ISOLATION, IDENTIFICATION AND ANTIBIOTIC SENSITIVITY OF *ESCHERICHIA COLI* AND *SALMONELLA* SPECIES FROM HOSPITAL WASTE WATER IN DHAKA CITY

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This is to certify that the thesis entitled ***"Isolation, Identification and Antibiotic Sensitivity of Escherichia coli and Salmonella species from Hospital Waste Water in Dhaka City"*** *submitted to the Department of Microbiology and Parasitology, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka-1207, as partial fulfillment for the requirements of the degree of Master of Science (MS) in Microbiology, embodies the result of a piece of bonafide research work carried out by* ***MD. SAIFULLAH MAHMOOD,*** *Registration No.:* ***14-06129,*** *Session:* ***JUL-DEC/2019*** *under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

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DEDICATED

TO

MY BELOVED PARENTS

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ABSTRACT

The use of antibiotics in the hospitals for patient care, and disinfection is a global scenario, but it has become a threatening issue for all. Antibiotics are partially metabolized and residual quantities reach into hospital waste water. A cross-sectional study was conducted between January and June 2021 from different hospitals waste water to identify the *Escherichia coli (E. coli)* and *Salmonella* spp. A total of 100 samples were collected for bacteriological analysis. Bacteria were identified using morphological, cultural and biochemical characterization. The *E. coli* showed metallic sheen and pink colored colonies on EMB agar and MacConkey agar respectively. It was gram negative and showed small rod shaped arranged in single or pair shaped. It was noticed as positive to Indole, MR and catalase test but negative to VP test. In case of *Salmonella* spp, it was observed as black smooth colonies on SS agar. These bacteria were observed as short rod-shaped gram-negative bacteria. The MR and catalase test were positive but Indole and VP test were negative. The occurrence of *E. coli* and *Salmonella* spp. were 80% and 87%, respectively. The antibiotic sensitivity test indicated that the both types of isolated *E. coli and Salmonella* spp. were highly resistant to tetracycline (100%). Besides, *E. coli* was resistant to ampicillin (93.75%) and sensitive to ciprofloxacin (65%), streptomycin (62.5%) and gentamycin (93.5%) but *Salmonella* spp. was highly resistant to ampicillin (100%) and sensitive to ciprofloxacin (73.86%), gentamycin (79.54%) and streptomycin (34%). Hospital waste water contained antibiotic resistant bacteria that was reported as alarming voice to public health.

Key Words: Hospital, Wastewater, Antibiotic, Public Health

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LIST OF ABBREVIATIONS

MAC	MacConkey
EMB	Eosin Methylene Blue
SSA	Salmonella-Shigella Agar
MSA	Mannitol Salt Agar
SAU	Sher-e-Bangla Agricultural University
NA	Nutrient Agar
MHA	Muller Hinton Agar
µg	Micro gram
MDR	Multiple drug resistant
NB	Nutrient Broth
%	Percentage
Gm	Gram
VP	Voges Proskauer
+ve	Positive
-ve	Negative
spp	Species
AMR	Anti-Microbial Resistance

CHAPTER 1

Introduction

CHAPTER 1

INTRODUCTION

Waste water refers to any water whose quality has been compromised by human activities. It includes liquid waste discharged from domestic homes, agricultural commercial sectors, pharmaceutical sectors, and hospitals. Hospitals consume an important volume of water a day. Drinking water taken by the people of Bangladesh has become an alarming issue as the sanitation and hygiene are not maintained properly. Contaminated water is a major causal agent for spreading a lot of infectious diseases which are responsible for severe illness even death throughout the country (Majumder *et al*., 2011; Uddin, 2018).

The water consumed in different units of hospital such as in patient wards, operating rooms, laboratories, laundries, kitchens, health services and administrative units decreases it's physical, chemical and biological quality and is converted to wastewater (Mahvi *et al.,* 2006). A variety of substances, such as pharmaceuticals, radionuclides, antiseptics, disinfectants, and solvents are used in hospitals for treatment, medical diagnostics, disinfection, and research. Many non-metabolized drugs excreted from patients and residual chemicals enter into waste water which finally interacts with the microflora of hospital sewage. Waste water causes several diseases like typhoid, fever, dysentery, cholera etc. which are frequently occurring in our country. Water is mainly contaminated by *Escherichia coli (E. coli), Salmonella* spp., *Shigella* spp., *Streptococcus* spp., *Vibrio* spp., *Bacillus* spp., *Pseudomonas* spp. and so on. These kinds of bacteria are causing the above-mentioned diseases and many other illness (Munshi *et al*., 2012). Besides these, antibiotic resistant has become an alarming issue all over the world including Bangladesh. To ensure healthcare facilities, the use of antibiotics has become more frequent and intensive. On the other hand, there has high pressure in the bacterial community are considered as important hotspots for the selection of resistant bacteria (Tesfaye *et al.*,2019). Hospitals also play a crucial role in the dissemination of antibiotic resistant bacteria into the environment. Antibiotic-resistant bacteria may spread through patients but also through wastewater (Hocquet *et al.*,2016).

Recently, in Bangladesh antibiotic resistant *Salmonella* spp. and *E. coli* were detected from pond water and sewage samples respectively by Mahmud *et al.* (2019) and Sobur *et al.* (2019). Previously Zahid *et al.* (2009) reported the occurrence of multidrug resistance (MDR) *E. coli*

in surface water in Bangladesh. Antibiotics have been instrumental for treatment and prevention of infectious diseases in veterinary and human treatment. The global need of antibiotics has continued to ameliorate since their introduction several years ago. It is observed that the global antibiotic consumption is increased by 65% over a period of 16 years between 2000 and 2015 (Klein *et al.,* 2018). The indiscriminate use of antibiotics has led to an increased global concern due to their detection in different environmental area frequently and the probable risks to humans and the environment. Antibiotics use in Bangladesh for both human medicine and livestock production is not well-regulated. This increases the drug misuse and consequent transfer to the environmental area particularly through hospital waste water. In Bangladesh, sewage and water treatment system is not well developed. Various types of clinics and hospitals are often established near the water body could be the major source of antibiotics in aquatic environments (Siddiqui *et al*., 2015).

Sewerage network of Sher-e-Bangla Nagar, an area having a number of hospitals located in the center of Dhaka City, consists of several drains containing waste water, connected with Turag and Buriganga river. Therefore, when the hospitals discharge their healthcare liquid waste into sewerage network is mixing with the domestic sewage before coming in contact with surface water without proper treatment. The poor people of Dhaka City are using these rivers' water for various purposes and suppling to their domestic animals. In majority cases, waste water containing multi drug resistant (MDR) *E. coli* and *Salmonella* can act as a vehicle to disseminate antibiotic resistance to other bacteria (Siddiqui *et al.,* 2015). On the other hand, arthropod vectors may transmit the resistance bacteria from drain to open food unhygienically prepared besides the roads, rivers or other natural water sources carrying waste water, which can definitely cause infection in human and animal bodies and become a threat for public health. However, there has not such reliable data related to this area regarding the issue of waste water to make people conscious.

On the above circumstances the present study was undertaken with the following objectives:

i. To isolate and identify the *E. coli* and *Salmonella* spp. from waste water samples

ii. To study the occurrence of zoonotic infection in waste water samples

iii. To assess the antibiotic resistance pattern of *E. coli*, and *Salmonella* spp.

CHAPTER 2

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

The purpose of this chapter is to provide a selective review of the works related to the present study characteristics and antibiogram profiles of *E. coli and Salmonella* recovered from hospital waste water. The literatures that were reviewed aiming to support the present research work are briefly mentioned here.

2.1 Water borne disease challenge

Outbreaks of waterborne pathogens are complex phenomena which is most often attributed to a wide variety of underlying etiologies related to climate, geography, and human factors. In under developed countries, waterborne outbreaks usually occur because of inadequacies in treatment of contaminated water or premise plumbing, with a large proportion of the recent outbreaks associated with *E coli* and *Salmonella.*

Collier *et al.* (2021) estimated total illnesses, emergency department (ED) visits, hospitalizations, deaths, and direct healthcare costs for 17 waterborne infectious diseases. About 7.15 million waterborne illnesses occur annually (95% credible interval [CrI] 3.88 million–12.0 million), results in 601,000 ED visits (95% CrI 364,000–866,000), 118,000 hospitalizations (95% CrI 86,800–150,000), and 6,630 deaths (95% CrI 4,520–8,870) and incurring US $3.33 billion (95% CrI 1.37 billion–8.77 billion) in direct healthcare costs.

Kim *et al.* (2010) stated *Salmonella enterica* has been one of the most widespread foodborne pathogens in Korea. Between 1998 and 2007, a total of 9,472 *Salmonella* isolates were identified from foodborne and waterborne illness patients.

Howard *et al*. (2004) stated that, though wastewater and drinking water are treated to eliminate pathogenic microorganisms and prevent waterborne transmission, numerous studies indicate that conventional wastewater treatment does not guarantee their complete elimination. Research group found treated water to contain a *Salmonella* MPN of 45/100 ml.

Sundstrom *et al.* (1997) stated that from the time immemorial, water has been both man's benefactor and his curse. He also mentioned water as a renewable resource delineating its supply, consumption, quality and analysis.

2.2 *Escherichia coli (E. coli)*

2.2.1 Historical background

E. coli which is known "Bacterium coli" was first isolated from a 2-3 days old new-born baby's stool and subsequently from young calves in 1885 by Theodore Escherich (Buxton and Fraser,1977; Sousa,2006). The name of bacteria was later transformed to the honor of its discoverer (Feng *et al.,* 2002).

2.2.2 Growth

E. coli is a facultative anaerobe growing from 7ºC to 50ºC with an optimum temperature of 37ºC. Although there have several reports of some strains which mainly grow at temperatures as low as 4ºC (Adams and Moss, 2008). The optimal pH for its growth is near neutral but growth of it is also possible down to pH 4.4 under optimal conditions. The minimum water activity for growth is 0.95 (Adams and Moss, 2008).

2.2.3 Biochemical properties

E. coli can be differentiated from other members of the Enterobacteriaceae on the basis of a number of biochemical and other sugar-fermentation tests. An important group of tests are maintained for this purpose are found by the acronym IMViC. These are tested for the ability to grow: indole from tryptophan (I); sufficient acid to decrease the medium pH under 4.4, the break point of the indicator methyl red (M); acetoin (acetyl methyl carbinol) (V); and the capability to use citrate (C) (Adams and Moss, 2008). Though *E. coli* can be determined with a variety of biochemical reactions, the indole test is found as the most useful method to separate lack of production of β-glucuronidase. Sorbitol non fermenting strains of *E. coli* O157:H7 have been associated with colitis and hemolytic uremic syndrome (HUS) (Besser *et al.,* 1999).

2.2.4 *Escherichia coli* and public health significance

Certain strains of *E. coli* produce toxins, which turns to hemorrhagic enteritis (Ramachandran and Varghese, 1987). Kulshrestra (1978) recovered a total of 240 *E. coli* isolates from fecal samples from four hospitals in 1995. It was noted that *E. coli* isolates produced LT (heat labile);

34 ST (heat stable) and 28 produced both LT and ST enterotoxins. In that study, *E. coli* serogroups O2, O7, O17, O35, O127 and O128 produced verotoxin, which were closed with the hemorrhagic enteritis. Serotype O9 has firstly been isolated from human peritoneum (most often appendicitis peritonitis), human urine, urinary infection, mastitis and coli granuloma disease (Anita Kumari *et al.*, 2002). Serotype O9 was found highly enterotoxigenic and produce enterotoxin (Moon and Whip, 1970; Harnet and Gyles, 1985). Serotypes O155, O156 and O109 have earlier been isolated from fecal samples of human being (Orskov *et al.*, 1977), serotype O156 has been reported to be enterotoxigenic. Serotypes O2, O4, O6, O18 and O75 are considered common in urinary tract infection (Cooke, 1985). *E. coli* is considered to be responsible for 70-95% of urinary tract infection (Delisle and Ley, 1989). Kapoor and Kul Shrestha (1998) isolated *E. coli* from 70 samples (60.68 %) out of 115 patients of urinary tract infection at different hospitals nearby IVRI (UP, India). It has been shown that *E. coli* can be isolated from as many as 77% cases of UTI. Only 57 isolates of *E. coli* (81.42 %) could be serotyped. The frequency of serotypes isolated in descending order was O6 (six isolates),O1, O2, O4 (five each), O8 (4), O20, O131 (three each) and O78, O26, O55, O69, O85,O11, O140 (two each) while the remaining 12 serotypes (O5, O11, O23, O24, O35, O41,O44, O53, O71, O75, O86 and O128) were one each (Kapoor and Kul Shrestha, 1998). Singh *et al.* (1996) isolated serotypes O8, O77, O112, O147 and O165 from 200 attendants in Bihar. Serotype O139 observed connected with oedema disease as first case of oedema was diagnosed in Denmark in 1994 caused by *E. coli* strain of serogroup O139 (Aarestrup *et al.*, 2001). Yasuoka *et al.* (2002) obtained vaginal *E. coli* (VEC) from Japanese women were distributed into 31 serotypes, including common serotypes O1, O4, O6, O18, O25 and O75 that were identified in three or more isolates, supporting the concept VEC are a reservoir along the faecal-vaginal urinary/neonatal‖ course of transmission in the extra intestinal *E. coli* infection.

2.3 *Salmonella spp.*

Salmonellosis is one of the most common and widely distributed food borne diseases. It constitutes a major public health burden and represents a significant cost in many countries. Millions of human cases are reported worldwide every year and the disease results in thousands of deaths (WHO, 2005). *Salmonella* infections are mainly asymptomatic in poultry, but are associated with widespread human illness from this source. Therefore, there is continuing

interest in finding ways of preventing flock infection and hence contamination of poultry products with *Salmonella* (Saeed *et al.*, 1999).

Salmonella enterica-associated gastroenteritis is an important food borne human disease. Most serotypes are capable of infecting a variety of animal species, including humans. There is considerable variation with time and geographical location in serotypes commonly associated with human Salmonellosis notably *S. enterica* serovar *Typhimurium* and *S. enterica* serovar Enteritidis (Cormican *et al.*, 2002), serotype Typhimurium is responsible for various disease manifestations, usually in the form of mild gastroenteritis with low mortality, but it can cause septicemia with high mortality (Scalzoe *et al.*, 2004).

2.3.1 *Salmonella* general characteristics, nomenclature and habitat

Salmonella is a Gram-negative facultative anaerobic rod-shaped bacterium in the family of Enterobacteriaceae which is known as enteric bacteria. *Salmonella* is a motile bacterium with the exception of *S. gallinarum* and *S. pullorum*; they are all non-spore forming. There are over 2500 serotypes of *Salmonella* (WHO, 2005). Different strains of *Salmonella*e have been determined and these are classified into groupings called serovars on the basis of their antigens (Snoeyenbos, 1994). The latest nomenclature which indicates recent advancement in taxonomy, in the genus *Salmonella* includes only two species: *S. enterica* and *S. bongoriS* (Cooper, 1994). *Salmonella* enterica is grouped into six subspecies which are separable by specific biochemical characteristics. Strains of *Salmonella* are classified into serovars based on extensive diversity of lipopolysaccharide (LPS) antigens (O) and flagellar protein antigens (H) in accordance with the Kauffmann–White scheme.

Salmonella has several hosts. Although primarily intestinal bacteria of animals and birds, *Salmonella* are widespread in the environment and commonly observed in farm effluents, human sewage and in any material closed to fecal contamination and are transmitted to humans by poisonous foods of animal origin (Acheson &Hohmann, 2001). Some serovars show mentionable host like *Salmonella* typhi and *Salmonella gallinarum* are generally found in humans and birds, respectively (Jorgensen, 2001). Epidemiological and bacteriological evidence mentioned that these animals may transmit the infection to human (Handeland *et al.*, 2002)

Cheesbrough (1985**)** noted that on SS and MAC agar *Salmonella* produce lactose nonfermenting colony. Most strains shows blackening of the colony due to Hydrogen Sulfide production.

2.3.2 Control of Salmonellosis

Salmonella enterica remains one of the most important food borne pathogens of humans and is often acquired through consumption of infected poultry meat or eggs. Control of Salmonella infections in chicken is therefore an important public health issue, three types of typhoid vaccines are currently available for use: (1) an oral live attenuated vaccine, (2) a parenteral heat-phenol-inactivated vaccine, (3) a newly developed capsular polysaccharide vaccine for parenteral use, a fourth vaccine, and an acetone-inactivated parenteral vaccine are available only to the armed forces in USA (Beal *et al.,* 2004). Hazards from *Salmonella* can be prevented by heating food sufficiently to kill the bacteria, holding chilled food below 4.4 ºC, preventing post-cooking cross contamination and prohibiting people who are ill or are carriers of *Salmonella* from working in food operations (Ward *et al.,* 1997). *Salmonella* surveillance and control of poultry industry at slaughter should be done to identify infected flocks as regulatory procedures for food safety and security program (Veling *et al.,* 2002).

2.3.3 **Public health importance of *Salmonella***

Bertrand *et al.* (2010) reported that all clinical isolates under study caused self-limiting gastroenteritis; both genders and all age groups. The isolates were distributed throughout Belgium but a cluster of several cases was observed around Brussels. At the same time, an increase in the incidence of this serovar was observed in the *Salmonella* isolates originating from the official surveillance campaign conducted by the Federal Agency for the Safety of the Food Chain, which identified pork as a likely source of the outbreak strain.

Kelly *et al.* (2009) constructed a quantitative model to estimate the probability of food containing eggs produced on the island of Ireland contaminated with *Salmonella*. Both external and internal contamination of the eggs by *Salmonella* was considered. The model estimates that there is a 90% chance that the probability of a serving of food being contaminated is between 0.0043% and 0.038%. These results indicate the importance of maintaining the low prevalence

of contaminated eggs at the time of lay to minimize the risk of human cases of Salmonellosis from consumption of eggs.

Kam *et al.* (2007) collected one hundred thirty-four isolates of *Salmonella* enterica serotype typhi from 17 public hospitals and clinics in Hong Kong from 2000 to 2004 were studied in relation to epidemiological and clinical events. Isolates originated from 80 patients, with 29 patients providing multiple isolates.

Zhang *et al.* (2006) stated that *Campylobacter* and *Salmonella* are the most commonly reported bacterial causes of human food borne infections, and increasing proportions of these pathogens become resistant to medically important antimicrobial agents, imposing a burden on public health.

Cherry *et al.* (2004) reported an outbreak of *S. enterica* serovar *typhimurium* in a veterinary clinic in New York, USA. They confirmed the bacteria in one cat, two veterinary technicians, and four persons associated with clinic patients and a nurse not related to the clinic. This outbreak emphasized the importance of strong public health ties to the animal health community.

Tsai *et al.* (1998) reported that the average numbers of micro-organisms in waste-water sludge from three local hospitals were: total count $8{\cdot}1\times10^{7}$ cfu g^{-1}, total coliform $1{\cdot}4\times10^{6}$ cfu g^{-1}, average faecal coliform $3{\cdot}6\times10^{5}$ $cfug^{-1}$ and faecal streptococci $1{\cdot}6\times10^{5}$ cfu g^{-1} where *Pseudomonas aeruginosa* $2{\cdot}2\times10^{5}$ $cfug^{-1}$ and *Salmonella* spp. $5{\cdot}5\times10^{5}$ cfu g^{-1}. The frequency of *Salmonella* spp. was found in sludge samples was 37% (10 out of 27 samples). For this reason, public health could be seriously threatened if the sludge from hospital waste-water was not properly treated.

2.4 Occurrence and Antimicrobial sensitivity pattern of *Escherichia coli and Salmonella spp.* isolates

Bojar Brandon *et al.* (2021) observed Antibiotic susceptibility of 201 isolates which were analyzed against eleven common antibiotics and the presence of twelve antibiotic resistant genes. AMR showed the following pattern: Hospital wastewater (37.8%) > urban post-chlorinated effluent (27.6%) > pre-chlorinated effluent (21.4%) > urban influent wastewater (13.3%). Besides, multi-drug resistance against three or more antimicrobial classes was more

prevalent among hospital wastewater populations (29.7%) compared to other sources. *E. coli* from wastewaters disinfected with chlorine were significantly correlated with increased trimethoprim-sulfamethoxazole resistance in *E. coli* compared to raw and treated wastewater populations.

Birosova *et al.* (2020) tested how the adaptability of the model microorganism *Salmonella enterica* is affected by wastewater full of pharmaceuticals, illicit drugs, and other micropollutants. Wastewater samples had been taken from effluent of hospitals and from wastewater treatment plant (WWTP) Petržalka influent and effluent. In these samples, presence of 38 substances was monitored. The highest concentration was observed in case of tramadol, citalopram, venlafaxine, cotinine, atenolol, valsartan, carbamazepine, azithromycin, and ciprofloxacin.

Akhter *et al. (2018)* identified E. coli from hospital waste water and their antibiotic resistant pattern against frequently used antibiotics. Out of 10 samples, they identified 10 isolates of *E. coli*. they used 8 commonly used antibiotics against the isolated bacteria. Finally, they observed that most of the isolated bacteria showed resistance to antibiotics. Among the 10 isolates, 100%, 80%, 80%, 70%, 30%, 20%, 20% and 10% isolates were resistant to ampicillin, ceftazidime, cefotaxime, tetracycline, chloramphenicol, gentamycin, ciprofloxacin and azithromycin antibiotics respectively.

Osinska *et al*. (2017) analyzed the virulence of isolated *E. coli* strains and determined their clonal relatedness with ERIC (enterobacterial repetitive intergenic consensus sequence) PCR. The highest counts of bacteria resistant to tetracyclines and fluoroquinolones were noted by them in UWW at 6.4×10^4, 4.2×10^4 and 3.1×10^3 CFU/Ml, respectively. They selected a total of 317 *E. coli* isolates resistant to at least one group of antibiotics among bacterial isolates from river water and wastewater samples. About 38% of those isolates were resistant to all of the tested antibiotics. The highest percent (43%) of MDR was noted in UWW samples from different sources.

Juthi *et al.* (2016) observed that, 9 tap water samples of 20 areas contained fecal coliform in summer and 13 were in fall where Tap water samples of 7 areas showed fecal coliform both in summer and in fall. Besides, out of 40 samples, 22 samples contained fecal coliform and fecal

coli-form bacteria, Coliform such as *Escherichia coli, Shigella* spp. and others bacteria were also present. Out of 40 samples of 20 areas, 23 samples of 16 areas, 7 samples of 5 areas and 3 samples of 3 areas showed the growth of *E. coli, Shigella* spp. and *Salmonella* spp. respectively.

Shokoohizadehd *et al.* (2015) collected 44 waste water samples in total from effluent and influent waste waters from three hospitals waste water treatment plant and transferred to the microbiology laboratory. They identified *E. coli* isolates with conventional microbiology methods. Genomic DNAs of *E. coli* were extracted; EHEC and ETEC strains were detected by PCR methods using specific primers. Based on microbiologic tests results, they found the frequency of *E. coli*, *Citrobacter* and *Enterobacter* in wastewater samples were 70.5, 8.82 and 20.58%. ETEC strains were found in 42.58, 28.6 and 33.3% wastewater samples in Imam, Kermanshahi and Farabi hospital respectively.

Hassan *et al. (*2015) investigated two bacterial isolates such as *E. coli* and *Salmonella* from six medical hospitals, five veterinary hospitals and five slaughter houses from Bangladesh were isolated to find out the antibiotic resistance pattern by using disc diffusion method. The antibiotic resistance patterns of identified isolates showed that Ampicillin, Ciprofloxacin, Enrofloxacin, Pefloxacin, Colistin, Erythromycin, Oxytetracycline were 100%, Doxycycline was 83%, Gentamycin was 50% and Neomycin was 33% resistance to medical isolates and Ampicillin, Enrofloxacin, Pefloxacin and Erythromycin were 100%, Ciprofloxacin was 40%, Colistin was 60%, Doxycycline was 80%, Gentamycin was 20%; Neomycin and Oxytetracycline 80% resistance to veterinary hospital isolates and Ampicillin, Enrofloxacin, Ciprofloxacin, Pefloxacin, Colistin, Oxytetracycline, Gentamycin, Doxycycline and Erythromycin were 100% and Neomycin was 40% resistance to slaughter houses isolates of *E. coli*. The level of resistance of *Salmonella* positive isolates was found Ampicillin, Enrofloxacin, Pefloxacin, Gentamycin and Erythromycin to 100%, Ciprofloxacin was 67%, Oxytetracycline was 33% but Colistin and Neomycin was found sensitive to the isolates from both medical and veterinary hospital. Results indicated that hospitals and slaughter houses waste effluent had multiple-antibiotic resistance against *E. coli* and *Salmonella.*

Fekadu *et al*. (2015) detected pathogenic (*Salmonella*, *Shigella*, and *S. aureus*) and potentially pathogenic (*E. coli*) bacteria from effluents of hospitals in Ethiopia. Dilution demonstrated tincture iodine to be the most effective agent, followed by sodium hypochlorite; the least active

was 70% ethanol. MIC for ethanol against S. aureus and Gram-negative rods from Yirgalem Hospital (YAH) showed 4 and 3.5 log reduction, respectively. Salmonella isolates from YAH effluent were resistant to ceftriaxone, tetracycline, and doxycycline. Isolates from Hawassa University Referral Hospital (HURH) effluent were resistant to the above three antibiotics as well as gentamycin.

Gupta *et al.* (2014) analyzed *Salmonella typhimurium* strains TA 98, TA 100 and TA 102 for their sensitivity to hospital waste waters. The results of the study showed that hospital waste water consists of mutagens causing frame shift mutations and base pair substitutions and amongst the three strains used in this study, TA 102 was most effective which along with TA 98 can be used for quick assessment of genotoxicity of hospital waste waters prior to its discharge.

Afroz *et al.* (2014) collected a total of 168 isolates of *S. enterica serovar* Typhi and 160 isolates of *S. enterica* . The average prevalence rate of *Salmonella* in the blood was 9.15%. Young patients, neonates, and elderly individuals were more prone to *Salmonella* infection than other patients, and females were more susceptible to *Salmonella septicemia* than males. Among *Salmonella* spp. isolates, 20.92% were multidrug resistant and showed high resistance against amoxicillin, ciprofloxacin, nalidixic acid, and chloramphenicol. Resistance rates to cefipime, cefixime, and ceftriaxone were increasing slowly.

Farhima *et al.* (2012) observed the influence of the most frequently prescribed antibiotic, fluoroquinolone (72%), on the development of antibiotic resistance in *Escherichia coli*. Out of 300, 24 ciprofloxacin resistant *E. coli* isolates from waste water were selected by them for the study that showed the MBC_{100} higher than expected (600 μg/mL).

Akubuenyi *et al.* (2011) observed the antibiotic resistance profile of bacterial isolates obtained from the wastewaters of the University of Calabar Teaching Hospital (UCTH) and the General Hospital Calabar (GHC), Cross River State, Nigeria, was determined using the disc-diffusion method. A total of 125 bacterial isolates from both hospitals` wastewater comprising of the following genera: *Escherichia*; *Salmonella*; *Shigella*; *Klebsiella; Pseudomonas*; *Streptococcus*; *Bacillus*; *Staphylococcus* and *Proteus*, were tested for their antibiotic resistance capability. Data obtained showed that all the isolates from both hospitals had multiple antibiotic resistance

(MAR). Over fifty five percent of the isolates from UCTH and 12.5% of the isolates from GHC exhibited resistance to the antibiotics tested (amoxycilin, gentamycin, augumetin, chloramphenicol, erythromycin, tetracycline, ciprofloxacin, streptomycin, and cotrimoxazole). Amongst the UCTH isolates, 5 different antibiotic patterns were observed ranging from 6-12 MAR combinations while 8 different antibiotic resistance patterns ranging from 4-12 MAR combinations were obtained from the GHC isolates. All the UCTH isolates were resistant to the antibiotics commonly used in the hospital (amoxicillin, augumentin, chloramphenicol, gentamycin, erythromycin, tetracycline, ciprofloxacin, streptomycin and cotrimaxazole) except Escherichia coli and Pseudomonas which were sensitive to ciprofloxacin. The same trend was obtained for the GHC isolates for the commonly used antibiotics (chloramphenicol, erythromycin, tetracycline, streptomycin, cotrimaxazole) in GHC.

Galvin *et al.* (2010) observed *E. coli* in effluent samples from a hospital (n = 17) and municipal sewers upstream (n = 5) and downstream (n = 5) from the hospital, effluent samples from throughout the treatment process (n = 4), and treated effluent samples (n = 13). Effluent downstream from the hospital mostly contained a higher proportion of antimicrobial-resistant *E. coli* than that upstream of the hospital. Wastewater treatment lessened the numbers of *E. coli* bacteria and antimicrobial-resistant *E. coli* were not eliminated. *E. coli* resistant to cefotaxime, ciprofloxacin, and cefoxitin were present in treated effluent samples.

Diwan *et al.* (2009) examined the occurrence of antibiotics in water associated with two hospitals in Ujjain district, India. The incoming safe water was free of antibiotics and metronidazole, norfloxacin, sulphamethoxazole, ceftriaxone, ofloxacin, ciprofloxacin, levofloxacin and tinidazole were found in the range of 1.4–236.6 μg^{-1} in hospital effluents. Contamination of aquatic environment by antibiotic usage in hospitals has serious implications on public health and so on.

Khan *et al.* (2004) tested twenty-four *Salmonella* isolates from hospital waste water in India for antibiotic sensitivity against eight commonly used antibiotics belonging to different groups. All of the isolates were highly sensitive to ciprofloxacin, moderately sensitive to chloramphenicol, ampicillin, nalidixic acid, less sensitive to cephalexin, kanamycin and resistant to cloxacillin and erythromycin. He also performed plasmid profile analysis of that 1415 isolates and found that all the isolates possessed multiple plasmids. Then concluded that

resistance pattern might bedue to the possession of multiple drug resistance factor (D'factor) in the plasmids.

Uzzau *et al.* (2000) reported that *Salmonella* constitutes a genus of zoonotic bacteria of worldwide economic and health importance. The current view of *Salmonella* taxonomy assigns the members of this genus into two species: *S. enterica* and *S. bongori*, *S. enterica* itself is divided into six subspecies, enterica, arizonae, diarizonae, indica, and houtenae also known as subspecies I, II, IIIa, IIIb, IV, and VI, respectively. Members of *Salmonella enterica* subspecies enterica are mainly associated with warm-blooded vertebrates and are usually transmitted by ingestion of food or water contaminated by infected feces.

Mezrioui *et al.* (1995) observed 118 *Salmonella* strains before and after treatment in stabilization ponds were tested for antimicrobial resistance. In the treatment plant, which decreases the abundance of *Salmonella* by 99%, a significantly lower level of antibiotic resistance ($P<0.01$) was identified at the system's inflow point (19%) than at its outflow (29%) where high tetracycline resistance was observed at all sampling points followed by resistance to ampicillin and streptomycin.

CHAPTER 3

Materials and Methods

CHAPTER 3

MATERIALS AND METHODS

This study was conducted at the Microbiology laboratory of the Department of Microbiology & Parasitology, Sher-e-Bangla Agricultural University, Dhaka, during the period of January to June, 2021.

3.1 Sample collection

A cross-sectional study was designed to investigate the bacteriological analysis of Hospital Waste water from Shaheed Suhrawardy Medical College Hospital**,** National Institute of Cardiovascular Diseases**,** National Institute of Traumatology & Orthopaedics Rehabilitation, National Institute of Ophthalmology and Bangladesh Shishu Hospital & Institute, Dhaka from January to June 2021. A total of 100 waste water samples were collected. Collected samples were immediately transported to the Microbiology and Parasitology laboratory of the Sher-e-Bangla Agricultural University for analysis. The samples were directly transferred in test tubes to the laboratory for further preparation and examination.

Table 1. Number of waste water samples collected from different hospitals of Dhaka

Sl. No.	Name of hospitals	No of samples
01.	ShSMCH	20
02.	NICVD	20
03.	NITOR	20
04.	NIO	20
05.	BSHI	20
Total		**100**

Legends: ShSMCH=Shaheed Suhrawardy Medical College Hospital, NICVD = National Institute of Cardiovascular Diseases, NITOR=National Institute of Traumatology & Orthopaedics Rehabilitation, NIO=National Institute of Ophthalmology and BSHI = Bangladesh Shishu Hospital & Institute.

3.2 Experimental Design

The whole experimental design was accomplished into two steps. The first step included isolation and identification of *Salmonella* spp., and *E. coli* from waste water by cultural and morphological characteristics. Biochemical tests were also done to confirm the isolated organism as *E. coli* and *Salmonella* spp. The second step included the study of response of the isolated bacteria against commercially available antibiotic discs.

Waste water samples were collected from the different hospitals of Dhaka. The samples were placed into Nutrient Broth (NB) and the stock culture were prepared using PBS. Primary growths of bacteria of each collected sample were performed in NB. 10-fold serial dilution was performed to get Total Viable Count. Each incubated broth sample was then streaked onto NA plates separately as to obtain individual colony. From individual colony subcultures were grown on NA, SS agar, EMB and MAC agar media for obtaining pure culture of the isolated organisms. After determining cultural character, these pure cultures of the organisms were subjected to staining and morphological examination for identification of organisms. The samples of NB were first inoculated on Nutrient Agar by spreading method. Then the isolated organism was inoculated on Eosin Methylene Blue (EMB) Agar, McConkey Agar by streak plate method for selection of *E. coli.* Similarly, then they are cultured on Salmonella Shigella (SS) Agar for selection of *Salmonella* spp. Then Gram's staining and biochemical tests were performed. Finally, the isolated organisms were subjected to antibiotic sensitivity test to observe the resistant characteristics of organism on some specific antibiotic discs.

3.3 Total viable count of the sample (TVC)

The collected samples were grown in NB for overnight in 37^0C. Then 10-fold dilutions of broth culture of samples were prepared using sterilized PBS and 0.1 ml of each dilution was inoculated to the Plate Count Agar plates using a fresh pipette for each dilution. The diluted samples were spread on the plate with a sterile L-shaped glass spreader. One sterile glass spreader was used for each plate. The plates were then incubated at 37ºC for 24 hours. Following incubation, plates those exhibited 30-300 colonies were counted. For each dilution three plates were used and the mean of the three plates were calculated. The number of bacteria

per ml of original sample was obtained by multiplying the number of colonies by diluting factor. The result of TVC was expressed as the CFU per ml of inoculums.

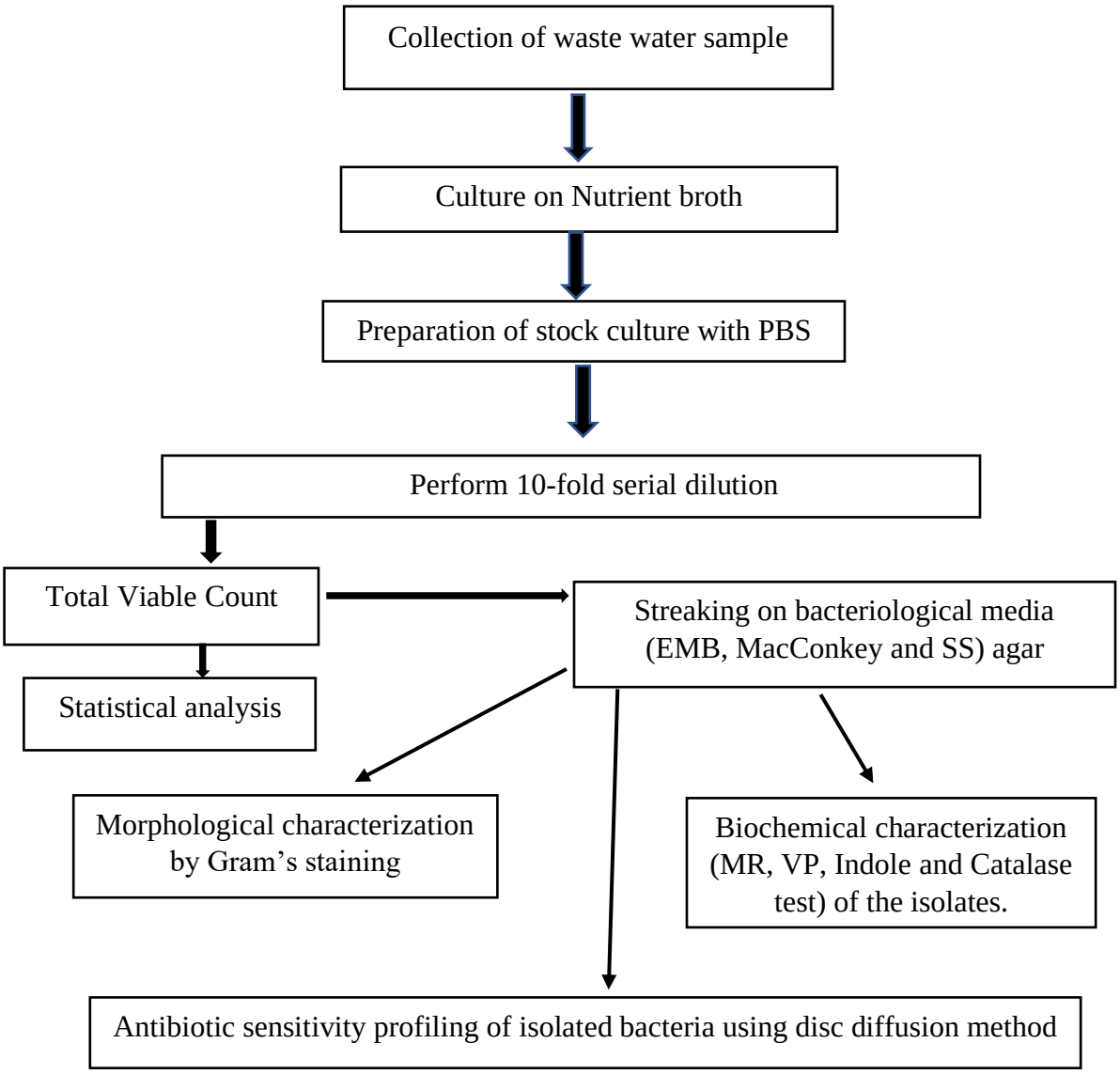

Fig 1: Schematic illustration of the experimental design

3.4 Statistical analysis

Occurrence of each organism was obtained by MS Excel dividing the number of positive samples with the total number of samples in each lot. Occurrence was expressed in percentage.

3.5 Materials

3.5.1 Media and reagents

3.5.1.1 Solid culture media

The media used for bacteriological analysis were Nutrient agar, Eosin-Methylene-Blue (EMB), MacConkey agar (MAC) and *Salmonella*-shigella (SS) from Himedia (India).

3.5.1.2 Liquid culture media (broth)

The liquid media used for this study were Nutrient broth (NB), Methyl-red and Voges-Proskauer broth (MR-VP).

3.5.1.3 Chemicals, reagents and solutions

The following reagent used during bacteriological study were phosphate buffered saline (PBS), reagents for Gram's staining like (Crystal violet, Gram's iodine, Safranine, Acetone alcohol, Alcohol solution (100 mL bottle) etc.

Reagents for methylene blue staining like (Methylene blue, ethyl alcohol, distilled water), Xylene, 4% sodium hydroxide, 3% hydrogen peroxide, oxidase reagent, Kovac's indole reagent (4 dimethyl amino benzaldehyde, concentrate HCL), mineral oil, normal physiological saline solution and other common laboratory chemicals and reagent.

3.5.1.4 Media used for biochemical test

In order to identify bacterial species Methyl Red and Voges-Proskauer broth (MR-VP broth), peptone broth were used and those were brought from Himedia (India).

3.5.2 Glass ware and other appliances

The different types of glass wares and appliances used during the course of the experiment were as follows:

Test tubes (with or without Durham's fermentation tubes and stopper), Petridishes, conical flask (100 ml, 500 ml, and 1000 ml), cotton, slides and coverslips, eppendrof tube, test tube stand, pipette, micropipette, incubator, refrigerator, sterilizing instruments, hot air oven, autoclave machine, electronic machine, glass bit, compound microscope, whirly mixture machine.

3.6 Materials required for anti-biogram study

3.6.1 Muller Hinton Agar (MHA)

Muller Hinton Agar plates were specially used for the antibiotic sensitivity test (Hi media, India).

3.6.2 Antibiotic discs

Commercially available antibiotic discs (Oxoid, England) were used to determine the drug sensitivity pattern. The mentioned antibiotics were widely used and was available in the market then. So, there had taken five antibiotics for determining sensitivity.

Table 2: Antimicrobial agent with their disc concentration

Antimicrobial agents with their disc concentration are presented below

Antimicrobial agents	Disc Concentration (µg)
Ciprofloxacin (CIP)	5
Streptomycin (S)	10
Ampicillin (AMP)	10
Gentamycin (GEN)	10
Tetracycline (TE)	30

3.7 Methods

3.7.1 Collection and transportation of sample

All waste water samples for this study were collected aseptically using sterile instruments and transferred carefully to appropriate containers. The samples were carefully handled and kept in ice box. Due aseptic care was taken during transportation and the samples were kept in sterile container of ice box until these are prepared for bacteriological analysis.

3.7.2 Cleaning and Sterilization of glass wares and other appliances

New and previously used glass wares and plastic wares were dipped into 2% sodium hypochlorite solution and left there until cleaned. After overnight soaking in a household dish washing detergent solution ('Trix', Reckitt and Colman Bangladesh Ltd.), the glass wares were cleaned by brushing and washed thoroughly in running tap water and rinsed four times in distilled water. The cleaned glass wares were then dried by keeping on a bench at room temperature or in an oven at 50-70°C. The Petri dishes were wrapped with brown paper. This glass wares were usually sterilized by dry heat at 160^0C for 1/2 hour in an oven. However, the bottles with plastic caps or rubber lined aluminum caps were sterilized by autoclaving at 121^0 C for 15 minutes less than 15 pounds pressure per square inch (1 kg/cm2). During autoclaving the caps were loosely fitted on the bottles. After autoclaving the glasses were immediately dried in an oven at 50-70 ^{0}C and the caps of the bottles were tightened after cooling.

3.8 Preparation of culture media

The commercial media were prepared according to the direction of the Manufacturers in the laboratory. The composition and the procedures for the preparation of media are presented in the appendix.

3.8.1 Nutrient broth media

Nutrient broth was used as primary growth media of Salmonella and E. coli from the collected waste water samples. Thirteen gm of dehydrated nutrient broth (NB) base (Himeda, India) was

dissolved in 1000 ml of distilled water, heated gently by an electric heater and then sterilized by autoclaving at 121°C under 15 lbs pressure per square inch (1kg/cm2) for 15 minutes (1 kg/cm2). 10 ml broth was transferred in sterile tubes and then stored at 4°C in the refrigerator until use.

3.8.2 Nutrient Agar Media

Nutrient agar was prepared according to the procedure of manufacturer; 2.5gms of Bacto-Nutrient Agar (Difco) was suspended in 100 ml distilled water and boiled to dissolve completely. The solution was sterilized by autoclaving at 121°C at 15 lbs. per sq. inch (1 kg/cm2) for 15 minutes. After autoclaving, the medium was poured in 10 ml quantities in sterile Petri dishes (75 mm diameter) to form a thick layer and allowed to solidify. The sterility of the medium was checked by incubating overnight at 37°Cand then the plates were stored at 4-8°C for future use.

3.8.3 Eosin Methylene Blue (EMB) agar media

Eosin Thirty-six grams powder of EMB agar base (Hi-media, India) was suspended in 1000 ml of distilled water. The suspension was heated to boil for few minutes to dissolve the powder completely in water. The medium was autoclaved for 15 minutes less than 15 lbs pressure per square inch (1 kg/ cm2) to make it sterile. After autoclaving the medium was put into water bath maintaining 45°C and 10-20 ml of medium was poured into small and medium size sterile Petri dish to make EMB agar plates. After solidifying the medium, the plates were kept in the incubator at 37°C for overnight to check their sterility.

3.8.4 McConkey (MC) agar

51 gms of dehydrated Bacto-MacConkey agar (Himedia, India) was suspended in 1000 mL of cold distilled water taken in a conical flask and heated up to boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in 10 ml quantities in sterile glass Petri dishes (medium sized) and in 15 ml quantities in sterile glass Petri dishes (large sized) to form a thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours and then the covers of the Petri dishes partially

removed. The sterility of the medium was judged and used for cultural characterization or stored at 4ºC in refrigerator for future use (Cowan, 1985).

3.8.5 Salmonella-Shigella (SS) agar media

63.03grams powder of SS agar base (Hi-media, India) was suspended in 1000 ml of autoclaved distilled water. The suspension was heated to boil for few minutes to dissolve the powder completely in water. The distilled water was autoclaved for 30 minutes less than 15 lbs pressure per square inch (1 kg/ cm^2) to make it sterile. Over heating or autoclaving may destroy the specificity of the medium. Then 10-20 ml of medium was poured into small and medium size sterile Petri dish to make SS agar plates. After solidifying the medium, the plates were kept in the incubator at 37°C for overnight to check their sterility.

3.8.6 Muller Hinton Agar (MHA)

Thirty-eighty grams of dehydrated Muller Hinton Agar Medium was suspended in 1000 ml cold distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 121°C and 15 lbs. pressure for 15 minutes. The autoclaved materials were allowed to cool to a temperature of 45°C in a water bath and distributed to sterile Petri dishes. After solidification Petri dishes were placed in an incubator for 24 hours at 37°C to check sterility and then placed in a refrigerator at 4°C until use.

3.9 Reagents preparation

3.9.1 Preparation of Methyl-Red Voges-Proskauer (MR-VP) broth

A quantity of 3.4 gm of MR-VP medium was dissolved in 250 ml of distilled water dispensed in 2 ml amount in each test tube; then the tubes were autoclaved at 121°C maintaining a pressure of 15 Ib/sq. inch for 15 minutes. After autoclaving, the tubes containing medium were incubated at 37°C for overnight to check their sterility and then stored in a refrigerator for future use.

3.9.2 Kovac's reagent

This reagent was prepared by dissolving 0.1 gm of Bacto methyl-red in 300 mL of 95% alcohol and diluted to 500 ml with the addition of distilled water.

3.9.3 Preparation of phosphate buffered saline (PBS)

For preparation of Phosphate buffered saline (PBS) solution, 8 gm of sodium chloride, 2.89 gm of disodium phosphate, 0.2 gm of potassium chloride and 0.2 gm of potassium hydrogen phosphate were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclave at 121ºC maintaining a pressure of 15 pounds per square inch for 15 minutes and stored at refrigerator until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 (Cheesbrough, 1985).

3.10 Processing of waste water samples

a. Collection of 10 ml of waste water.
b. Mixed well properly with PBS.
   (WW : PBS=1 ml : 9 ml)

3.11 Methodology followed for isolation and identification of *E. coli and Salmonella*.

3.11.1 Isolation of bacteria by culturing of sample into different bacteriological media

3.11.1.1 Primary culture

Primary growth was performed in nutrient broth followed by inoculation at 37℃ for overnight.

3.11.1.2 Method for obtaining pure culture

Enriched culture from nutrient broth was streaked on to selective agar media and incubated at 37ºC for 24 hours. Single colony appeared on the selective media was further streaked onto selective media to obtain pure cultures.

3.11.1.3 Identification of isolated bacteria

The cultural examination of different sources of waste water samples for bacteriological analysis was done according to the standard method (ICMSF, 1985). Identification of bacteria was performed on the basis of colony morphology; Gram's staining reaction and biochemical test.

3.11.1.4 Colony characteristics

Colony characteristics such as shape, size, surface texture, edge and elevation, color and opacity developed on various selective media after 24 hours of incubation at 37°C were recorded.

3.11.1.5 Morphological identification of bacteria by Gram's staining

Gram's staining of the pure culture was performed according to method described by Cheesbrough (2006). Briefly a single colony was picked up with a bacteriological loop, smeared on a glass slide and fixed by gentle heating. Crystal violet was then applied onto smear to stain for two minutes and then washed with running tap water. Few drops of Gram's iodine were then added for few seconds. After washing with water, Safranin was added as counter stain and allowed to stain for 2 minutes. The slides were then washed with water, blotted and dried in air and then examined under light microscope (400X) using immersion oil.

3.11.2 Biochemical tests

3.11.2.1 Catalase test

This test was used to differentiate those bacteria that produced the enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci. To perform the test an amount of 2-3 ml of 3% hydrogen peroxide solution was poured into a test tube. Using a sterile wooden stick or a glass rod, a good growth of the test organism was immersed into the solution. If the organisms are catalase producer, bubbles of oxygen are released.

3.11.2.2 Indole test

Two milliliter of peptone water was inoculated with the 5ml of bacterial culture and incubated at 37°C for 48 hours. Kovac's reagent (0.5 ml) was added, shaked well and examined after one minute. A red color in the reagent layer indicated indole. In negative case there is no development of red color (Cheesbrough, 2006).

3.11.2.3 Voges-Proskauer test

Two milliliter of sterile glucose peptone water was inoculated with the 5 ml of test organisms. It was incubated at 37°C for 48 hours. A very small amount of creatine was added and mixed. Three milliliter of sodium hydroxide was added and shacked well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink color for positive cases. In case of negative reaction there was no development of pink color (Cheesbrough, 2006).

3.11.2.4 Methyl-red test

The test was conducted by inoculating a colony of the test organism in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37^0C, a drop of methyl red solution was added. A red coloration was positive and indicates an acid P^H resulting from the fermentation of glucose. A yellow coloration indicated negative result (Cheesbrough, 2006).

3.12 Maintenance of stock culture

Stock culture was mixed with a medium prepared by adding one ml of 50% sterilized glycerol in one ml of pure culture in nutrient broth and this was stored at -20°C for further use.

3.13 Antibiotic sensitivity test

The disc diffusion method was used to detect antimicrobial susceptibility assay according to the recommendation of Clinical and Laboratory Standards Institute (CLSI). Antimicrobial drug susceptibility against five commonly used antibiotics were performed by disc diffusion. The procedure of disc diffusion method is presented below:

i. One well isolated colony was selected from the S-S and EMB agar plate.

ii. Colony was touched with a sterile loop and streaked onto nutrient agar and incubated overnight at 37°C.

iii. 4 or 5 well isolated colonies were transferred into a tube of sterile physiological saline and vortex thoroughly.

iv. The bacterial suspension was compared with 0.5 McFarland standard. The comparison was made by viewing this tube against a sheet of white paper on which black lines were drawn.

v. A sterile cotton swab was dipped into the bacterial suspension. The excess fluid of swab was removed by pressing firmly against the inside of the tube just above the fluid level.

vi. The swab was streaked over the entire surface of Mueller-Hinton agar (Himedia, India) medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculums.

vii. The antimicrobial discs were placed individually using sterile forceps and then gently press down onto the agar.

viii. The plates were inverted and incubated at 37°C temperature for overnight. After incubation the diameter of the zone of complete inhibition (including diameter of the discs) was measured in millimeters with a ruler.

3.13.1 Interpretation of the results

After the discs were placed on the plate, the plates were inverted and incubated at 37°C for 12 hours following which the diameter of the zones of complete inhibition (including the diameter of the disc) was measured and recorded in millimeters. The measurements were made with a ruler on the under surface of the plate without opening the lid. The zones of growth inhibition were compared with the zone-size interpretative table provided by Clinical and Laboratory Standards Institute (CLSI, 2016). Antimicrobial testing results were recorded as susceptible,

intermediate and resistant according to zone diameter interpretive standards provided by CLSI (2016).

Table 3: The zone-size of *E. coli* and *Salmonella* spp interpretative table provided by Clinical and Laboratory Standards Institute (CLSI, 2016).

Antimicrobial agents	Resistant	Intermediate	Sensitive
Ciprofloxacin	≤20	21-30	≥ 31
Streptomycin	≤15	16-21	≥22
Ampicillin	≤12	13-19	≥20
Tetracycline	≤ 11	12-14	≥ 15
Gentamycin	≤ 12	13-14	≥ 15

CHAPTER 4

Results & Discussion

CHAPTER 4

RESULTS & DISCUSSION

The aim of the present research was the isolation, identification and determination of antibiotic of *E. coli* and *Salmonella* spp. isolated from waste water from different hospitals close to Sher-e-Bangla Agricultural University, Dhaka, Bangladesh. The isolates were confirmed as *E. coli* and *Salmonella* spp. by cultural and staining characteristics through media culture and biochemical test. This study had shown the differences in occurrence of *E. coli* and *Salmonella* spp. in different sources and locations. Finally, the antibiotic sensitivity and resistance patterns of the isolates of different sources were identified in this study.

4.1 Total Viable Count from the isolated samples

Table 4: Total Viable Count

Name of the Hospital	Number of Samples Tested	Total Viable Count (cfu/ml) (mean ± SD)
ShSMCH	20	$(7.2 \pm 4.3)x10^9$
NICVD	20	$(2.8 \pm 2.1)x10^9$
NITOR	20	$(2.7 \pm 1.9)x10^9$
NIO	20	$(1.4 \pm 1.2)x10^9$
BSHI	20	$(1.9 \pm 2.4)x10^9$

Legends: ShSMCH=Shaheed Suhrawardy Medical College Hospital, NICVD = National Institute of Cardiovascular Diseases, NITOR=National Institute of Traumatology & Orthopaedics Rehabilitation, NIO=National Institute of Ophthalmology and BSHI = Bangladesh Shishu Hospital & Institute, SD= Standard Deviation

Total viable count was different among selected hospitals. The highest average TVC was found in ShSMCH ((7.2 ± 4.3) x10^9) cfu/gm comparing to NICVD ((2.8 ± 2.1) x10^9) cfu/gm, NITOR ((2.7 ± 1.9) x10^9) cfu/ml. These three hospitals mainly provide treatment to adults where ShSMCH treats infectious diseases as general hospital. The lowest average TVC was found in NIO ((1.4 ± 1.2) x10^9) cfu/ml which was slightly lower than BSHI ((1.9 ± 2.4) x10^9) cfu/ml. Like these Tsai *et al*. (1998) found that the average TVC in waste water sludge from three local hospitals was $8{\cdot}1\times10^7$ $cfug^{-1}$ where *Salmonella* spp. was $5{\cdot}5\times10^5$ $cfug^{-1}$. These results indicated severe threat to public health. Here the standard deviation was higher than average which could be happened due to collection of samples from different sources within the hospital where every source did not contain same number of infectious agents. As all the data of different sources were taken for calculation without considering extreme values, standard deviation became higher than the average. These results indicated severe threat to public health.

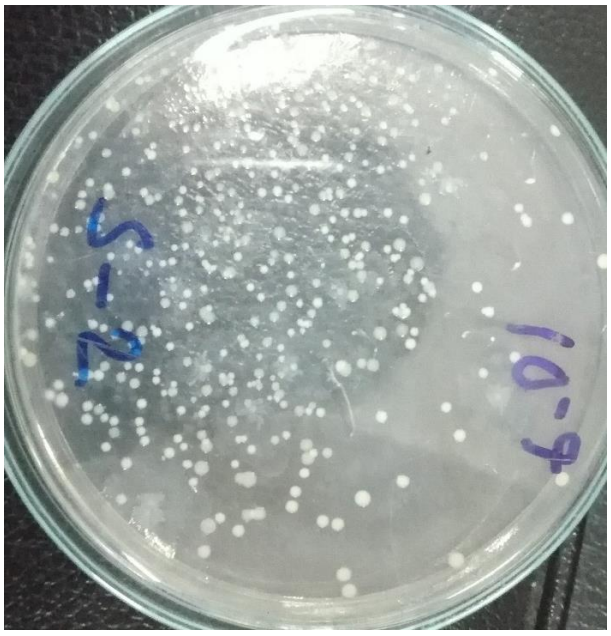

Fig 2: Total Viable Count by 10-fold dilution

4.2 Identification of *E. coli*

4.2.1 Cultural examination

4.2.1.1 Solid media

Cultural characteristics on different solid media are shown in Figure 3.

Table 5. Summary of cultural characteristics of *E. coli* isolated from hospital waste water

MacConkey agar	Nutrient agar	EMB agar	Remarks
bright pink or red colonies	smooth, circular, white to grayish white colony	greenish-black colonies with metallic sheen	*E. coli*

Legend: EMB= Eosin Methylene Blue agar

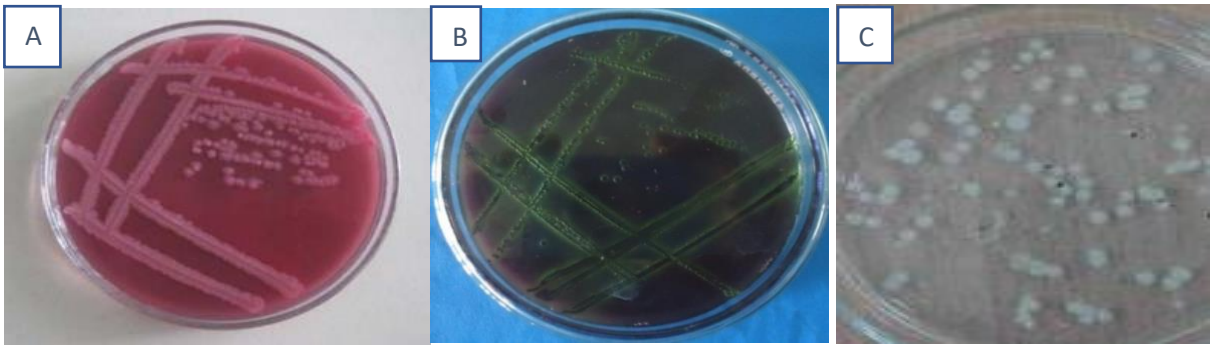

Fig 3: Colonies characteristics of *E. coli* showed in MacConkey agar (A), EMB agar (B) and Nutrient agar (C)

4.2.2. Biochemical tests

All the isolates were Methyl-red positive, Voges-Proskauer negative, Indole positive and Catalase negative

Results of these tests are shown in Table.6 and Fig. 4 & 5

Table. 6: MR-VP and Indole test profiles of *E. coli* isolated from waste water

Sl. No.	Name of Biochemical Tests	Results	Interpretation
1	MR	+	*E. coli*.
2	VP	-	
3	Indole	+	
4	Catalase	-	

MR = Methyl red; VP = Voges- Proskauer; + = positive reaction; - = negative reaction

MR+ve VP -ve Indol +ve Control

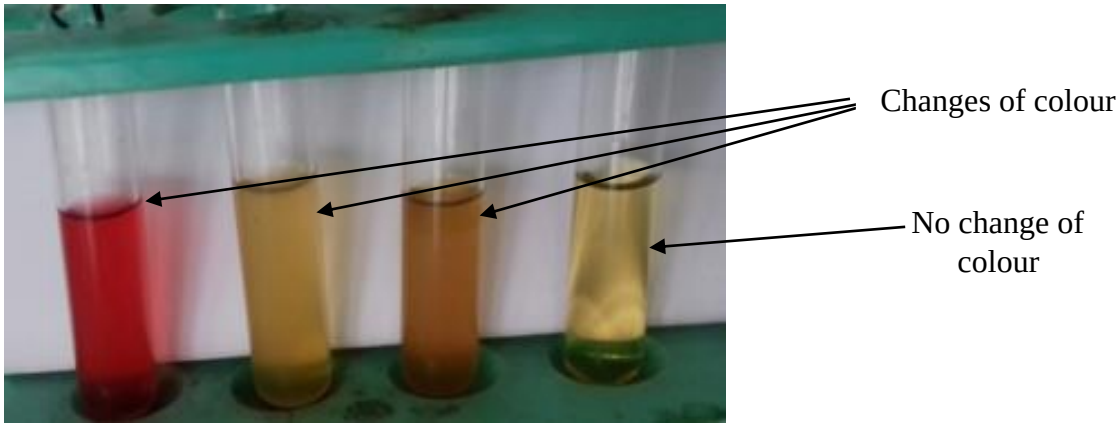

Fig 4. Results of Indole, Voges-Proskauer and Methyl Red tests

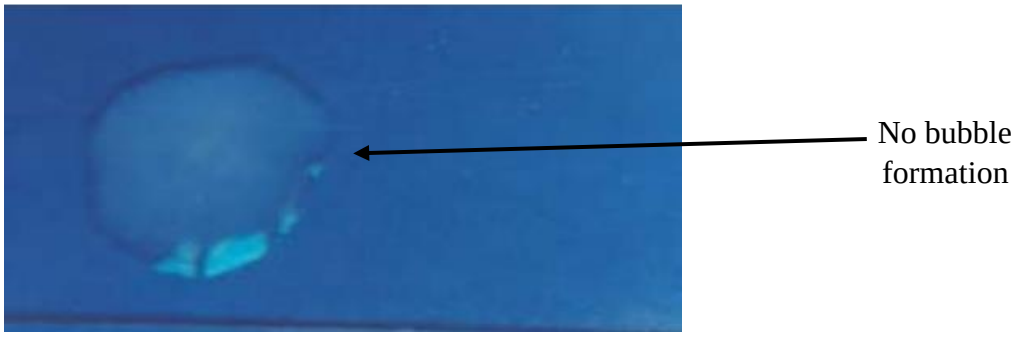

Fig 5: Catalase Negative

4.3 Identification of *Salmonella spp.*

4.3.1 Cultural examination

Cultural characteristics on different solid media are shown in Figure 6.

Table 7. Summary of cultural characteristics of *Salmonella spp.* isolated from hospital waste water

SS agar	EMB agar	Remarks
Black centered, smooth, small round colony	Colorless, circular and smooth colony	*Salmonella* spp.

Legends: SS= Salmonella Shigella agar, EMB= Eosin Methylene Blue agar

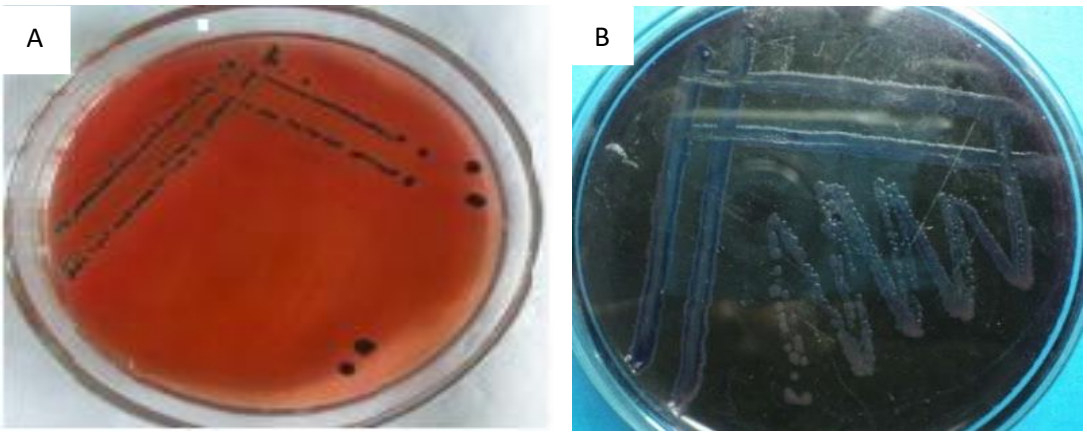

Fig 6: Colonies characteristics of *Salmonella spp.* showed in SS agar (A) and EMB agar (B)

4.3.2. Biochemical tests

All the isolates were Methyl-red positive, Voges-Proskauer, Indole negative and Catalase positive.

Results of these tests are shown in Table.8 and Fig. 7 & 8

Table.8: MR-VP and Indole test profiles of *Salmonella* isolated from waste water

Sl. No.	Name of biochemical tests	Results	Interpretation
1	MR	+	*Salmonella* spp.
2	VP	-	
3	Indole	-	
4	Catalase	+	

VP = Voges Proskauer; + = positive reaction; - = negative reaction MR = Methyl red

MR+ve VP -ve Indol -ve Control

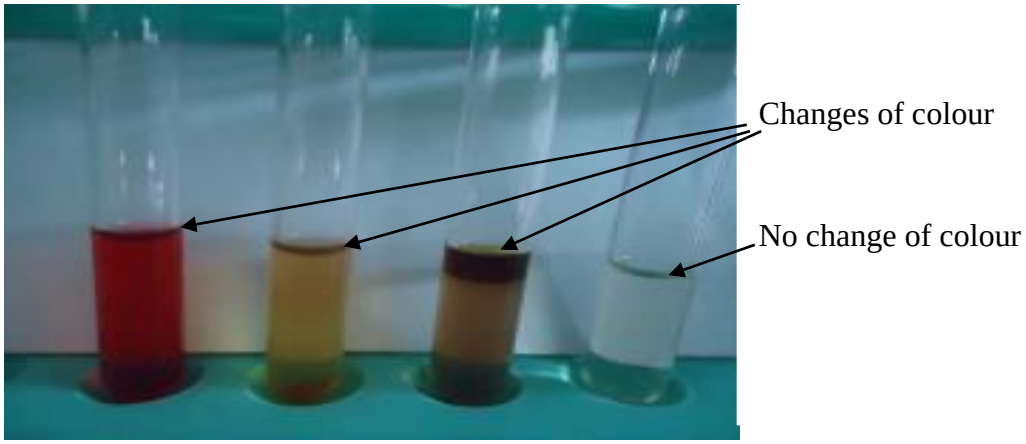

Fig 7: Results of Indole, Voges-Proskauer and Methyl Red tests

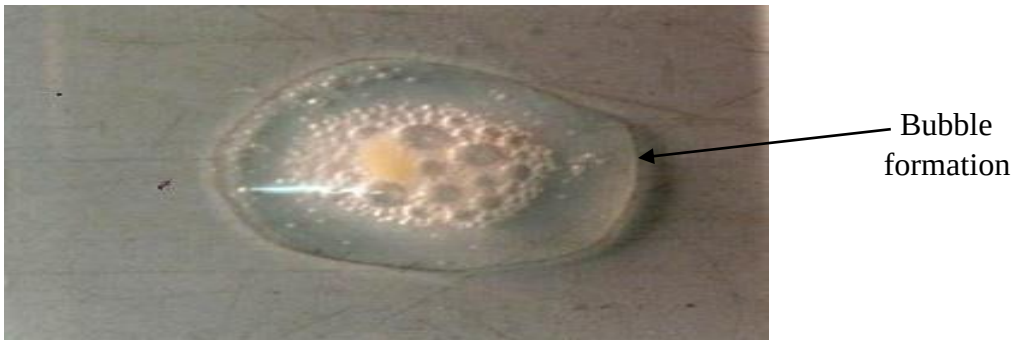

Fig 8: Catalase Positive

The media used in this study were selected considering the experience of Buxton and Fraser (1977) and Nazir *et al*. (2005). Specific enrichment media and biochemical tests were used for the isolation and identification of *Salmonella* which was previously suggested by a number of researchers (Robenson *et al*., 2003; Dhruba *et al.;* Buxton and Fraser, 1977). In this study the colony characteristics of Salmonella observed on SS agar and NA agar has similarity with the findings of other authors (Buxton and Fraser, 1977; Rahman, 1977). Again, in this study, colony characteristics of *E. coli* observed in NA and EMB were similar to the findings of Buxton and Fraser (1977) and Nazir *et al.* (2000).

4.4 Gram Staining Properties

4.4.1 *E. coli* and *Salmonella* spp.

In Gram's staining and observation under compound light microscope both organisms revealed Gram-negative character where red as well as the morphology small bacilli shaped arranged in single or paired which is the characteristics of *E. coli* and single or paired short plump rods which is the characteristics of *Salmonella* spp.

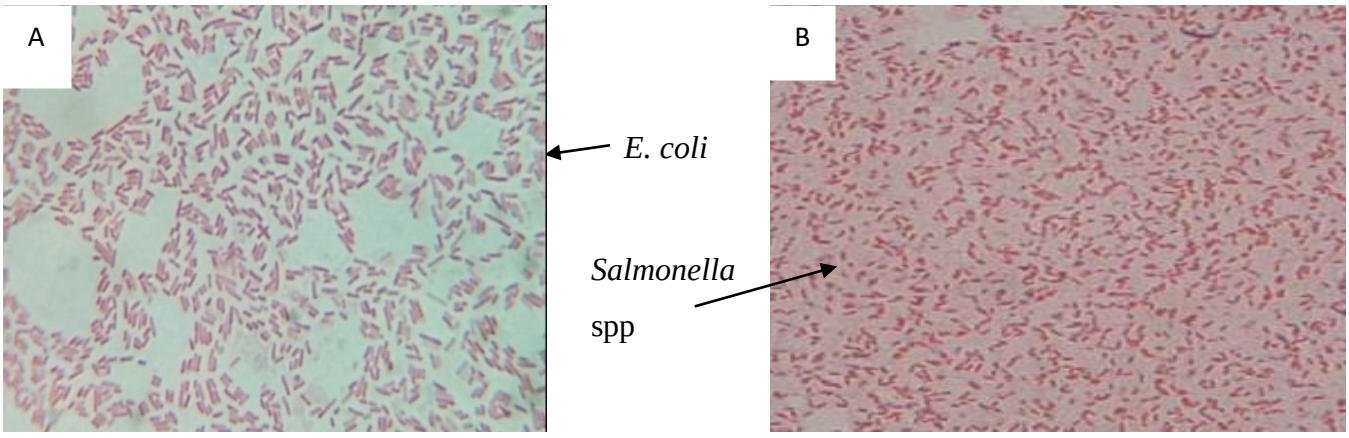

Fig. 9**:** Gram negative, pink colored rod-shaped *E. coli* (A) and single or pair shaped *Salmonella* (B) under the light microscope (100 x)

In Gram's staining, the morphological characteristics of the isolated *Salmonella* showed Gram negative, small rod shaped, single or paired in arrangement under microscope which was supported by other researchers (Gene, 2002; Jones *et al.*, 1997 and Freeman 1985). The morphology of the isolated *E. coli* exhibited Gram negative character with short rod arranged in single or paired and motile which was supported by several authors Buxton and Fraser (1977) and Freeman (1979). The staining properties of these *E. coli* and *Salmonella* bacteria were similar according to the description of Ronald Atlas (2015) in his book.

4.5 Occurrence of *Salmonella* spp and *E. coli*

Table 9: Summary of occurrence of Bacteria from waste water.

Sl. No.	Name of the Hospitals	Total no. of Samples	*E. coli*		*Salmonella* spp.	
			+Ve samples	Occurrence *(%)*	+Ve samples	Occurrence (%)
1.	ShSMCH	20	17	85	20	100
2.	NICVD	20	18	90	20	100
3.	NITOR	20	18	90	16	80
4.	NIO	20	12	60	15	75
5.	BSHI	20	15	75	16	80
	Total	100	80	80	87	87

Legends: ShSMCH=Shaheed Suhrawardy Medical College Hospital, NICVD = National Institute of Cardiovascular Diseases, NITOR=National Institute of Traumatology & Orthopaedics Rehabilitation, NIO=National Institute of Ophthalmology and BSHI = Bangladesh Shishu Hospital & Institute.

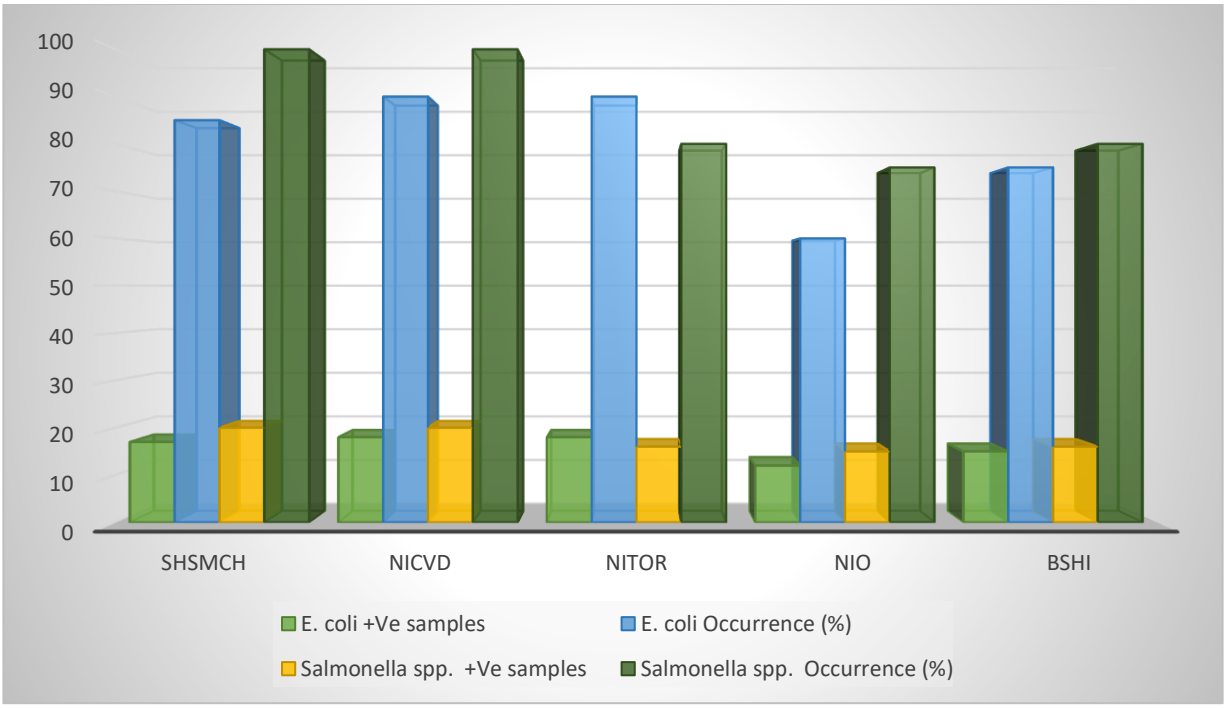

Fig 10: Occurrence of *Salmonella* spp and *E. coli* in waste water

In this study *E. coli* and *Salmonella* spp. were found to be distributed widely in the waste water samples analyzed. The overall occurrence of *E. coli (E)* and *Salmonella spp.(S)* were 80% and 87% respectively. The occurrence was higher in Suhrawardy Hospital (E-85%, S-100%) and Cardiovascular Hospital (E-90%, S-100%) comparing to Shishu Hospital (E-75%, S-80%) and Eye Institute Hospital (E-60%, S-75%) due to the number of admitted patients was much higher in first two hospitals than last two ones where there had severe lack of hygenic measures. In Eye hospital, the occurrence was the least due to shortage of long time infected admitted patients rather there had a lot of patients taking treatment for emergency basis. Though the occurrence of bacteria should less in Shishu hospital as we thought considering hospital as children's hospital, the occurrence of bacteria was out of imagination. But visiting the Shishu hospital, it could be told that the increased prevalence in waste water did not show the actual condition of the child health as a lot of attendants and guardians stayed with children and used hospital basins, toilets and so on. According to Nahar *et al*. (2019) where they isolated 47 samples and tested in laboratory in Mymensingh. *E. coli* and *Salmonella* spp. were found to be

widely distributed in the environmental samples analyzed. The overall occurrence of *E. coli* and *Salmonella* spp. were 76.59% and 89.36%, respectively. According to Shokoohizadehd *et al.* (2015) where he collected 44 waste water samples in from effluent and influent waste waters from three hospitals waste water treatment plant. Based on microbiologic tests results, they found the occurrence of *E. coli, Citrobacter* and *Salmonella* in wastewater samples were 70.5%, 8.82% and 81.58%. in Imam, Kermanshahi and Farabi hospitals respectively. Kam *et al.,* (2007) examined one hundred thirty-four isolates of *Salmonella* enterica serotype typhi from 17 public hospitals and clinics in Hong Kong from 2000 to 2004. Isolates originated from 80 patients, with 29 patients providing multiple isolates. According to Fekadu *et al* (2015), the prevalence is similar in hospital waste water from influent but there has seen the 99% reduction of bacteria by using effluent plant and chlorine treatment where there has not a single treatment plant in any mentioned hospitals of Dhaka. Infection by *E. coli* and *Salmonella* is a common cause of food poisoning in humans (Hobbs and Robert, 1993). Human might get *Salmonella* and *E. coli* infection during coming contact with hospital's waste water mixed with river water or washed vegetables beside infected drain. So, it had great public health significance to study the prevalence of *Salmonella* and *E. coli* in the waste water to assess the risk originated from mentioned hospitals. Thereby, this study was aimed to create public awareness revealing the risks of *Salmonella* and *E. coli* infection in human and animal.

4.6 Results of antibiotic sensitivity tests

A total of two isolates such as. *E. coli, Salmonella* spp. were subjected to antibiotic sensitivity assay because these two isolates have severe public health importance and cause different diseases. Besides, necessary media and reagents were available in the laboratory. The results of antibiotic sensitivity assay are presented below:

4.6.1 Antimicrobial profile of *E. coli*

Positive *E. coli* isolates were tested against 5 different antibiotics. Among them Gentamycin showed the highest susceptibility pattern followed by the Ciprofloxacin and Streptomycin in this study. Highest resistant pattern was showed by Tetracycline and then Ampicillin.

Table 10: Antimicrobial profile of *E. coli*

Name of antibiotic disc	Interpretation	Number of Sensitivity	Percentage (%)
Ciprofloxacin N=80	S	52	65.00
	I	28	35.00
	R	0	0.00
Streptomycin N=80	S	50	62.50
	I	18	22.50
	R	12	15.00
Ampicillin N=80	S	0	0.00
	I	5	6.25
	R	75	93.75
Tetracycline N=80	S	0	0.00
	I	0	0.00
	R	80	100.00
Gentamycin N=80	S	75	93.75
	I	5	6.25
	R	0	0.00

Legends: R=Resistant, S=Sensitive, I=Intermediate.
Ciprofloxacin: R (≤20), I (16-21), S (≥31)
Streptomycin: R (≤15), I (21-30), S (≥22)
Ampicillin: R (≤12), I (13-19, S (≥20)
Tetracycline: R (≤11), I (12-14), S (≥15)
Gentamycin: R (≤12), I (13-14), S (≥15)
*Diameter of zone of inhibition expressed in mm.

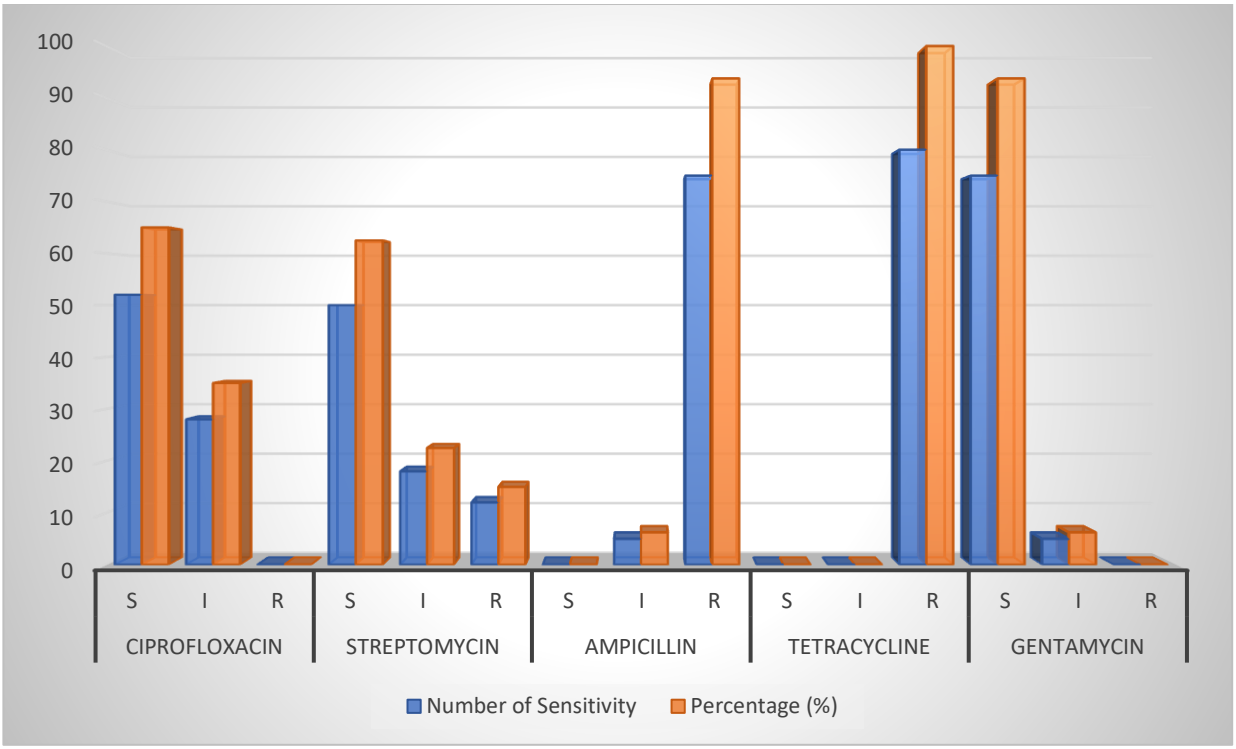

Fig 11: Antibiogram of *E. coli*

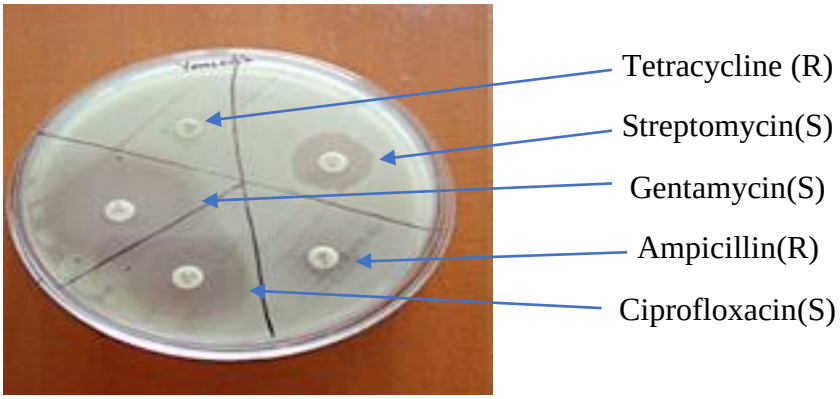

Fig 12: Antibiotic sensitivity of *E. coli*

4.6.2 Antimicrobial profile of *Salmonella* spp.

Positive *Salmonella* isolates were tested against 5 different antibiotics. Among them Gentamycin showed the highest susceptibility pattern followed by Ciprofloxacin and Streptomycin. Highest resistant pattern was showed by Tetracycline and Ampicillin.

Table 11: Antimicrobial profile of *Salmonella* spp.

Name of antibiotic disc	Interpretation	Number of Sensitivity	Percentage (%)
Ciprofloxacin N=88	S	65	73.86
	I	17	19.25
	R	6	6.89
Streptomycin N=88	S	30	34.00
	I	42	47.82
	R	16	18.18
Ampicillin N=88	S	0	0.00
	I	0	0.00
	R	88	100.00
Tetracycline N=88	S	0	0.00
	I	0	0.00
	R	88	100.00
Gentamycin N=88	S	70	79.54
	I	18	20.46
	R	0	0.00

Legends: R=Resistant, S=Sensitive, I=Intermediate.
Ciprofloxacin: R (≤20), I (16-21), S (≥31)
Streptomycin: R (≤15), I (21-30), S (≥22)
Ampicillin: R (≤12), I (13-19, S (≥20)
Tetracycline: R (≤11), I (12-14), S (≥15)
Gentamycin: R (≤12), I (13-14), S (≥15)
*Diameter of zone of inhibition expressed in mm.

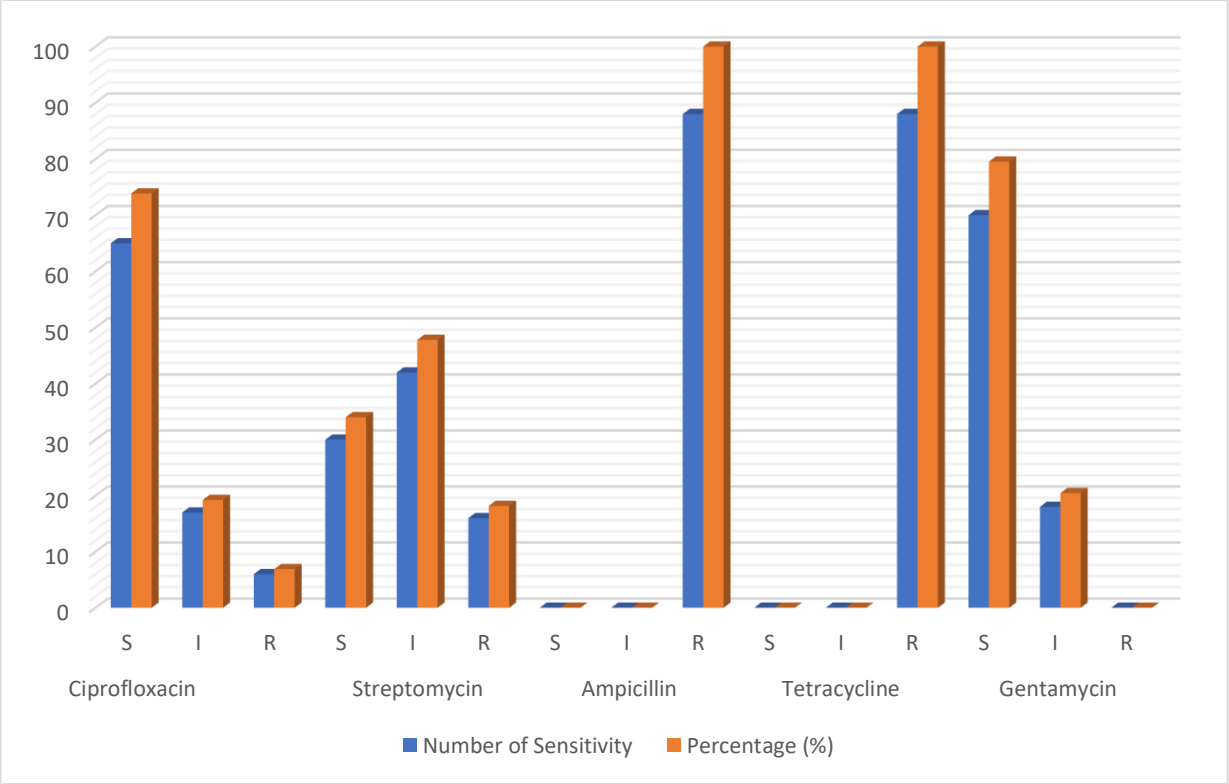

Fig 13: Antibiogram of *Salmonella* spp.

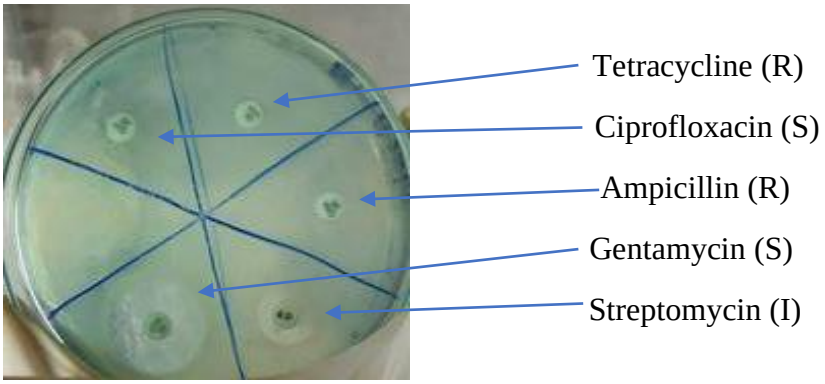

Fig 14: Antibiotic Sensitivity *Salmonella* spp.

Because of the rapid spread and emergence of antibiotic resistant bacteria and their resistance genes among humans, animals and the environment at global scale, antibiotic resistance is now considered as a one health challenge. Hospital waste water is a major source for antibiotic resistant bacteria that are of great public health concern. Most of the researches on AMR emphasized on human and animal and there is a lack in AMR situation in the waste water of hospitals in Sher-e-Bangla Nagar area.

One of the most alarming findings of this study is that all the isolates of *E. coli* and *Salmonella* spp. were found Multi Drug Resistant (MDR) to tetracycline and ampicillin mostly. Tetracycline is used widely (Hassan *et al.*, 2015) in Bangladesh. Long time wide spread use of tetracycline in veterinary and human medicine could be lined with this resistant result against tetracycline. Peak *et al.* (2007) and Huang *et al.* (2019) also found tetracycline resistance genes in various types of waste water. It was also important to note that isolates of *E. coli* and *Salmonella* spp. in this study were found to be resistant in nature. For example, waste water is resistant against tetracycline, ampicillin, ciprofloxacin and streptomycin. Rashid *et al*. (2015) observed the presence of MDR *E. coli* in various aquatic sources in Bangladesh earlier. Like these evidences, we found both resistant and sensitive bacteria from hospitals. Waste water containing *E. coli* where tetracycline was resistant (100%). Ampicillin (93.75%) was close to tetracycline considering resistant. On the other hand, gentamycin (93.75%) was more sensitive than ciprofloxacin (73.86%) and streptomycin is intermediate (62.5%). Waste water containing *Salmonella spp* where tetracycline and ampicillin are100% resistant. On the other hand, gentamycin (79.54%) was more sensitive than ciprofloxacin (65%) and streptomycin (47.82%) is sensitive. In Bangladesh, Zahid *et al.* (2009) went through an investigation on the occurrence of multiple ARB and their chromosomal determinants in surface water. From 147 samples, they isolated 103 bacterial species where 65% were *E. coli* including isolates resistant to tetracycline. While Siddiqui *et al*. (2015) showed presence of antibiotic resistant *Salmonella* spp. in hospital waste and many of which truly ended up in the sewage in Bangladesh. Same type of result was also shown by another group (Ashfaq KMA, Pijush S *et al,.2013*) where Ampicillin was the antibiotic to which highest number of resistant bacteria (93.1%) was found. In the study, *Salmonella spp.* were 100% resistant to Ampicillin by *E. coli* (75%). Among all isolates of Gram-negative bacteria, 75% of isolates were found to be resistant to Amoxicillin followed by

Ampicillin (63.5%), Chloramphenicol (30.7%), Gentamycin (28.8%) and least being Ciprofloxacin (23.1%). Previously, similar Ciprofloxacin resistant *E. coli* was found and characterized from the Chittagong Medical College Hospital & Dhaka Medical college Hospital liquid waste. Nahar *et al. (*2019) found *E. coli* were resistant streptomycin (16.6%) and ampicillin (97.2%) while *Salmonella* spp. to ciprofloxacin (07.1%), streptomycin (19.1%) and ampicillin (100%) by observing 47 samples from sewage water in Mymensingh. According to Alam *et al*. (2013) examined sixty-nine isolates for antibiotic sensitivity where 73.9% strains were resistant to ampicillin. However, resistance to streptomycin, tetracycline, and doxycycline was recorded in less than 13% of the strains. Some variations of antibiotic sensitivity with others were also observed. Most of the hospitals of abroad used ETP strictly which is completely rare in mentioned hospital. Some results were recorded as different from others mostly abroad because of indiscriminate use of antibiotics in Bangladesh where there had a lack of proper law and order to control excess use of antibiotics. The diagnosis process was not developed and the hygenic measures were taken in the mentioned hospital like others in the world. Besides, as we completed this research amid of covid19 with some limitations and collected water from different points of hospitals, some data were not similar but close to previous research. So, the present study findings support maximum of the earlier observations.

CHAPTER 5

Summary & Conclusion

CHAPTER 5
SUMMARY AND CONCLUSION

Water borne pathogens are harmful for human and they cause fever, dysentery, typhoid, cholera, diarrhoea, abdominal pain in human. *Escherichia coli, Salmonella spp*. like bacteria are commonly found in hospital waste water due to not having any treatment plant in hospital.

A cross-sectional study was designed to isolate the bacteria from waste water at Suhrawardy Hospital, National Cardiovascular Hospital, Orthopaedics Hospital, National Eye Institute Hospital and Shishu Hospital, Dhaka from January to June 2021. A total of 100 waste water samples were collected. Collected samples were immediately transported to the Microbiology & Parasitology laboratory of the Sher-e-Bangla Agricultural University for analysis.

After processing of samples primary culture was done in nutrient broth and nutrient agar then pure culture was obtained from different selective media. The pure culture staining and biochemical tests were done by maintaining standard procedures. The prevalence of *Escherichia coli* and *Salmonella spp.* was 80%, and 87% respectively.

All pure isolates were subjected to antibiotic sensitivity test by disc diffusion method against 5 different antibiotics discs. *E. coli* isolates were showed sensitive to Gentamycin (93.75%), Ciprofloxacin (65%), streptomycin (62.5%) and resistant to Tetracycline (100%) and Ampicillin (93.75%). Among all Salmonella *spp.* isolates Gentamycin (79.54%) showed the highest susceptibility pattern followed by the ciprofloxacin (73.86%) and Streptomycin (34%). Highest resistant pattern was showed by Tetracycline (100%) and Ampicillin (100%).

Overall, the prevalence of *Escherichia coli* and *Salmonella* spp. in hospital's waste water and their drug resistance is very alarming for public health. Government should give extra care for the hospitals throughout the country because of the extreme weakness in every hospital experienced in the time of ongoing Covid19 pandemic. Setting Effluent Treatment Plant (ETP) is mandatory for every hospital to stop the mixing of untreated waste water with other water bodies and infecting people through food.

Besides, the reckless use of antibiotics should be controlled with proper legislative forces as it is an alarming issue which can cause severe pandemic if we do not control it properly. Government and we all need to come forward to stop quacks giving antibiotic unnecessarily without the permission and prescription of registered doctors.

LIMITATIONS

Amid of the outbreak of Corona pandemic throughout the world, we started our research work. There was strict lockdown in our country which made us about to close our research work. By maintaining every safety measures, it was so tough to conduct the research as we collected samples from hospitals. There had limitation of instruments and reagents in our lab and we had the shortage of funds.

RECOMMENDATION

The widespread emergence of drug-resistance among pathogens has become one of the most serious challenges worldwide. Therefore, reduction of selective pressure by regulating the use of antibiotics is a key step to undermine the spread of resistance in hospital wastewater. Proper management of hospital wastewater should be also practiced in every health institution in order to reduce the problem. Treatment of waste water must be made mandatory for all hospitals in Dhaka. All hospitals should have ETP and it is needed to monitor by the legislative authority so that no water can come out of hospitals without treatment. Medical waste disposal vehicles can be used properly so that no medical waste can mix with water. Besides, we all have to be concerned about reckless use of antibiotics and keep every medical waste in specific place of the hospitals. Dhaka city dwellers and slum dwellers should take drinking water after heating and supply this water to animal farms also and try to avoid using river water within the city.

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APPENDICES

APPENDIX I

COMPOSITION OF DIFFERENT MEDIA

1. Nutrient broth (NB)

Peptic digest of animal tissue	……	5.00gm
Sodium chloride	……	5.00gm
Beef extract	……	1.50gm
Yeast extract	……	1.50gm
Distilled water	……	1000 ml
Final pH (at 25ºC)	……	7.4+0.2

2. Eosin Methylene Blue (EMB) agar

Peptone	……	10.00gm
Dipotassium Phosphate	……	2.00gm
Lactose	……	5.00gm
Sucrose	……	5.00 gm
Agar	……	13.50 gm
Eosin	……	0.40 gm
Methylene blue	……	0.065 gm
Distilled water	……	1000 ml
Final pH (at 25ºC)	……	7.4+0.2

3. MacConkey (MC) agar

Lactose	……	10.00gm
Peptone	……	20.00gm
Sodium chloride	……	5.00gm
Bile salts	……	5.00gm
Neutral red	……	0.075 gm
Agar	……	12.00gm
Distilled water	……	1000 ml

4. Mannitol salt agar

Lab-Lemco Powder	……	1.00gm
peptone	……	10.00 gm
Mannitol	……	10.00 gm
Phenol red	……	0.025 gm
Agar	……	15.0 gm
Distilled water	……	1000 ml

5. Salmonella-Shigella (SS) agar

Peptic digest of animal tissue	……	5.00 gm
Beef extract	……	5.00 gm
Lactose	……	10.00 gm
Bile salts mixture	……	8.50 gm
Sodium citrate	……	10.00 gm
Sodium thiosulfate	……	.8.50 gm
Ferric citrate	……	1.00 gm
Brilliant green	……	0.00033 gm
Neutral red	……	0.025 gm
Agar	……	15.00 gm
Distilled water	……	1000 ml
Final pH (at 25ºC)	……	7.0 ± 0.2

6. Nutrient agar (NA)

Beef extract	……	3.00 gm
Peptone	……	5.00 gm
Sodium chloride	……	5.00 gm
Agar	……	20.00 gm
Distilled water	……	1000 ml
Final pH (at 25ºC)	……	7.1 ± 0.1

7. MR-VP medium (Himedia, India)

Buffered peptone	……	7.00 gm
Dextrose	……	5.00 gm
Dipotassium phosphate	……	5.00 gm
Final pH(at 25ºC)	……	6.9 ± 0.2

APPENDIX II

COMPOSITION OF DIFFERERNT REAGENTS

1. Peptone water

Peptone	……	1.00 gm
Distilled water	……	1000 ml

2. Kovac's reagent for indole preparation

Pdimethyl amino benzaldehyd	……	5.00gm
Amyl alcohol	……	75.00 ml
Conc. HCl	……	25.00 ml

3. V-P reagent-1

5% alpha-naphthol in absolute ethyl alcohol

4. V-P reagent-2

Creatine	……	0.30%
Cotton blue	……	0.05g

5. Phosphate buffered saline

Sodium chloride	……	8.00gm
Disodium hydrogen phosphate	……	2.80gm
Potassium chloride	……	0.20gm
Potassium hydrogen phosphate	……	0.20gm

6. Methyl red solution **1000 ml**

Methyl red	……	0.05 gm
Ethanol (absolute)	……	28.00 ml
Distilled water	……	22.00 ml

7. Phenol red

Phenol red dye	……	0.20gm
Distilled water	……	100 ml

APPENDIX III

TOTAL VIABLE COUNT

Serial No	Name of the Sample	Total Viable Count (TVC) (CFU/ml)
1	WW1	$3.6x10^9$
2	WW2	$3.4x10^9$
3	WW3	$2.4x10^9$
4	WW4	$2.9x10^9$
5	WW5	$2.7x10^9$
6	WW6	$3.5x10^9$
7	WW7	$1.1x10^8$
8	WW8	$3.2x10^8$
9	WW9	$2.7x10^8$
10	WW10	$3.8x10^8$
11	WW11	$3.1x10^8$
12	WW12	$2.9x10^8$
13	WW13	$1.3x10^{10}$
14	WW14	$1.5x10^{10}$
15	WW15	$2.1x10^9$
16	WW16	$2.6x10^9$
17	WW17	$3.1x10^9$
18	WW18	$3.7x10^8$
19	WW19	$3.9x10^7$
20	WW20	$4.6x10^7$
21	WW21	$2.6x10^8$
22	WW22	$2.5x10^8$
23	WW23	$2.8x10^8$
24	WW24	$2.1x10^8$

25	WW25	$3.2x10^{9}$
26	WW26	$3.5x10^{9}$
27	WW27	$3.9x10^{9}$
28	WW28	$3.4x10^{9}$
29	WW29	$2.1x10^{9}$
30	WW30	$2.9x10^{10}$
31	WW31	$2.7x10^{9}$
32	WW32	$1.6x10^{8}$
33	WW33	$1.8x10^{8}$
34	WW34	$4.1x10^{10}$
35	WW35	$2.3x10^{9}$
36	WW36	$2.8x10^{9}$
37	WW37	$3.6x10^{8}$
38	WW38	$3.4x10^{8}$
39	WW39	$2.4x10^{9}$
40	WW40	$2.3x10^{9}$
41	WW41	$2.8x10^{9}$
42	WW42	$3.6x10^{8}$
43	WW43	$3.4x10^{8}$
44	WW44	$2.4x10^{9}$
45	WW45	$2.9x10^{8}$
46	WW46	$2.7x10^{9}$
47	WW47	$3.5x10^{8}$
48	WW48	$3.2x10^{8}$
49	WW49	$1.1x10^{8}$
50	WW50	$2.4x10^{9}$
51	WW51	$2.7x10^{9}$
52	WW52	$3.8x10^{8}$
53	WW53	$3.7x10^{8}$

54	WW54	$3.3x10^{8}$
55	WW55	$2.9x10^{9}$
56	WW56	$2.2x10^{9}$
57	WW57	$2.5x10^{9}$
58	WW58	$3.9x10^{8}$
59	WW59	$3.6x10^{8}$
60	WW60	$3.4x10^{8}$
61	WW61	$3.9x10^{8}$
62	WW62	$4.1x10^{7}$
63	WW63	$4.5x10^{7}$
64	WW64	$2.8x10^{9}$
65	WW65	$2.7x10^{9}$
66	WW66	$3.1x10^{7}$
67	WW67	$3.8x10^{7}$
68	WW68	$2.2x10^{9}$
69	WW69	$2.6x10^{9}$
70	WW70	$2.9x10^{9}$
71	WW71	$2.4x10^{8}$
72	WW72	$1.2x10^{8}$
73	WW73	$1.5x10^{8}$
74	WW74	$1.8x10^{10}$
75	WW75	$1.7x10^{10}$
76	WW76	$3.3x10^{8}$
77	WW77	$3.5x10^{8}$
78	WW78	$3.9x10^{8}$
79	WW79	$3.2x10^{8}$
80	WW80	$2.9x10^{9}$
81	WW81	$3.1x10^{9}$
82	WW82	$2.7x10^{9}$

83	WW83	$1.4x10^{9}$
84	WW84	$2.9x10^{9}$
85	WW85	$3.7x10^{8}$
86	WW86	$3.2x10^{8}$
87	WW87	$2.8x10^{9}$
88	WW88	$2.1x10^{9}$
89	WW89	$2.6x10^{9}$
90	WW90	$3.4x10^{8}$
91	WW91	$3.8x10^{7}$
92	WW92	$3.5x10^{7}$
93	WW93	$4.1x10^{7}$
94	WW94	$2.6x10^{9}$
95	WW95	$2.9x10^{9}$
96	WW96	$2.6x10^{9}$
97	WW97	$3.5x10^{8}$
98	WW98	$3.8x10^{8}$
99	WW99	$3.7x10^{8}$
100	WW100	$3.1x10^{8}$