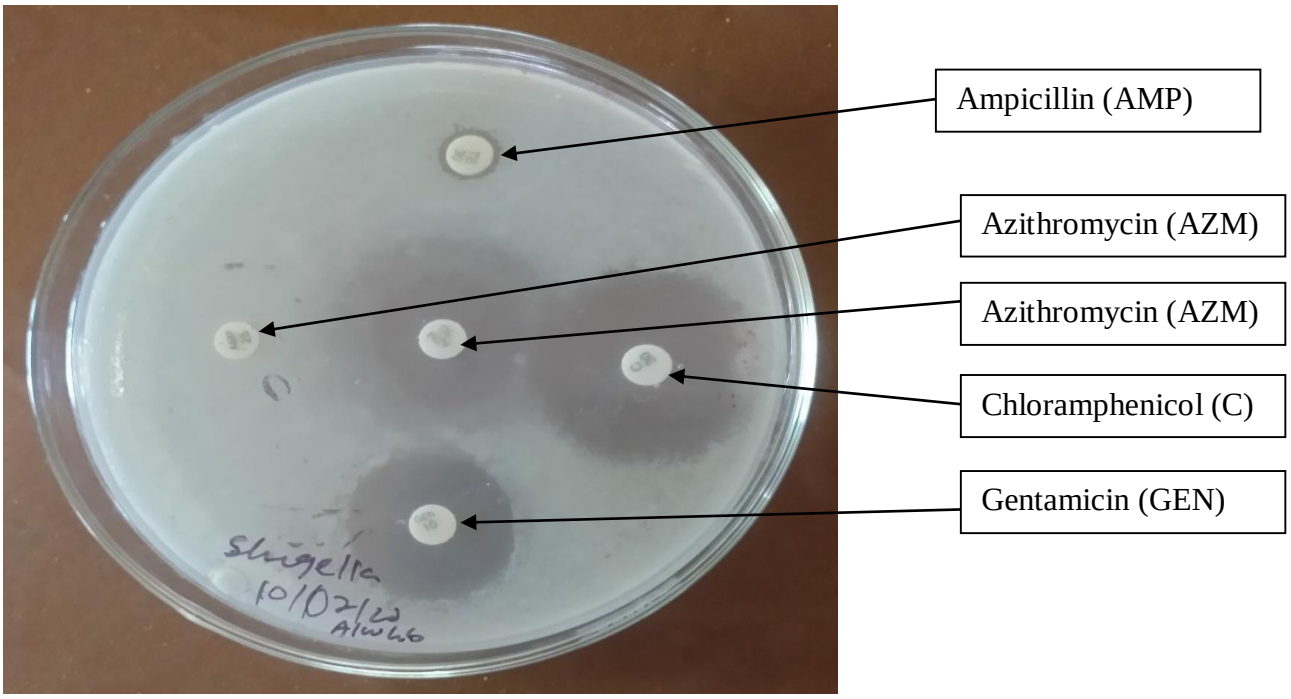

Plate 26: Antibiotic sensitivity test result of *Shigella* on Muller-Hinton agar

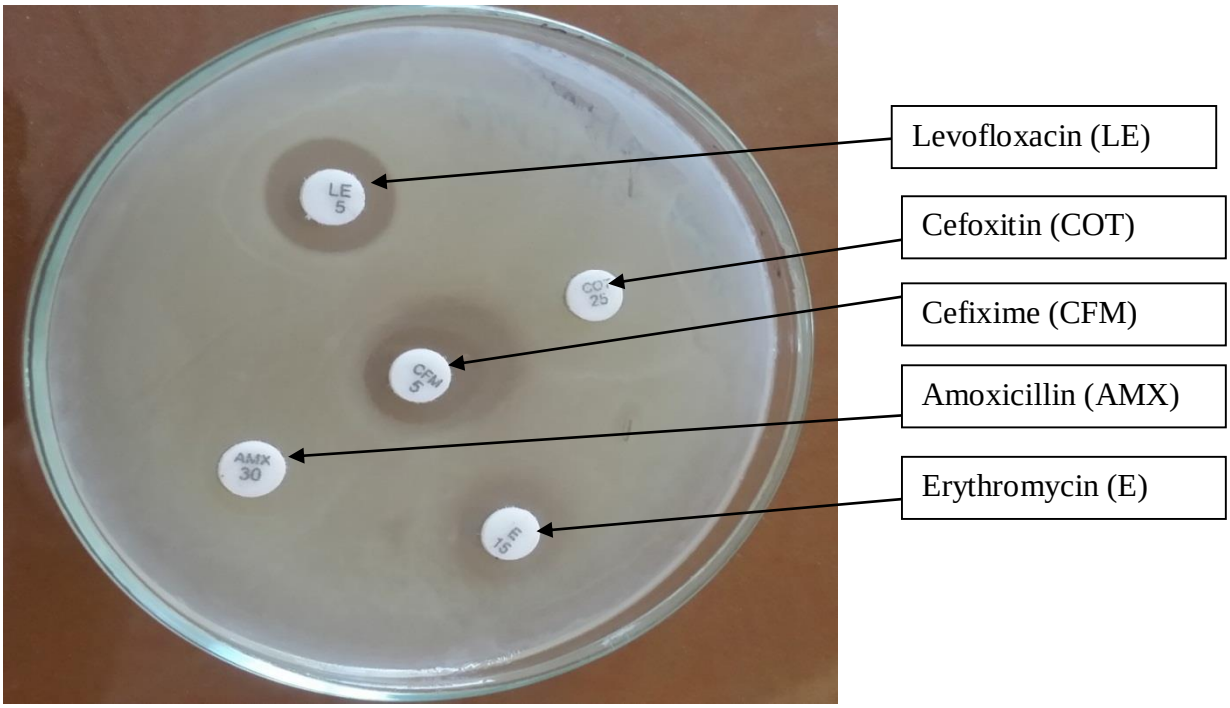

Plate 27: Antibiotic sensitivity test result of *Shigella* on Muller-Hinton agar

CHAPTER 5

DISCUSSION

The present study was undertaken to determine the bacteriological assessment of human stool samples in selected areas in Dinajpur district, during the period from January to June 2020. In this study 33 samples of stool were collected from patients visited at Dinajpur General Hospital in Bangladesh. After the collection, all samples were transported to the bacteriological laboratory of the Department of microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for bacteriological examinations, isolation and identification of microorganisms were confirmed by their colony of different cultural media, staining characteristics and biochemical tests. Similar finding of bacteriological media used in this study were found according to (Buxton and Frazer, 1977).

The current study was used differential and selective media to isolate and characterized of isolated bacteria various based on their growth characteristics on specific culture media (biochemical and gram staining reactions). Accordingly, in this study were isolated two bacterial species of *Salmonella* and *Shigella* were identified as shown (Table 7). This with agreement of Rahman *et al*. (2006) who conducted similar studies that found bacterial pathogens associated with *Salmonella* and *Shigella* can be transmitted to human beings. In Gram's staining, the organisms revealed gram negative, pink colored with rod shaped appearance and arranged in single or in pair were suspected as *Salmonella* and *Shigella* which supported by Daniels, *et al.* (2007) who exhibit gram negative, small rod, single or paired in arrangement under microscope.

According to (Table 5 & 6) the present study indicated that different biochemical tests were used for characterization and further confirmation of two bacterial pathogens of *Salmonella* and *Shigella* was presented positive and negative reactions to indole, Motility Indole Urease, Methyl red, Voges Proskauer, Triple Sugar Iron agar, catalase test. This is inline agreement of Kara *et al.* (2004) who obtained similar findings.

The present study revealed that the prevalence of *Salmonella* and *Shigella* isolated from human stool samples related to male and female was (50% and 36.4%) respectively, presented (Table 8). The current study indicated that the male was higher prevalence than the female, which also indicated the most patients collected from the sample were

female. Out of 17 patients 6 were positive according to their age. The age of the participants was ranged 5-15 years found (28.5%) (n=2) and (14.2%) (n=1) and 16-45 years of age obtained of result (30%) (n=3) and (0.0%) (n=0) (Table 8) was found positive in *Salmonella* and *Shigella* respectively. Also the overall prevalence related to age was (42.7% and 30%) of *Salmonella* and *Shigella* respectively. The present study was partially supported by Akter *et al. (*2012) who found the prevalence of *Salmonella – Shigella* in Bangladesh.

Disc diffusion result of *Salmonella* and *Shigella* were analyzed by using CLSI guideline -2013 and BSAC -2013 Guideline as comparative analysis of antibiotic sensitivity (Table 10 and 11). According to the antibiotic, *Salmonella* species were sensitive to Gentamicin, Cefixime and Chloramphenicol. The isolates were resistant to Cefoxitin, Erythromycin, Azithromycin, Amoxicillin, Tetracycline and Ampicillin while intermediate to Levofloxacin. This is with agreement of (Chiu *et al.,* 2004) who studied antibiotic sensitivity. Similarly, *Shigella* species were sensitive to Gentamicin, Azithromycin and Chloramphenicol. The isolates were resistant to Cefoxitin, Erythromycin, Levofloxacin, Amoxicillin, Tetracycline and Ampicillin while intermediate to Cefixime. This study was strong supported by the (Sheikh *et al.,* 2010).

CHAPTER 6

SUMMARY AND CONCLUSION

This present study was performed in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur. This newly study was conducted for the isolation, identification and determination of cultural characteristics, gram staining, biochemical properties, serological and antibiotic sensitivity of the bacteria isolated from human stool. Samples were inoculated into various types of ordinary and selective media such as Nutrient agar (NA), Brilliant Green Agar, MacConkey (MA) agar, *Salmonella Shigella* (SS) agar and Hektoen Enteric (HE) agar.

Salmonella lives in the intestines of animals or humans. It can be found in water, food, soil, or surfaces that have been contaminated with the feces of infected humans or animals.

Shigella can be passed through direct contact with the bacteria in the stool. For example, this can happen in a child care setting when staff members don't wash their hands well enough after changing diapers or helping toddlers with toilet training. *Shigella* bacteria also can be passed in contaminated food or by drinking or swimming in contaminated water.

Based on the findings of the present study, the following conclusions were made;

I. It is clear that Salmonellosis and Shigellosis are still serious health problems, therefore, it is recommended that the public should be made aware of the modes of transmission, and the need for proper hygiene and sanitation should be emphasized.

II. Since both gender is susceptible to infection by *Salmonella* and *Shigella* species, there is need to target both males and females by public health authorities in control.

III. The present study revealed that the most of antibiotics are resistant to *Salmonella* and *Shigella* patients such as; Cefoxitin, Erythromycin, Amoxicillin, Tetracycline and Ampicillin.

IV. This study suggested that Widal test is not very reliable for the diagnosis of *Salmonella* since false positive and false negative results are common.

V. A national wide study should be carried out to investigate the true prevalence of Salmonellosis and Shigellosis in Bangladesh.

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APPENDICES

APPENDIX 1

Composition of Different Media

1. Nutrient agar (Hi Media)

Ingredients:	**g/L**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 250C)	7.4 ± 0.2

2. MacConkey agar (Hi Media)

Ingredients:	**g/L**
Peptic digest of animal tissue	17.0
Protease peptone	3.0
Lactose monohydrate	10
Bile salt	1.5
Sodium chloride	5.0
Agar-agar	15.0
Neutral red	0.03
Final pH (at 250C)	7.1 ± 0.2

***3. Salmonella Shigella* Agar (Hi Media)**

Ingredients	**Gms / Litre**
Beef Extract	5.00
Enzymatic Digest of Casein	2.50
Enzymatic Digest of Animal Tissue	2.50
Lactose	10.00

Bile Salts	8.50
Sodium Citrate	8.50
Sodium Thiosulfate	8.50
Ferric Citrate	1.00
Brilliant Green	0.00033
Neutral Red	0.025
Agar	13.50

4. Hektoen Enteric Agar (Hi Media)

Ingredients	**Gms/Litre**
Proteose peptone	12.000
Yeast extract	3.000
Lactose	12.000
Sucrose	12.000
Salicin	2.000
Bile salts mixture	9.000
Sodium chloride	5.000
Sodium thiosulphate	5.000
Ferric ammonium citrate	1.500
Acid fuchsin	0.100
Bromothymol blue	0.065
Agar	15.000
Final pH (at 25°C)	7.5±0.2