Isolation and Characterization of Multiple Drug Resistant Bacteria from Waste Water of Hospital and Non-Hospital Environment

A THESIS
BY

MAHMUDUL HASAN

REGISTRATION NO.:1605459

SEMESTER: JULY–DECEMBER 2017

SESSION: 2016

MASTER OF SCIENCE (MS)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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DECEMBER, 2017


Dedicated
To
My Beloved Parents

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ABSTRACT

A huge amount of antibiotics are normally used in hospital for patient care and disinfection which potentially raising antibiotic resistant bacteria in the liquid hospital waste that can also transmitted to non-hospital environments through drainage system. The current study was aimed to isolate and characterize multiple drug resistant bacteria from waste water of hospital and non-hospital environments. For this purpose the present research work was conducted during the period of (July-December) 2017 from untreated hospital and non-hospital waste water of different areas of Dinajpur district of Bangladesh. A total 20 samples were randomly collected and analyzed through different bacteriological, biochemical, molecular and antibiotic susceptibility testing. All 20 sample were 100% positive with one or more isolates, total 55 bacterial isolates were isolated, among them 32(58.2%) were from hospital environment and 23(42.1%) were from non-hospital environment. Result of total viable count showed that maximum countable bacteria (2.20×10^{10}) CFUs/ml that were from MARMCH Site-2 and minimum number of countable bacteria (1.0×10^{10}) CFUs/ml was isolated from sample of Kalitola. Among the isolates *E.coli* 16(29%), *Pseudomonas spp* 12(21.8%), *Klebsiella spp* 9(16.4%), *Salmonella* spp 8(14.5%), *Staphylococcus* spp 5(9%) and *Vibrio* spp 5(9%) were identified. Multi drug resistant (MDR) *Pseudomonas aeruginosa* was characterized from hospital waste water by 16s rRNA sequencing. The antibiotic sensitivity study revealed that among the hospital isolates, about (83.3%) were resistant against Ampicillin, followed by Amikacin, Kanamycin and Penicillin (77.8%). On the other hand, non- hospital isolates were resistant against Amoxicillin and Penicillin (66.7%) followed by Ampicillin and Vancomycin (58.3%). Both hospital and non-hospital isolates were sensitive to Gentamycin respectively 72.5% and 75%. The findings of the experiment clearly suggested that hospital waste water contained more MDR bacteria than non-hospital waste water which are released into receiving water bodies that may cause serious threat to public health. Reducing indiscriminate use of antibiotics in both hospital and non-hospital settings and use of waste water treatment plant (WTP) in hospital may reduce this problem.

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LIST OF ABBREVIATIONS AND SYMBOLS

-	Negative
#	Identifying number
%	Percentage
@	At the rate of
+	Positive
µg	Microgram
µl	Microlitre
^0C	Degree of celcius
Assist	Assistant
BA	Blood Agar
BD	Bangladesh
EMB	Eosin Methylene Blue
ER	Erythromycin
et al.	Associated
etc	Etcetera
Gm	Gram
H.S	Hemorrhagic septicemia
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
HSTU	Hajee Mohammad Danesh Science and Technology University
MARMC	M. Abdur Rahim Medical College
SHD	Sadar hospital of Dinajpur
IBCH.	Islami bank community hospital, Dinajpur

LIST OF ABBREVIATIONS AND SYMBOLS (Cont.)

HSTU	**Hajee Mohammad Danesh science and Technology University, Dinajpur**
M.S	**Master of Science**
MC	**Mac-Conkey Agar**
MSA	**Mannitol Salt Agar**
MI	**Milliliter**
MIU	**Motility Indole Urease**
MR	**Methyl Red**
NA	**Nutrient Agar**
NB	**Nutrient Broth**
No.	**Number**
PBS	**Phosphate Buffer Saline**
Prof.	**Professor**
PSS	**Physiological Saline Solution**
RPM	**Rotation Per Minute**
SC	**Subcutaneous**
SE	**Standard Error**
SL	**Serial number**
spp	**Species**
SSA	**Salmonella- Shigella Agar**
v/v	**Volume by volume**
vp	**Voges-Proskauer**
w/v	**Weight by volume**

CHAPTER 1

INTRODUCTION

Antibiotics are a class of naturally-occurring, semi-synthetic and or chemically synthesized compounds with antimicrobial activity. They are widely used in human and veterinary medicine to treat and prevent diseases and as growth promoters in animal intensive industries. The increasing incidence of resistance to a wide range of antibiotics by microorganisms is a major concern facing modern medicine. Clinical infections, disease and death caused by resistant bacteria are increasingly common. We know for a fact that antibiotic resistance can be established and propagated in human and animal digestive systems (Launay *et al.*, 2014; Chopra *et al*., 2001). Antibiotic resistance has become a major clinical and public health problem within the life time of most people living today (Stuart *et al.*, 2002). Confronted by increasing amounts of antibiotics over the past 60 years, bacteria have responded to the deluge with the propagation of progeny no longer susceptible to them. While it is clear that antibiotics are pivotal in the selection of bacterial resistance, the spread of resistance genes and of resistant bacteria also contributes to the problem (Stuart *et.al.* 2002). Possible mechanisms by which humans enhance the spread of antibiotic resistance among environmental bacteria include the deliberate or accidental introduction of antibiotics, resistant bacteria and resistance genes into the environment. Antibiotics exert a selection in favor of resistant bacteria by killing or inhibiting growth of susceptible bacteria; resistant bacteria can adapt to environmental conditions and serve as vectors for the spread of antibiotic resistance (Wegener *et al*., 1999; Kruse, 1999). The main risk for public health is that resistance genes are transferred from environmental bacteria to human pathogen (Wegener *et al.,* 1999; Kruse, 1999**).**

Hospital waste waters are composed of the effluents of different services: kitchen, internal laundry, heating and cooling systems, laboratories, radiology departments, outpatients departments, transfusion centers and wards. Due to the nature and quantity of the micro-pollutants they harbor, such as active substances of medicines and their metabolites, chemicals, heavy metals, disinfectants, sterilizers, and radioactive markers (Emmanuel *et al*., 2005 ; Boillot *et.al*.,2008) and the proliferation of drug-resistant microorganisms (Hawkshead *et al.,* 2008). Hospital waste water is considered a hot spot for antibiotic

resistance (AR) as a consequence of receiving a cocktail of antibiotic compounds, disinfectants, and inputs of bacterial shadings and metabolized drugs from patient excrement, which potentially contain multidrug-resistant (MDR) pathogens (Chagas *et al*., 2010, 2011; Galvin 2010) As such, hospital waste waters provide an environment for the exchange of antibiotic resistance genes (ARGs) between clinical pathogens and other environmental bacteria in recipient sewers, which could result in broader epidemiological consequences extending beyond the hospital setting. Integrons enable the recombination and expression of mobile gene cassette arrays which contain ARGs and are used as indicators to gather information on trends of AR development and dissemination in bacterial communities (Bengtsson *et al.,* 2015; Stalder. *et al*., 2014). Hospital waste effluents, even if it is treated, may contain pathogenic drug- resistant bacteria, which constitute the most dangerous single risk factor for dissemination of pathogenic and drug resistant organisms to the environment (Rahman *et al*., 2005).

Microorganisms that are important causes of infection in humans, such as gram negative bacilli (GNB) that include *Enterobacter spp.* and *Pseudomonas aeruginosa,* are able to survive for long periods of time in the environment, thus contributing to the selection of resistant pathogens disseminated in the environment, as well as in hospitals, industry and veterinary facilities. These natural reservoirs of resistant genes may contribute to the appearance of resistant bacteria due to gene transfer mechanisms (Aygen *et al.*, 2000, Alp *et al.,* 2002; Sader *et al.,* 1997). Visitors to developing countries passively acquire antibiotic-resistant gut *Escherichia coli*, even if they are not taking prophylactic antibiotics, which suggests that they encounter a reservoir of antibiotic-resistant strains during travel (Murray *et al.,* 1990). Peoples of developing countries often bear antibiotic-resistant fecal commensal organisms (Calva *et al*., 1996; Lamikanra 1989). Apparently healthy people in developing countries carry potentially pathogenic, antibiotic-resistant organisms asymptomatically (Woolfson *et al.,* 1997). It has been shown that most multiple antibiotic resistances in clinical isolates of Gram negative bacteria are plasmid borne. Moreover, animal and human faeces have been recognized as reservoirs of the resistance plasmid (Shanahan 1994; Woodford 1998). Faecal indicator bacteria (FIB) (*Escherichia coli (E. coli)* and *Enterococcus spp*., (ENT)) residing in the gastrointestinal tract of warm blooded animals are generally used to monitor the microbial quality of water sources. Additionally, polluted surface waters and sediments can contain a variety of pathogenic microbes including bacteria, viruses and protozoa (Haller *et al*., 2009; Mwanamoki *et al*.,

2014). The choice of bacterial indicators is thus very important. Bacteria belonging to the *Pseudomonas* genus are extensively present in the environment, such as water soil and sediment. Being known for its innate resistance mechanisms, *Pseudomonas* spp. are capable of staying viable in the aquatic environment for long periods (Spindler *et al*.,2012) which carries the hazard of spreading ARGs and mobile genetic elements and can cause infections in humans (Spindler *et al*.,2012 ; Quinteira *et al*.,2005). The occurrence of emerging biological contaminants including antibiotic resistance genes and faecal Indicator Bacteria is still little investigated in developing countries under tropical conditions. The ARGs *aad*A and bla_{TEM} were most frequently detected in higher concentration than other ARGs and The ARGs bla_{SHV} and bla_{NDM} were identified in CRB sediments contaminated by hospital and urban wastewaters. (Devarajan *et al*., 2016)

From above discussion it seems that drug resistance is now a big threat to our whole ecology, so this problem should not be overlooked at all. If this problem cannot be solved very soon it will cause serious health hazard throughout the world. As the resistant gene from resistant organisms are spreading through the untreated hospital and in some circumstances from on hospital waste water, considering all above facts; the objectives of the current study were;

- To isolate and identify public health important bacteria from waste water in hospital and non- hospital environments.
- Molecular characterization of important pathogenic bacteria.
- To compare and understand the drug resistance pattern of pathogens from hospital and non hospital waste water.

CHAPTER 2

REVIEW OF LITERATURE

The study was conducted to isolate and characterize drug resistant bacteria from hospital and non-hospital waste water of Dinajpur. Similar study was conducted in various part of the world, their study was reviewed bellow.

2.1 Isolation of multiple drug resistant bacterial pathogens from hospital waste water

Rabbani *et al.*, (2017) studied antibiotic resistant bacteria in untreated hospital waste water in Dhaka City, Bangladesh. They isolated 59 *Escherichia coli* and 29 *Klebsiella pneumonia* isolates from two renowned hospital of Dhaka city. Their study demonstrated that the sites that were at the disposal point of hospital waste have higher degree of resistance and high degree of resistance also observed when 23 high-resistant *E. coli* isolates were further tested with 15 additional antibiotics. Their study revealed a significant rise of multiple drug resistant (MDR) bacteria in the hospital waste and underscore necessity of hospital waste treatment.

Onuoha *et al.*, (2017) studied isolation and characterization of multi-drug resistant bacterial pathogens from hospital effluents, south eastern, Nigeria. He processed a total of 60 waste water samples for the presence of drug resistant pathogens, out of which 61 bacterial isolates were recovered. The most frequently identified bacterium was *Pseudomonas* spp 17 (27.9%) followed by *E. coli* 16(26.2%), *Staphylococcus aureus* 15(24.6%) and *Salmonella* spp 13(21.3%). His study showed that prevalence of multiple drug resistance (MDR) bacterial isolates exhibited resistance to more than three 3 antibiotics, although their patterns of resistance varied, and all isolated organisms were resistant to clindamycin and ampicillin. He also added that the most effective antibiotic was the quinolones where there was some level of susceptibility among many of the isolates. He suggested that the presence of these multi-drug resistant strains from hospital waste waters could act as a vehicle to disseminate antibiotic resistant bacteria in the environment; therefore, there is need for hospital wastewater to be treated before they are released into the environment.

Kalaiselvi *et al.*, (2016) conducted a study to highlight the incidence of antibiotic resistant bacteria in hospital-generated recycled water. They mostly identified *Staphylococcus aureus* (11.42%), *Pseudomonas aeruginosa* (9.28%), *Enterococcus faecalis* (10%) and *Bacillus subtilis* (8.57%) which were removed by treatment, but *Escherichia coli* (16.42%), *Klebsiella pneumonia* (8.57%), and *Proteus mirabilis* (11.42%) survived in the final sedimentation tank (lagoon) from where this water will be used for gardening purposes. Their study showed that isolated pathogens were resistant to first-line antibiotics. They suggested that effluent treatment plants in hospitals should be monitored for the fulfillment of the guidelines and quality control of treated water to stop the emergence of multi-drug resistant bacteria in the hospital environment.

Thai-Hoang *et al.*, (2016) examined waste water discharged from clinical isolation and general wards at two hospitals in Singapore. They phylogenetically classified 119 antibiotic-resistant isolates and tested for the presence of antibiotic resistant genes (ARGs) through different antibiotics. Among these resistant isolates, 80.7% were detected with *intI1* and 66.4% were found to carry at least 1 of the tested ARGs. Among 3 sampled locations, the clinical isolation ward had the highest concentrations of antibiotic resistant bacteria (ARB) and the highest levels of ARGs linked to resistance to β-lactam **(***blaKPC***)**), co-trimoxazole (*sul1, sul2, dfrA*), amikacin [*aac(6=)-Ib*], ciprofloxacin (*qnrA*), and *intI1*.They found strong positive correlations ($P<0.05$) between concentrations of bacteria resistant to meropenem, ceftazidime, amikacin, co-trimoxazole, and ciprofloxacin and abundances of *blaKPC, aac(6=)-Ib, sul1, sul2, dfrA, qnrA*, and *intI1* genes.

Devarajan *et al.*, (2016) investigated the occurrence of antibiotic resistance genes and bacterial markers in a tropical river receiving hospital and urban waste waters. They quantified total bacterial load, the abundance of FIB (*E. coli and Enterococcus spp.* (ENT)), *Pseudomonas spp*. and ARGs (blaTEM, blaCTX-M, blaSHV, *bla*NDM and *aad*A) using quantitative PCR in the total DNA extracted from the sediments recovered from hospital outlet pipes (HOP) and the Cauvery River Basin (CRB), Tiruchirappalli, Tamil Nadu, India. ARGs aadA and blaTEM was the most frequently detected in higher concentration than other ARGs at all the sampling sites. They suggested that Tropical aquatic ecosystems receiving wastewaters can act as reservoir of ARGs, which could potentially be transferred to susceptible bacterial pathogens at these sites.

Youngho *et al.*, (2016) investigated the community of whole microbes and antibiotic resistance bacteria (ARB) in hospital waste water treatment plants (WWTP) receiving domestic waste water (DWW) and hospital waste water (HWW). They characterized the whole microbial community and ARB on the antibiotic resistance database from an influent of a secondary clarifier, at each treatment train, based on high-throughput pyrosequencing. Their pyrosequencing analysis revealed that the abundance of *Bacteroidetes* in the DWW sample was higher (Approximately1.6 times) than in the HWW sample, whereas the abundance of *Proteobacteria* in the HWW sample was greater than in the DWW sample.

Jamali *et al.*, (2015) conducted a study on emergence of drug resistance in bacterial isolates from hospital waste water: a potential health hazard. They isolated 50 *enterobacteria* from hospital waste water; all the isolates were further tested for their antibiotic susceptibility. They observed an elevated level of resistance against penicillin-G (90%) in strains isolated from hospital wastewater. All the isolates were sensitive to erythromycin, doxycycline, gentamycin, kanamycin and chloramphenicol. They observed seven different resistance patterns in *enterobacteria* isolated from hospital wastewater among the fifteen antibiotics they tested. They concluded that their study may be beneficial to design environment-friendly techniques for the removal of antibiotics from wastewater to overcome the problem of antibiotic resistance development in the aquatic environment.

Fekadu *et al.*, (2015) conducted a cross-sectional study for the assessment of antibiotic- and disinfectant-resistant bacteria in hospital waste water, south Ethiopia. They detected pathogenic (*Salmonella*, *Shigella*, and *S. aureus*) and potentially pathogenic (*E. coli*) bacteria from effluents of both hospitals. Their study demonstrated that 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. They explained that hospital effluents tested contained antibiotic-resistant bacteria, which are released into receiving water bodies, resulting in a threat to public health.

Iweriebor *et al*., (2015) assessed the antimicrobial resistance and virulence profiles of some common *Enterococcus spp* that are known to be associated with human infections that were recovered from hospital waste water and final effluent of the receiving wastewater treatment plant in Alice, Eastern Cape. They confirmed 62 belong to the *Enterococcus* genus of which 30 were identified to be *E. faecalis* and 15 *E. durans*. The remaining isolates were not identified by the primers used in the screening procedure. Their study indicated that hospital wastewater may be one of the sources of antibiotic resistant bacteria to the receiving waste water treatment plant (WWTP). Their findings also revealed that the final effluent discharged into the environment was contaminated with multi-resistant enterococci species thus posing a health hazard to the receiving aquatic environment as these could eventually be transmitted to humans and animals that are exposed to it.

Harris *et al.*, (2014) conducted a study on antimicrobial resistant *Escherichia coli* in the municipal wastewater system: Effect of hospital effluent and environmental fate. Their study suggested that waste water treatment plat (WWTP) influent containing hospital effluent had a higher mean percentage of AMR *E. coli*; although, there appeared to be no within treatment plant effect on the prevalence of antimicrobial resistant (AMR) *E. coli* and examination of WWTP sludge showed a similar variation. They concluded that it appears that hospital effluent is not the main contributing factor behind the development and persistence of AMR *E. coli* within WWTPs, although resistance may be too well-developed to identify an influence from hospital effluent.

Moges *et al*., (2014) conducted a cross-sectional study at Gondar (Ethiopia) to study the antibiotic resistant pattern of pathogenic bacteria from hospital and non-hospital environment. They recovered 113 bacterial isolates among them 65 (57.5%) were from hospital environment and 48 (42.5%) were from non-hospital environment. *Klebsiella* spp. 30 (26.6%) was the most frequently identified bacteria in their study, followed by *Pseudomonas spp*. 19(16.8%), *E. coli* (11.5%) and *Citrobacter spp* (11.5%), and *Staphylococcus aureus* (8.2%). The overall prevalence of multiple drug resistance (MDR) in their study was 79/113 (69.9%). They found higher rates of MDR bacteria in hospital environment (81.5%) than non-hospital environment (54.2%). They suggested that multiple drug resistance to the commonly used antibiotics is high in the study area. They described that the contamination of waste water by antibiotics or other pollutants lead to

the rise of resistance due to selection pressure therefore presence of antibiotic resistance organisms in this waste water should not be overlooked.

Khan *et al*., (2013) investigated the incidence of antibiotic resistant bacteria in hospital generated effluent discharged into municipal sewage system of Sylhet city, Bangladesh. They isolated and identified 29-gram negative bacteria from 6 effluent samples. Their study showed ten of the isolates resistance to three or more commonly used antibiotics. Plasmid profiles of the multi-drug resistant isolates showed to harbor two or more plasmids and almost all of them showed a common band for plasmid DNA size of 24.5kb. They concluded that resistance to the bacterial pathogens may cause community acquired infections, thus, exert a serious public health threat through confining the antibiotic pool, so they suggested that hospitals should follow, monitor and regulate proper sanitary measures of hospital generated effluents.

Varela *et al.*, (2013) investigated the occurrence of VRE in a hospital effluent and in the receiving urban waste water treatment plant. They isolated vancomycin and ciprofloxacin resistant enterococci from hospital and urban waste water. They explained that hospital effluent and raw urban waste water had identical counts of vancomycin resistant enterococci. They also added that Vancomycin resistant enterococci were significantly more prevalent in the hospital effluent than in the urban waste water. Their study observed similar multidrug-resistance phenotypes in isolates from patients, hospital effluent and urban waste water. Their study suggested that hospital effluents may contribute to spread vancomycin resistant enterococci to the environment.

Zubair *et al.*, (2013) analyzed the incidence and transferability of antibiotic resistance in the enteric bacteria isolated from hospital waste water. They tested sixty-nine isolates for antibiotic sensitivity; among them 73.9% strains were resistant to ampicillin followed by nalidixic acid (72.5%), penicillin (63.8%), co-trimoxazole (55.1%), norfloxacin (53.6%), methicillin (52.7%), cefuroxime (39.1%), cefotaxime (23.2%) and cefixime (20.3%). They noted multiple antibiotic resistances in both among extended spectrum β-Lactamase (ESBL) and non-ESBL producers. They founded fifteen isolates that are multidrug resistant producing ESBLs. Four ESBL producing isolates could transfer their R-plasmid to the recipient strain *E.coli* K-12 with conjugation frequency ranging from 7.0 x 10-3 to 8.8 x 10-4. Their findings indicated that ESBL producing enteric bacteria are common in

the waste water. They described that such isolates may disseminate the multiple antibiotic resistances traits among bacterial community through genetic exchange mechanisms and thus require immediate attention.

Guessennd *et al.*, (2013) assessed that untreated effluents generated by hospital activities can contribute significantly to the spread of multiresistant bacteria (MRB) in the environment. They mostly isolated *E. coli, K. pneumoniae, P. aeruginosa, Staphylococcus aureus* and *A. baumanii*. Most of the strains were resistant to three classes of antibiotics (beta-lactams, aminoglycosides and fluoroquinolones). Their study suggested that the effluent of University of Cocody teaching hospital (Abidjan, Côte d'Ivoire) contains most of multi-resistant bacteria involved in nosocomial infections such as *Acinetobacter baumanii* and *Enterobacteriaceae producing* beta-lactam antibiotics in expanded spectrum (ESBL) *Pseudomonas* resistant to ceftazidime (PARC), *Staphylococcus aureus* resistant to methicillin (MRSA).

Essam *et al*., (2012) analyzed twelve isolates out of twenty-eight were selected from three hospital drains (four isolates from each hospital drain) in Cairo, Egypt. He tested these isolates with thirteen different antibiotics and the sensitivity of antibiotic resistant bacteria was expressed as 42.86%. Only three out of twelve isolates were identified as *Staphylococcus aureus* and the relationship between the plasmids and these isolates exhibited that two isolates detected two plasmids and one detected four plasmids are responsible for the resistant toward antibiotics.

Katouli *et al.*, (2012) investigated the presence and survival of antibiotic resistant bacteria in untreated hospital wastewaters (UHWW) and their transmission to the receiving sewage treatment plant (STP) in South East Queensland (SEQ), Australia. They collected 245 *Escherichia coli* and 167 *Staphylococcus aureus* strains from UHWW. There data suggested that some MDR bacterial strains found in UHWW may have the ability to survive transmission to the sewage treatment plant (STP) and then through to the final treated effluent before being released into surface waters.

Thompson *et al.*, (2012) investigated the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in untreated hospital waste waters (UHWW), their transmission into the receiving sewage treatment plant (STP) and survival through the STP treatment. They

isolated 224 *Staphylococcus aureus* and strains these strains were typed using the PhP typing method and RAPD-PCR and tested for their antibiotic resistance patterns. Resistance to cefoxitin and the presence of *mec*A gene identified MRSA isolates. Among the 131 (68%) MRSA strains, 24 were also vancomycin resistant. MDR strains (including MRSA) were more prevalent in hospital waste waters than in the STP. Their study provides evidence of the survival of MRSA strains in UHWWs and their transit to the STP and then through to the final treated effluent and chlorination stage.

Bolaji *et al.*, (2011) observed that *Enterobacter aerogenes, Pseudomonas putida. Pseudomonas fluorescens, Klebsiella edwardsii, Proteus mirabilis, Pseudomonas aeruginosa, Shigella spp and Flavobacterium meningosepticum* were all 100% resistant to all 8 antibiotics he used. All these isolates he was collected from selected hospital waste water of Nigeria. He concluded that these organisms have been well exposed to the tested antimicrobials and they have developed mechanisms to avoid them. He also added that antibiotic resistant bacteria are also present in hospitals where people go to for the treatment of infections.

Sharma *et al*., (2010) investigated that multiple drug resistant bacteria in the hospital effluent were alarmingly high. Drug resistant hospital effluent isolates showed simultaneous resistance for most of the antibiotics including Penicillin, Cephalosporin, Cotrimoxazole, Gentamycin and Quinolones. They concluded that liquid wastes from healthcare were laden with MDR bacteria and seemed to pose a huge public health threat and transfer of these resistances to the bacterial pathogens causing community acquired infections, which are limiting our antibiotic pool.

Sandra *et al*., (2010) assessed effluent from municipal, hospital, and secondary treatment facility sources and they enumerated and characterized antimicrobial-resistant *Escherichia coli.* Their result suggested that downstream Effluent from the hospital contained a higher proportion of antimicrobial-resistant *E. coli* than that upstream from the hospital. They also described that waste water treatment reduced the numbers of *E. coli* bacteria (total and antimicrobial resistant); however, antimicrobial resistant *E. coli* was not eliminated, and *E. coli* resistant to cefotaxime (including extended-spectrum beta lactamase [ESBL] producers), ciprofloxacin, and cefoxitin was present in treated effluent samples.

Diwan *et al.*, (2010) conducted a cross-sectional study in a teaching hospital outside the city of Ujjain in India to find out antibiotic resistant bacteria in hospital waste water. They described Ciprofloxacin was the highest prescribed antibiotic in the hospital and its residue levels in the hospital waste water were also the highest. They did not find any antibiotic in samples of the municipal water supply and the groundwater. They suggested that there may be a positive correlation between the quantity of antibiotics prescribed in the hospital and antibiotic residue levels in the hospital waste water. They also found both higher number of antibiotic resistant bacteria and higher level of antibiotics from samples that are collected afternoon hour. They confirmed that although ciprofloxacin was the most prevalent antibiotic detected in the waste water, *E.coli* was not resistant to it. They concluded that antibiotics are entering the aquatic environment of countries like India through hospital effluent.

Ibrahim *et al.*, (2010) investigated the antibiotic resistance in gram-negative pathogenic bacteria in hospitals' drain in Al-Madina Al-Munnawara. They collected 21 distinct species and 13 genera belonging Gram negative rods and the most common isolates were belonging to *Escherichia coli* (32.05%) followed by *Enterobacter cloacae* (17.9%). They 234 identified Gram-negative rods isolates that were tested for their susceptibility for seven antibiotics. Gentamicin was the most effective antibiotic, as only 2.6% of these isolates were resistant to 10µg/ml, while 2.1% were resistant up to 256µg/ml. The least effective antibiotic tested was sulphamethoxazole, as 82.9% of the isolates were resistant to 25µg/ml of this antibiotic, while 62.8% were resistant to 1024µg/ml. The second less effective antibiotic was ampicillin.

Islam *et al.*, (2008) assessed that *E.coli* those were isolated from hospital waste was multidrug resistant and also there plasmid containing multidrug resistant genes. These *E.coli* and there genes from hospital wastes can be the possible source of transferring the highly resistant pathogens to the human that could be threat for the treatment of disease by commercially available antibiotics.

Yang *et al.*, (2008) examined bacterial populations and antimicrobial resistance patterns between clinical and sewage isolates from a regional hospital in northern Taiwan. They recovered 87.2% gram negative bacteria from clinical samples and 91%, from swage samples and *Escherichia coli* were the leading bacterial isolates in both groups. Their

study suggested that sewage isolates had higher antimicrobial resistance rates than the clinical isolates from the same hospital. They hypothesized that the low efficacy of the hospital sewage treatment may contribute to the dissemination of multidrug resistant bacteria from this hospital compartments to the environment.

Diab *et al.*, (2008) surveyed bacterial population including, total viable bacteria (TVB) and total coliform (TC) counts in the final effluent for 5 hospitals (3 governmental and 2 private) at Al-Madinah Al- Mounwwarah. Their Study founded that Gram negative bacterial strains dominated specially those of family Enterobacteriaceae and Pseudomonadaceae. *E.coli* was mostly founded in their study followed by *Enterobacter cloacae* and *Chryseobacterium meningiosepticum.* All their studied strains exhibited resistance to at least 3 of the 9 tested antibiotics. Many bacterial isolates resist the whole 9 antibiotics and to a concentration of more than 100 µg/ml. their study revealed that most of the resistance strains were gram negative and the mechanism of action proved to be inhibitory.

Tumeo *et al.*, (2008) conducted a study in France to trace whether antibiotic-resistant *Pseudomonas aeruginosa* isolated from hospitalized patients recovered in the hospital effluents, the result showed genotyping of both clinical and wastewater isolates was determined by using pulsed-field gel electrophoresis (PFGE). There was no common PFGE pattern in antibiotic-resistant *P. aeruginosa* from humans and waste water therefore antibiotic resistance profile of waste water isolates were different from that of clinical isolates.

Ekhaise and Omavwoya, (2008) conducted a study in Nigeria demonstrated that hospital waste water was observed to play a significant role in the influence on the qualities of the bacteriological and physiochemical parameters on the receiving environment due to increased amount of organic matter and essential nutrients in hospital wastewater.

Naeem *et al.*, (2007) carried out a study to estimate the spread of multidrug resistant (MDR) bacterial from hospital effluent to the municipal sewage system. They founded total viable count of bacterial populations in hospital effluents ranging from 10 colony forming unit (CFU)/ml for *Serratia marcescens* to 500 CFU/ml for *Escherichia coli* in three hospitals; the percent of MDR bacteria population in all these hospitals ranged from (5%) *Serratia marcescens* to (97%) *Escherichia coli*. Their study revealed that the MDR

bacteria carried simultaneous resistance for most commonly used antibiotics and obviously the spread of such MDR bacteria to the community is a matter of grave concern.

Elmanama *et al*., (2006) analyzed to study the resistance profile of bacterial isolates from Al-Shifa hospital in Gaza as a health institution and comparing their profile to a non-health institution. Total of 45 samples were collected and 154 different bacteria were isolated from these samples. They collected total of 45 samples and 154 different bacteria were isolated from these samples. From their isolated bacteria 30.5% were *E. coli*, 33.1% *Pseudomonas* sp., 10.4% *Klebsiella* sp., 4.5% *Proteus* sp. and 21.4% *Enterococcus* sp. Their isolates were subjected to antimicrobial susceptibility testing with different antibiotics. They founded the percent of resistance for Gram-positive bacteria (*Enterococcus*) to 5 antibiotics were as the following: Streptomycin (91.0%), Vancomycin (75.8%), Erythromycin (60.6%), Teicoplanin (9.1%) and Ampicillin (6.1%).

Ajamaluddin *et al*., (2000) investigated the prevalence of multiple antibiotic resistance and r-plasmid in *Escherichia coli* isolates of hospital sewage of aligarh city in India. They have tested 30 *Escherichia coli* isolates from hospital sewage for their resistance and sensitivity against 10 antibiotics. Their study showed that 90% isolates were resistance against ampicillin and sulphamethizole. They also showed that the total 30 *E. coil* isolates 86.6% were resistant to erythromycin and rifampicin but none of them was resistant to kanamycin and streptomycin. They isolated Plasmids (tool. wt. 16.5 mega daltons) from five different *E. coil* strains which harbored only a single plasmid and were characterized on the basis of antibiogram. They concluded that multiple drug resistance among most of the *E. coil* isolates is plasmid borne.

Chitnis *et al*., (2000) studied the spread of multiple drug-resistant (MDR) bacteria from hospital effluent to the municipal sewage system. They founded the MDR bacterial population in hospital effluents ranged from 0.58 to 40% for ten hospitals studied while it was less than 0.00002 to 0.025% for 11 sewage samples from the residential areas. They also added that the MDR bacteria carried simultaneous resistance for most of the commonly used antibiotics and obviously the spread of such MDR bacteria to the community is a matter of grave concern.

Luca *et .al*., (1998) investigated possible increase of antibiotic resistant bacteria in sewage associated with the discharge of wastewater from a hospital and a pharmaceutical plant by using *Acinetobacter* species as environmental bacterial indicators. He isolated 385 *Acinetobacter* strains from samples collected upstream and downstream from the discharge points of the hospital and the pharmaceutical plant. His result indicated that while the hospital waste effluent affected only the prevalence of oxytetracycline resistance, the discharge of wastewater from the pharmaceutical plant was associated with an increase in the prevalence of both single- and multiple-antibiotic resistance among *Acinetobacter* species in the sewers.

Michael *et al.*, (1985) conducted a study in Philadelphia to examine transfer of plasmids pBR322 andpBR325 in wastewater from laboratory strains of *Escherichia coli* to bacteria indigenous to the waste disposal system showed that bacterial strains isolated from raw wastewater or a plasmid free *E. coli* laboratory strain served as recipients. Transfer of the pBR plasmid into the recipient strain occurred during a 25-h co-incubation in either L broth or sterilized wastewater.

Dubreuil *et al.*, (1984) conducted a study in France to compare vitro activity of Cefoxitin with Metronidazole and Clindamycin against 322 strains of anaerobic bacteria collected from several hospitals during 1982 and tested by an agar dilution method showed that Metronidazole and Cefoxitin inhibited at least 89% of strains tested, whereas Clindamycin was less active.

Grabow *et al.*, (1972) studied and compared the number and properties of drug-resistant coliform bacteria in hospital and city sewage. About 26% of coliforms in hospital waste water and 4% coliforms in city sewage were transferable resistance to at least one of the drugs ampicillin, chloramphenicol, streptomycin, sulfonamide, or tetracycline. In both effluents, the occurrence of fecal *Escherichia coli* among R+ coliforms was twice as high as among coliforms with nontransferable resistance. He discussed significance of the results with regard to environmental pollution with R+ bacteria and the dissemination of these organisms.

2.2 Isolation of multiple drug resistant bacterial pathogens from non-hospital/environmental waste water

Mutuku, (2017) investigated antibiotic resistance profiles among enteric bacteria isolated from wastewater in septic tanks. They isolated predominantly *E.coli* (41 isolates), following organisms were *Salmonella* (19), *Proteus* (13) and *Klebsiella* (22). They tested the strains to different classes of antibiotics including β-lactams, macrolide, tetracycline and sulfa drugs. They recorded all the strains as multiple drug resistant with *E.coli* being the most resistant to the antibiotics, followed by *Salmonella, Klebsiella* and *Proteus.* They suggested that the findings of their study will provide an understanding of the changing antibiotic resistance trends in enteric bacteria from sewage effluent and help design improved sewage treatment strategies to contain the spread of drug resistance which poses a great public health risk.

Osińska *et al.*, (2016) experimented the Prevalence of plasmid-mediated multidrug resistance determinants in fluoroquinolone-resistant bacteria isolated from sewage and surface water. They investigated 116 multiresistant isolates and their study showed that bacteria from *Escherichia* (25.0 %), *Acinetobacter* (25.0 %), and *Aeromonas* (6.9 %) genera were predominant in the FQRB group. They described that Fluoroquinolone resistance was mostly caused by the presence of the gene aac (6)-1b-cr (91.4 %) and the most prevalent bacterial genes connected with beta-lactams resistance in FQRB were blaTEM, blaOXA, and blaCTX-M. They detected highest number of multidrug-resistant (MDR) microorganisms in TWW and DRW samples. Their study indicated that discharged TWW harbors multiresistant bacterial strains and that mobile PMQR and ARGs elements may have a selective pressure for species affiliated to bacteria in the river water.

Martins *et al.*, (2016) the occurrence of multidrug resistance (MDR) in Escherichia coli strains isolated from discharged final effluents of two wastewater treatment facilities in the Eastern Cape Province of South Africa. Their study revealed five pathotypes of *E. coli* in the following proportions: enterotoxigenic ETEC (1.4%), enteropathogenic EPEC (7.6%), enteroaggregative EAEC (7.6%), neonatal meningitis (NMEC) (14.8%), uropathogenic (41.7%), and others (26.9%) through Molecular characterization. Their isolates showed varying (1.7–70.6%) degrees of resistance to 15 of the test antibiotics. They concluded that municipal wastewater effluents are important reservoirs for the dissemination of

potentially pathogenic *E. coli* (and possibly other pathogens) and antibiotic resistance genes in the aquatic milieu of the Eastern Cape and a risk to public health.

Bäumlisberger *et al*., (2015) assessed influence of a non-hospital medical care facility on antimicrobial resistance in wastewater. They analyzed Microbial composition, ARGs and MGEs using different annotation approaches with various databases, including Antibiotic Resistance Ontologies (ARO), integrons and plasmids. Their analysis identified seasonal differences in microbial communities and abundance of ARG and MGE between samples from different seasons. However, they did not detected any obvious differences between up- and downstream samples. Their results suggested that, in contrast to hospitals, sewage from the nursing home does not have a major impact on ARG or MGE in wastewater, presumably due to much less intense antimicrobial usage. They discussed possible limitations of metagenomic studies using high-throughput sequencing for detection of genes that are seemingly confer antibiotic resistance.

Kappell *et al.*, (2015) investigated study was to evaluate antibiotic resistance of *Escherichia coli* is isolated from the urban water ways of Milwaukee, WI. They isolated 259 *E.coli* and all obtained isolates were determined to be multi-drug resistant. Their study showed that *E.coli* from urban water ways a greater incidence of antibiotic resistance to 8 of 17 antibiotics tested compared to human derived sources and these *E.coli* isolates also demonstrated a greater incidence of resistance to higher numbers of antibiotics compared to the human derived isolates. Their study these results indicate that Milwaukee's urban water ways may select or allow for a greater incidence of multiple antibiotic resistance organisms and likely harbor different antibiotic resistance gene pool than clinical sources.

Blaak *et al*., (2015) assessed the prevalence and concentrations of antimicrobial resistant (AMR) *Escherichia coli* in Dutch surface water. Among their surface water isolates, 26% were resistant to at least one class of antimicrobials, and 11% were multidrug-resistant (MDR). The different resistance types occurred with similar frequencies among *E. coli* from surface water and *E. coli* from municipal waste water. By contrast, among *E. coli* from HCI waste water, resistance to cefotaxime and resistance to ciprofloxacin were significantly overrepresented compared to *E. coli* from municipal waste water and surface water. They concluded that MDR *E. coli* are omnipresent in Dutch surface water, and indicate that municipal waste water significantly contributes to this occurrence.

Franz *et al.*, (2015) investigated the pathogenic *Escherichia coli* producing extended-spectrumβ□lactamases isolated from surface water and waste water. They characterized 170 ESBL-producing *E. coli* from Dutch waste water (n= 82) and surface water (n=88) with respect to ESBL-genotype, phylogenetic group, resistance phenotype and virulence markers associated with enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), extraintesinal *E. coli* (ExPEC), and Shiga toxin-producing *E. coli* (STEC). Overall, 17.1% of all ESBL-producing *E. coli* were suspected pathogenic variants. They concluded that their study demonstrates the aquatic environment is a potential reservoir of *E. coli* variants that combine ESBL-genes, an elevated level of multi-drug resistance and virulence factors, and therewith pose a health risk to humans upon exposure.

Luczkiewicz *et al.*, (2015) studied the antimicrobial resistance of *pseudomonas spp*. isolated from waste water and waste water-impacted marine coastal zone. Among their identified species, resistance to all antimicrobials but colistin was shown by *Pseudomonas putida*, the predominant species in all sampling points. They observed in other species, resistance was mainly against ceftazidime, ticarcillin, ticarcillin-clavulanate, and aztreonam, although some isolates of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas pseudoalcaligenes,* and *Pseudomonas protegens* showed multidrug-resistance (MDR) phenotype. Their obtained data suggested that Obtained that *Pseudomonas spp*. are equipped or are able to acquire a wide range of antibiotic resistance mechanisms, and thus should be monitored as possible source of resistance genes.

Rahim *et al.*, (2014) monitored and compared antibiotic resistant bacteria and their resistance genes in municipal and hospital wastewaters. Their study revealed that there is a variation in prevalence of different groups of antibiotic resistant bacteria (ARB) in municipal waste (MWs) and hospital waste (HWs). They described that all waste water Treatment plant (WWTPs) decreased the concentration of ARB, However, high concentration of ARB was found in the final effluent of WWTPs. Similar to ARB, different groups of ARGs were found frequently in both MWs and HWs. All genes also detected with a relative high frequency in effluent samples of MWs WWTPs. They suggested that discharge of final effluent from conventional WWTPs is a potential route

for dissemination of ARB and ARGs into the natural environment and poses a hazard to environmental and public health.

Abhay, (2012) studied the antibiotic resistance, plasmid and RAPD profiles of the multidrug resistant (MDR) Coliform bacteria isolated from raw and treated sewage of Ghaziabad city, India. They isolated 7.5% and 19.1% of the total Coliform bacteria respectively in the raw and treated sewage. Five MDR Coliform bacteria (2 from raw and 3 from treated sewage) were *Enterobacter spp* and these MDR strains were resistant to most of the commonly used antibiotics including amikacin. Plasmid isolation studies showed that all MDR strains harboured a single plasmid of approximately 54.4 kb size. Further, MDR strain R1 that was resistant to all 16 antibiotics tested showed plasmid-mediated resistance which was confirmed by plasmid curing study.

Łuczkiewicz *et al.*, (2011) conducted a study Identification and Antimicrobial Susceptibility of Fecal Coliforms Isolated from Surface Water. They isolated 274 fecal coliforms from two watercourses influencing the costal water quality of the Gdańsk and Puck bays, 265 were identified as *Escherichia coli*. The remained strains belonged to: *Acinetobacter* spp. (n=1), *Enterobacter* spp. (n=3), *Klebsiella* spp. (n=4), and *Shigella* spp. (n=1). They tested their isolated *E. coli* against 19 antimicrobial agents. In their study the highest number of isolates was resistant to penicillin (ampicillin 21% and piperacillin 14%), as well as to tetracycline (16%). Up to 19% of *E. coli* isolates were resistant to 3 or more of the analyzed antimicrobial agents, and 9% were regarded as multiple-antibiotic-resistant (MAR) strains. Two of the analyzed isolates were regarded as extended-spectrum β-lactamase – producing strains.

Pandey *et al*., (2011) conducted a study on isolation and characterization of multi drug resistance cultures from waste water. They identified 3 bacterial isolates, they included; *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus* species. They performed antibiotic sensitivity tests through Amoxicillin, Ampicillin, Tetracycline, Ofloxacin, Chloramphenicol, Ciprofloxacin at lower to higher conc. (10µg- 10mg) and best result obtained for *Ofloxacin, Ampicillin, Chloramphenicol* antibiotics (10µg-1mg). Their study was showed resistance for all cultures against *Ofloxacin,* it was 100% and for *Chloramphenicol,* the resistance activity was measured 80%. The MIC and MBC were also performed for identified cultures.

Gómez *et al*., (2010) assessed the number of both extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* and vancomycin-resistant enterococci (VRE) present in the inflow (hospital and urban sewage) and effluent waste water treatment plant (WWPT) and receiving waters. Their study showed that the average counts of ESBL producing *Enterobacteriaceae* in hospital sewage were much higher (more than 2.5 logs) than those observed in urban waste water. On the other hand, the sewage treatment plant studied did not perform well in removing pathogenic microorganisms. They described that *Enterobacteriaceae* concentrations were reduced in the final effluent by almost two log units as compared with the crude sewage concentrations, a high number of *Enterobacteriaceae* were detected in the effluent, representing a risk for microbiological pollution of water resources. They concluded that sewage (particularly hospital sewage) contains a high proportion of ESBL producing *Enterobacteriaceae* and vancomycin resistant enterococci, implying a potential risk of spreading these resistant bacteria and resistance genes to the environment and possibly to the human and animal population.

Iversen *et al*., (2002) conducted a study on high prevalence of vancomycin-resistant enterococci (VRE) in Swedish sewage. They screened samples from urban raw sewage, treated sewage, surface water, and hospital sewage in Sweden (*n* _ 118) for VRE. They isolated VRE from 21 of 35 untreated sewage samples (60%), from 5 of 14 hospital sewage samples (36%), from 6 of 32 treated sewage samples (19%), and from 1 of 37 surface water samples. They further characterized thirty-five isolates from 33 samples were by geno- and phenotyping, MIC determination, and PCR analysis. They founded that most isolates (30 of 35) carried the *vanA* gene, and the majority (24 of 35) of the isolates were *Enterococcus faecium*. They also added that all VRE from hospital sewage originated from one of the two hospitals studied. That hospital also had vancomycin consumption that was 10-fold that of the other. They conclude that VRE were commonly found in sewage samples in Sweden.

CHAPTER 3

MATERIALS AND METHODS

3. Materials

This study was conducted from July- December 2017 at different hospitals and non-hospital sites of Dinajpur district of Bangladesh. All microbiological analysis was carried out in microbiology laboratory of Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur.

3.1.1 Study area and period

The hospital samples were collected from three main hospitals of Dinajpur during the period of July- December 2017. These hospitals are selected because of their importance in Dinajpur and their lack of wastewater treatment plant. All three hospitals are located inside the Dinajpur (Municipality) and people of Dianjpur mainly dependent on these hospitals for their all kinds of health care. Non-hospital wastewater samples were collected from different site of HSTU campus and from different bazar of Dinajpur district.

3.1.2 Collection of Hospital Waste Water Sample

Ten untreated wastewater samples were collected from three hospitals of Dinajpur district. Four samples were collected from M. Abdur Rahim Medical College Hospital (MARMCH) including medicine ward (site-1), Pathology Laboratory (site-2), surgery ward (site-3) and general ward (site-4). Three samples were collected from Sadar hospital of Dinajpur (SHD), including general ward (site-1), Gynecology and Obstetrics ward (site-2); Pathology Laboratory (site-3). Rest of three hospital samples were collected from Islami bank community hospital, Dinajpur (IBCH). Including; Pathology Laboratory (site-1), general ward (site-2) and surgery ward (site-3)

Figure 1: Collection of Sample

Figure 2: Sample collecting box with sample

3.1.3 Collection of Non- Hospital Wastewater Sample

Ten untreated wastewater samples were collected from different sites of HSTU campus and different Bazar area of Dinajpur city. Three samples were collected from HSTU campus including; cow farm (site-1), Ostrich farm (site-2) and Poultry farm (site-3). One sample was collected from Baser Hat Bazar. Two samples were collected from each of the following area; Kalitola (site 1&2), Lilir Mor (site1&2) and Bahadur Bazar (site 1&2).

3.1.4. Glassware and Appliances

The different types of important equipment used for this work are listed in following; Distilled water, Sterile bent glass or plastic spreader rods, Micropipette (5-50µl; 10-100µl; 50-500µl; 100-1000µl), Freeze (-20°C), Refrigerator (4°C), Spirit lamp, Vortex Mixture, Labeling tape**,** Experimental test tube, Stopper, Petri dish, Conical flask, Durham's tube, Slide, Microscope, Cotton, Immersion Oil**,** Autoclave**,** Thermometer, Incubator**,** Jar, Beaker, Cylinder, Electric Balance, Filter paper, Bacteriological loop etc.

3.1.5. Bacteriological Media

3.1.5. 1. Plate Count Agar (HI-MEDIA, India)

Plate Count Agar (PCA), also called Standard Methods Agar (SMA), is a microbiological growth medium commonly used to assess or to monitor "total" or viable bacterial growth of a sample. PCA is not a selective medium (Cheesbrough, 1985).

3.1.5.2. Nutrient Agar Medium, (HI-MEDIA, India)

Nutrient agar is a basic media that is used for cultivating of non-fastidious microorganisms (Cheesbrough, 1985).

3.1.5.3. Eosin Methylene Blue, (EMB) (HI-MEDIA, India)

EMB contains dyes that are toxic for Gram positive bacteria and bile salt which is toxic for Gram negative bacteria other than coliforms. EMB is the selective and differential medium for coliforms. *Escherichia coli*: Blue-black bulls eye; may have green metallic sheen. *Escherichia coli* colonies grow with a metallic sheen with a dark center, *Aerobacter aerogenes* colonies have a brown center, and nonlactose-fermenting gram-negative and *Klebshella spp* bacteria appear pink (Cheesbrough, 1985).

3.1.5.4. MacConkey Agar medium, (Difco)

A differential medium for the isolation of coliforms and intestinal pathogens in water and biological specimens. Mac-Conkey agar is a culture medium designed to selectively grow Gram-negative bacteria and differentiate them for lactose fermentation. Lactose-fermenting organisms grow as pink to brick red colonies with or without a zone of precipitated bile. Non-lactose fermenting organisms grow as colorless or clear colonies (Cheesbrough, 1985).

3.1.5.5 Manitol Salt Agar (HI-MEDIA, India)

Mannitol salt agar or MSA is a differential medium and commonly used growth medium in microbiology. Gram positive *Staphylococcus*: Fermenting mannitol: Media turns yellow. Gram positive *Staphylococci*: Not fermenting mannitol. Media does not change color (Cheesbrough, 1985).

3.1.5.6 *Staphylococcus* Agar No. 110 (HI-MEDIA, India)

Staphylococcus Agar No. 110 is used to isolate *Staphylococci* and formation of golden yellow colonial pigments. (Cheesbrough, 1985).

3.1.5.7 Salmonella-Shigela Agar (Difco)

Salmonella Shigella Agar (SS Agar) is a selective and differential medium widely used in sanitary bacteriology to isolate *Salmonella* and *Shigella* from feces, urine, and fresh and canned foods. *Shigella* and the major part of *Salmonella*: Clear, colorless and transparent (Cheesbrough, 1985).

3.1.5.8 Cetrimide Agar Base (Difco)

Cetrimide agar base promotes the production of pyocyanin a water-soluble pigment as well as fluorescence, under ultraviolet light, of *Pseudomonas* spp which constitutes a presumptive identification. Cetrimide is the selective agent as it inhibits the growth of the accompanying microbial flora. Typical *Pseudomonas* spp colonies are greenish or yellowish green in color (Cheesbrough, 1985).

3.1.5.9 Blood Agar Medium, (HI-MEDIA, India)

For the isolation, cultivation and detection of hemolytic reaction of fastidious microorganisms. Blood Agar Base is suitable to isolate and cultivate a wide range of microorganisms with difficult growth. Upon adding blood, it can be utilized for determining hemolytic reactions *Staphylococcus aureus* gives beta hemolysis. *Streptococcus pneumoniae* gives alpha hemolysis. *Streptococcus pyogenes* gives beta hemolysis (Clin. Path, 1951).

3.1.510 Simmons Citrate Agar Medium, (HI-MEDIA, India)

Simmons citrate agar is used for determination the ability of bacteria to ferment citrate as a sole source of carbon.

3.1.5.11. Triple Sugar Iron Agar (HI-MEDIA, India)

Triple sugar iron agar is used for identification of gram negative bacteria that capable of fermenting sugar. Triple sugar iron agar contains lactose, sucrose and glucose.

3.1.5.12. Motility Indole Urease (MIU) test

MIU medium is a semisolid medium used in the qualitative determination of motility, production of indole and ornithine decarboxylase. MIU medium is used for the differentiation of the Family Enterobacteriaceae. The organisms tested must ferment glucose for proper performance of the medium. MIU medium contains dextrose as fermentable sugar, ornithine as an amino acid, bromcresol purple as pH indicator, casein peptone as a source of tryptophan, and other essential nutrients for growth. The medium contains a small amount of agar allowing for detection of motility (Cheesbrough, 1985).

3.1.5.13 Liquid Media

- Nutrient broth (Difco)
- Methyl Red-Voges Proskauer (MR-VP) broth (Difco)
- 1% Pepton Water (Difco)
- Buffered peptone water Broth

3.1.5.14 Reagent

- Crystal violet dye
- Grams iodine
- Alcohol
- Safranin
- Saline
- Iodine solution
- Kovac's reagent
- Methyl- red solution
- 3% H_2O_2
- P –Amino dimethylanilin oxalate
- Phenol red
- Phosphate buffered saline (PBS) solution

2. METHOD

3.2.1 Study design

A cross-sectional study design was employed and hospital and non-hospital waste water samples were collected at different intervals during the study period.

3.2.2 Experimental Layout

The present study was conducted into three phases; in first phase all hospital and non-hospital sites were selected by visiting the area according to their possibility to getting the multiple drug resistant bacterial pathogens. In the second phase isolation and identification of the organisms from the collected sample using cultural, staining and biochemical characteristics was done. In third phase drug resistant pattern of the selected isolates were determined by using different antibiotic discs available in the market. The experimental layout of the present study was shown in figure 3.

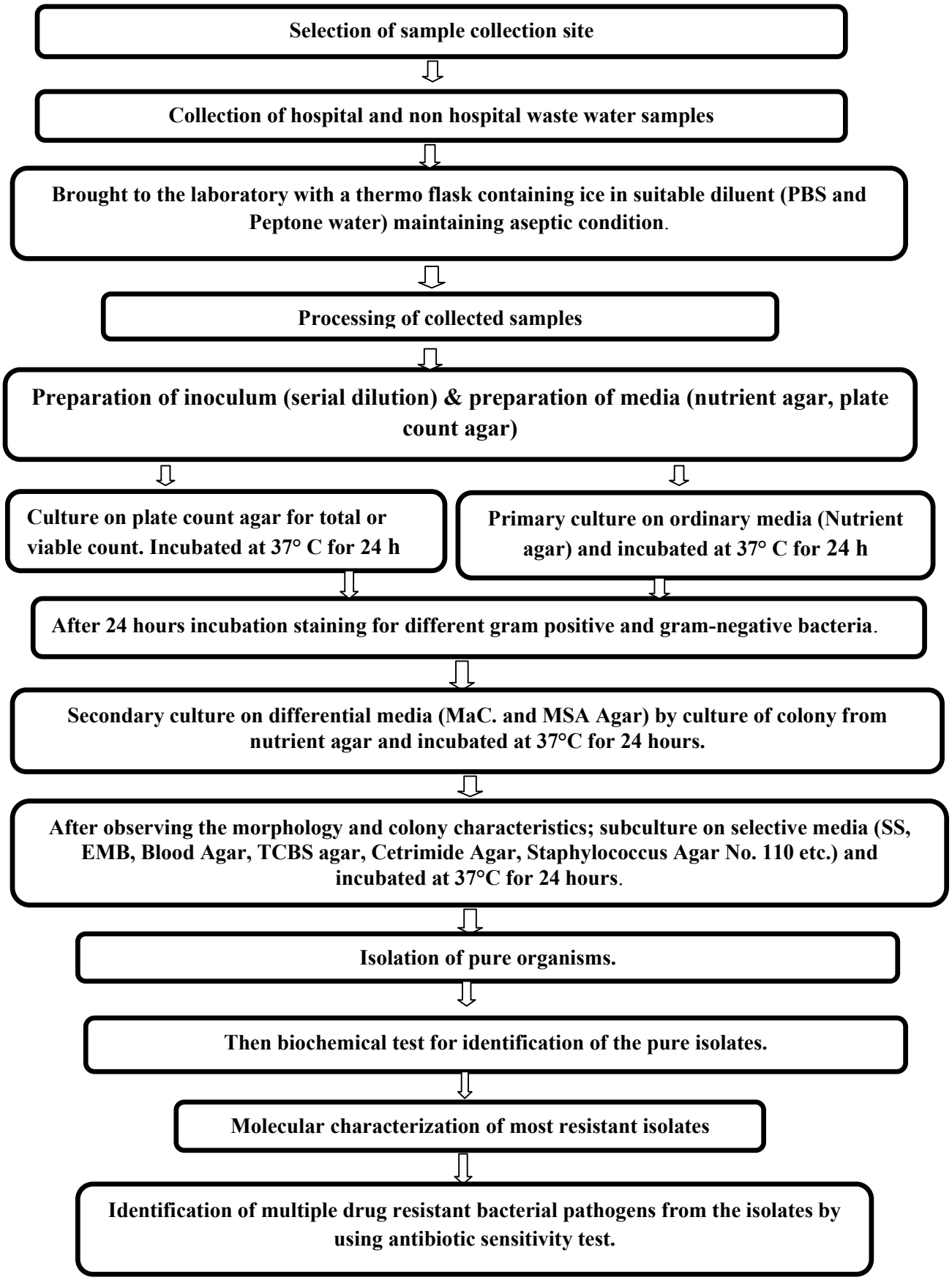


Figure 3: The schematic illustration of the experimental layout

3.2.3 Sample processing and isolation bacteria

All of those hospital and non-hospital samples were collected from different areas of Dinajpur sadar were transported to the microbiological laboratory of department of Microbiology, HSTU, and Dinajpur, Bangladesh in cool conditions and processed within two hours of collection. To determine the total viable plate count, serial 10-fold dilutions of samples were prepared in physiological saline, and 50 µl (0.05 ml) of aliquot was spread plated on plate count agar (PCA). Plates were incubated for 24 hours at 37ºC before bacteriological counts were done. The number of colonies on each plate having 30–300 colonies was counted by using a digital colony counter or by conventional plate count method. Finally, the bacterial count was reported CFU/mL as follows:

$$CFU/mL = \frac{colonies\ counted \times \text{Dilution factor}}{Actual\ volume\ of\ sample\ in\ plate,(mL)}$$

Calculation:

Colonies per plate=45

Dilution factor=1:1×10^{10}(1:100, 00000000)

Volume of dilution added to plate= 0.05 ml

So, 45 ×10^{10} /50 = 9 ×10^{12} CFUs/ml (Colony-forming units).

After that, based on colony morphology representative colonies were picked and sub-cultured on different selective and differential media such as blood agar, MacConkey agar, EMB agar, SS agar, TCBS agar, Cetrimide agar base etc. After obtaining pure colonies and recording key features such as hemolysis on blood agar isolated organisms were identified biochemically in a systematic way following standard methods (Holt JG *et.al.)*

Figure 4: Colony count by conventional method

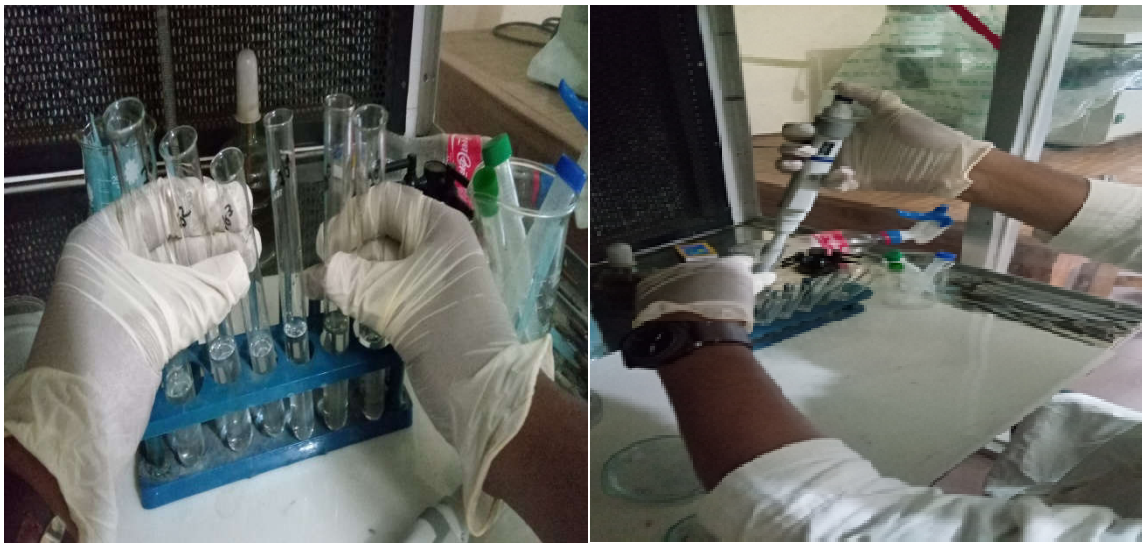


Figure 5: Serial dilution of collected sample

3.2.4 Laboratory Preparation

All items of glassware including test tubes, pipettes, cylinders, flasks conical flasks, glass plates, slides, vials and other necessary instruments cleaned by detergents powders. The glassware then cleaned by brushing, washed thoroughly and finally sterilized by autoclaving for 15 minutes at 121°c under 15 lbs. pressure per square inch. Autoclaved items were kept in oven at 50°c for further use.

Preparation of Culture Media

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer.

3.2.4.1 Nutrient broth media

Thirteen grams of dehydrated nutrient broth was suspended into 1000 ml of distilled water and boil to dissolve it completely (necessary calculation was done for required number of tubes). The solution was then distributed in tubes, stopper with cotton plugs and sterilized in autoclaving at 121° C and 1.2 kg/cm^2 pressure for 15 minutes. The sterility of the medium was checked by incubating at 37° C for overnight and stored at 4° C in aerator for further use (Cater 1979).

3.2.4.2 Plate Count Agar (PCA)

Add 17.5 g to 1 liter of distilled water. Dissolve by bringing to the boil with frequent stirring, mix and distribute into final containers (necessary calculation was done for required number of plates). Sterilize by autoclaving at 121°C for 15 minutes. After autoclaving, the medium was poured into each sterile petri dish 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 cultural characterization or stored at 4°C refrigerator for future use (Cater 1979).

3.2.4.3 Nutrient agar (NA) media

28 grams of nutrient agar powder was dissolved in 1000 ml of cold distilled water in a flask (necessary calculation was done for required number of plates). The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petri dish 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 cultural characterization or stored at 4°C refrigerator for future use (Cater 1979).

3.2.4.4 Eosin Methylene Blue (EMB) agar

Thirty six grams of EMB agar base was added to 1000 ml of water in a flask and boil to dissolve the medium completely (necessary calculation was done for required number of plates). The medium was sterilized by autoclaving at 1.2 kg/cm2 pressure and 121°C for 15 minutes and I to 50° C and shake the medium in order to oxidize the methylene blue

(i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile Petri dish sized 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 petri dishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.4.5 Mac-Conkey agar

51.5 grams Mac Conkey agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely (necessary calculation was done for required number of plates). The medium was then sterilized by autoclaving at 1.2 kg/cm2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C to decrease the temperature. Then 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 thick layer their in. 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 checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.4.6 Cetrimide Agar Medium

46.7 grams of cetrimide agar base powder was added in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely(necessary calculation was done for required number of plates). The medium was then sterilized by autoclaving for 15 minutes at 121°c maintaining a pressure of 1.2 kg/. Then 20/10 ml of medium was poured into each sterilized petri dishes and allowed to cool and to solidify. After solidification all petri dishes was incubated at 37°c for overnight to check their sterility. The sterile medium was then used for differential cultural characterization.

3.2.4.7 Blood agar media

Forty grams of Blood agar base (HI-MEDIA, India) powder was dissolved in 1000 ml of distilled water (necessary calculation was done for required number of plates). The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes and 45° C. Then 5-10 % sterile defibrinated blood was added to the medium and

distributed to sterile petri dishes 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 cultural characterization or stored at 4°C refrigerator for future use. (Cater 1979).

3.2.4.8 Salmonella Shigela (SS) Agar

Suspend 63.02g in 1 liter of distilled water. Bring to the boil to dissolve completely (necessary calculation was done for required number of plates). Sterilize by boiling for 5 minutes. After boiling the medium was put into water bath at 450- 500C to decrease the temperature. Then 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 thick layer there in. 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 checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.4.9 Mannitol Salt Agar (MSA)

111 grams Mannitol Salt Agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely (necessary calculation was done for required number of plates). The medium was then sterilized by autoclaving at 1.2 kg/cm2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 450- 500C to decrease the temperature. Then 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 thick layer there in. 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 checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. Petri dishes, these were incubated at 37° C for overnight to check their sterility and used for cultural characterization or stored at 4°C in refrigerator for future use (Cater 1979).

3.2.4.10 TCBS Agar Medium

89.8 grams powder of TCBS Agar base was added in 1000 ml of cold distilled water in a flask and heated to boiling for dissolving the medium completely (necessary calculation was done for required number of plates). The medium was not sterilized as the instruction given in the cover of the media box. After solidification all petri dishes was incubated at 37°c for overnight to check their sterility. The sterile medium was then used for differential cultural characterization. (Cater 1979).

3.2.4.11 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 maintaining a pressure of 1.2 kg/.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.

3.2.4.12 Methyl Red

A quantity of 17 gm of MR-VP medium was dissolved in 1000 ml of distilled water, dispensed in 2 ml amount in each tube and the tubes were autoclaved (necessary calculation was done for required number of test tubes). After autoclaving, the tubes containing medium were incubated at 37°C for overnight o check their sterility and then in refrigerator for future use.

3.2.5 Preparation of reagents

3.2.5.1 Methyl- Red solution

The indicator MR solution 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.

3.2.5.2 Alpha- Naphthol solution

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

3.2.5.3 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals in100 ml of cooled water.

3.2.5.4 Phosphate Buffered Saline solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate Na2HPO4, 12H2O), 0.2 gram of potassium chloride (KC1) and 0.2 gram of potassium hydrogen phosphate (KH2PO4) were suspended in 1000 ml of distilled for the preparation of phosphate buffered saline solution. The solution was heated to dissolve completely. Then the solution was sterilized by autoclaving at 1.2 kg / cm2 pressure and 121° C for 15 minutes and stored for future use.

3.2.5.5 Indole reagent (Kovac's reagent)

This solution was prepared by dissolving 25 ml of concentrated Hydrochloride acid in 75 ml of amyl alcohol and to the mixture 5 grams of paradimethyl –amino- benzyldehyde crystals were added. This was then kept in a flask equipped with rubber cork for future use.

3.2.6. Morphological characterization of bacteria by Gram staining method:

The most widely used staining procedure in 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-positives and gram-negatives. 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. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolorization step and a counterstain is used to impart a pink color to the decolorized gram- negative organisms.

3.2.6.1 Preparation of Gram's staining solution

The four solutions;

- ✓ Crystal violet
- ✓ Gram's iodine
- ✓ 95% alcohol
- ✓ Safranins needed for the Gram staining procedure.

3.2.6.2 Gram staining procedure

1. Obtain clean glass slides were taken.

2. A sterile technique was used; a smear of each of the organisms was prepared. Smear made of a drop of water on the slide was placed then each organism separately to the drop water with a sterile was transferred. A circular motion of the inoculating loop of organism was mixed and spreads.

3. Air–dry the smears were allowed then heat-fixed in the usual manner.

4. Smears were flooded with crystal violet and let stood for 1 min then slides were washed with tap water.

5. Smears were flooded with grams iodine mordant and let stood for 1 min. Slides were washed with tap water.

6. Smears were decolorized with 95% ethyl alcohol. Slides were washed with tap water.

7. Smears were counter stain with safranin for 30 sec. Slides were washed with tap water.

8. Slides were examined under oil immersion. (Cuppuccion and Natalie Sherman, 1996).

3.2.7. Biochemical test

Isolated organism with supporting growth characteristics of suspected identified by biochemical test. Several types of biochemical tests were performed in this study to confirm the specific bacteria that are as follow down:

- ✓ Catalase test
- ✓ Indole test
- ✓ Methyl Red I(MR) Test
- ✓ Voges-proskauer (VP) test
- ✓ Simmon's citrate
- ✓ Triple Sugar Iron (TSI) agar
- ✓ Motility Indole Urease (MIU) test

3.2.7.1 Catalase test

This test was used to differentiate bacteria which produce the enzyme catalase. To perform this test, a small colony of good growth pure culture of test organism was smeared on a slide. Then one drop of catalase reagent (3% H_2O_2) was added on the smear. The slide was observed for bubbles formation. Formation of bubble within few seconds was the indication of positive test while the absence of bubble formation indicated negative result (Cheesbrough, 1985).

3.2.7.2 Indole test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial culture and incubated at 37°c for 48 hours. Kovac's reagents (0.5ml) were added, shake well and examined after 1 minute. A red color in the reagent layer indicated indole test positive. In negative case there is no development of red color (Cheesbrough, 1985).

3.2.7.3 Methyl Red test (MR)

Sterile MR-VP broth was inoculated with the test organism and following incubation at 37°c for 24 hours. if the organism would ferment glucose via the mixed acid fermentation pathway like lactic, acetic, which decreases the PH, hence upon the addition of the indicator methyl red the broth becomes red in color and yellow color indicated a negative result (Cheesbrough, 1985).

3.2.7.4 Voges-Proskauer test (VP)

Voges Proskauer Test – If the organism would ferment glucose via the butylenes glycol pathway, an intermediate product, acetyl methyl carbinol or acetone which is neutral is converted to diacetyl upon the addition of the VP – Reagent –B (40% KOH with 0.3% creatine) in the presence of VP – Reagent – A (5% alpha- naphthol in abs. methyl alcohol). Diacetyl is red in color. Negative is yellow color (Cheesbrough, 1985).

3.2.7.5 Simmon's Citrate Agar (SCA)

This tube medium is used to identify Gram negative enteric bacilli based on the ability of the organisms to utilize citrates the sole source of carbon. (Citrate utilization test). The organism which utilizes citrate as its source of carbon degrades it to ammonia and subsequently converts it to ammonium hydroxide. The pH of the medium is then increased, and this is indicated by a change in color from green to blue (Cheesbrough, 1985).

3.2.7.6 Triple Sugar Iron Agar (TSI)

- This tube medium is used to identify Gram negative enteric bacilli based on the following biochemical characteristics (Cheesbrough, 1985)
- Glucose fermentation – indicated by yellow butt
- Lactose fermentation – indicated by yellow slant
- Hydrogen sulfide production – indicated by blackening of the medium
- Gas production – indicated by presence of a crack, bubble or gas space
- pH indicator – phenol red
- Hydrogen sulfide indicator – ferric ammonium citrate with sodium thiosulfate.

3.2.8. PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Pseudomonas aeruginosa*

3.2.8.1 Basic protocol of bacterial genomic DNA isolation

Bacteria from saturated liquid nculture are lysed and portions are removed by digestion with protease-k. Cell wall debris, polysaccharides and remaining proteins are removed by Phenol-chloroform extraction and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Procedure

- Inoculate a 25 ml of liquid culture with *Pseudomonas spp.* Grow in conditions appropriate for *Pseudomonas spp.* until the culture is saturated.
- Spin 1.0 ml of the overnight culture in a micro centrifuge tube for 5 minutes at 10000 rpm.
- Discard the supernatant.
- Repeat this step. Drain well onto a kimwipe.
- Re-suspend the pellet in 467 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ ml Proteinase k to give a final concentration of 100 µg/mg Proteinase k in 0.5% SDS. Mix thoroughly and incubate 30 min for 1 hr at 37^0C.
- Add an approximately equal volume (500 µl) of Phenol/Chloroform/Isoamyl alcohol. Mix thoroughly but very gently to avoid shearing the DNA, by inverting the tube until the phase are completely mixed.
- Then centrifuge the tubes at 12000 rpm for 10 minutes.
- Remove aqueous, viscous supernatant (~ 400 µl) to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of Phenol/Chloroform/Isoamyl alcohol extract thoroughly and spin in a microcentrifuge at 10000 rpm for 5 min.
- Transfer the supernatant to a fresh tube (~400 µl).
- Add $1/10^{th}$ volume of 3 M sodium acetate and mix.
- Add 0.6 volumes of isopropanol to precipitate the nucleic acids, keep on ice for 10 minutes.
- Centrifuge at 13500 rpm for 15 minutes.
- Discard the supernatant.
- Wash the obtained pellet with 1 ml of 95% ethanol for 5 minutes. Then centrifuge at 12000 rpm for 10 minutes.

- Discard the supernatant.
- Dry the pellets as there is no alcohol.
- Resuspend the pellet in 50 µl of TE and then 7.5 µl of RNase. Store DNA at 4^0C for short term and at -20^0C for long term.

3.2.8.2 PCR amplification and sequencing of 16S rRNA

PCR Condition:

Table 1: Condition of PCR.

Step	Temperature	Duration	Cycles
1. Initial denaturation	95ºC	5 min	01
2. Denaturation	95ºC	30 Sec	35
3. Annealing	56ºC	30 Sec	
4. Extension	72ºC	1.5 min	
5. Final extension	72ºC	10 min	01
6. Holding	4ºC	hold	-

3.2.8.3 Electrophoresis

Process of Electrophoresis:

- Preparation of gel.
- Sample application in the gel.
- Adjustment of voltage or current (gel-electrophoresis about 70-100 volts).
- Set up run time about 30-60 minute
- When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.
- The gel stained in ethidium bromide (0.5µg/ml) for 10 minutes, in a dark place.
- The gel was distained in distilled water for 10 minutes. The distained gel was placed on the imaging system in the dark chamber of the image documentation system.
- The UV light of the system was switched on; the image was viewed on the monitor, focused, acquired and saved in an USB flash drive.

3.2.8.4 Nucleotide sequence accession number and BLAST analysis

The nucleotide sequence 16S rRNA gene region data was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogenic tree, primer pairs were designed from NCBI database search tool

3.2.8.5 Chain-termination methods (Sanger sequencing)

Steps of Sanger sequencing using ABI 3130 Genetic analyzer

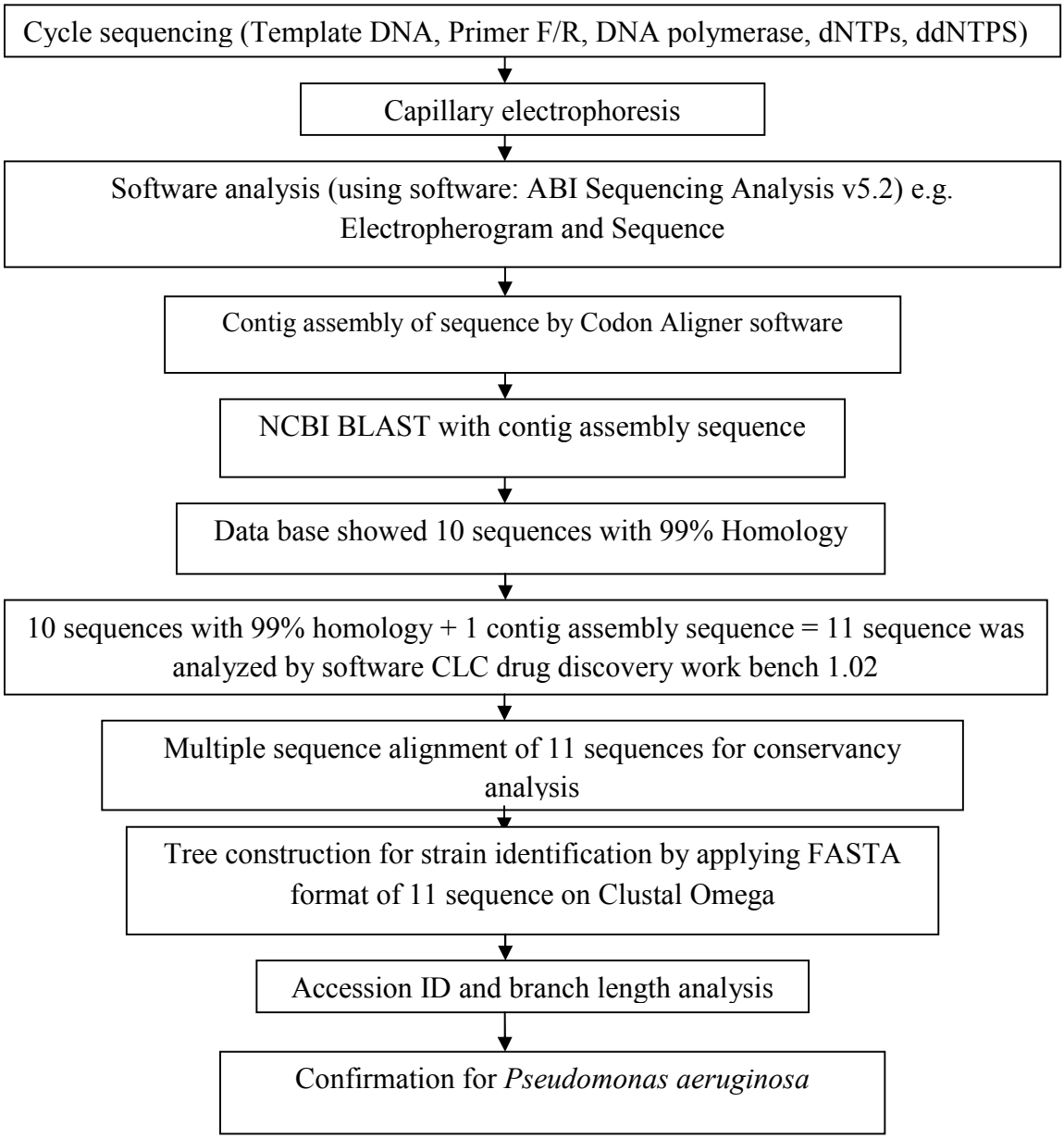


Figure 6: Steps of Sanger sequencing using ABI 3130 Genetic analyzer.

3.2.9 Antibiotic susceptibility testing

The standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility profiles of the isolates (Bauer, 1999) according to the recommendations of National Committee for Clinical Laboratory Standards (CLSI- 2015). Bacterial inoculums was prepared by suspending the freshly grown bacteria in 4–5 ml sterile nutrient broth and the turbidity was adjusted to that of a 0.5 McFarland standard. The antimicrobial susceptibility testing was performed using Mueller-Hinton medium, Antibiotic disks were applied using some sterile forceps. Agar plates were incubated at 37°C for 18 hours. After overnight incubation at 37 °C, the diameter in millimeters of the zones of inhibition around each of the antimicrobial discs was recorded and categorized as resistant or sensitive in accordance with company recommendations. All isolates were tested for sensitivities to 10 of routine and practical antibiotics. antibiotic disks are used shown in table 2;

Table 2: Antimicrobial agents with their disc concentration

Antimicrobial agents	Symbol	Disc concentration (µg/disc)
Ampicillin	**AMP**	**25 µg**
Amoxicillin	**AMX**	**30 µg**
Amikacin	**AK**	**30 µg**
Chloramphenicol	**C**	**30 µg**
Ciprofloxacin	**CIP**	**5µg**
Gentamycin	**GEN**	**10 µg**
Kanamycin	**K**	**30 µg**
Penicillin	**P**	**10 µg**
Tetracycline	**TE**	**30 µg**
Vancomycin	**VA**	**30 µg**

3.2.9.1 Zone diameter interpretative standards for all isolates

All isolated organisms were compared with the CLSI 2015 standard for antibiotic susceptibility testing and interpreted properly.

Table 3: Zone diameter interpretative standards for all isolates

Antimicrobial agent active against bacteria	Disc Code	Potency	Resistant	Intermediate	Susceptible
Amoxycillin ***Enterobacteriaceae*** ***Staphylococcus spp.***	AX	20/10 µg	≤13	14-17	≥18
			≤19	—	≥20
Ampicillin ***Enterobacteriaceae*** ***P. aeruginosa***	AMP	10 µg	≤13	14-16	≤17
Amikacin ***Enterobacteriaceae,*** ***P. aeruginosa*** **and** ***Staphylococcus spp.***	AK	30 µg	≤14	15-16	≤17
Gentamycin ***Enterobacteriaceae,*** ***P. aeruginosa*** **and** ***Staphylococcus spp***	GEN	10 µg	≤12	13-14	≤15
Kanamycin ***Enterobacteriaceae*** ***Staphylococcus spp.***	K	30 µg	≤13	14-17	≤18
Ciprofloxacin ***Enterobacteriaceae,*** ***P. aeruginosa*** **and** ***Staphylococcus***	CIP	5 µg	15	16-20	≥21

spp.					
Chloramphenicol ***Enterobacteriaceae, Staphylococcus spp.***	C	30 µg	≤12	13-17	≥18
			≥16	—	≥17
Vancomycin ***Enterococcus spp.*** **and** ***Staphylococcus spp.***	VA	30 µg	≤14	15-16	≥17
Tetracycline ***Enterobacteriaceae and Staphylococcus spp***	TE	30 µg	≤11	12-14	≥15
			≥14	15-18	≥19
Penicillin ***Staphylococcus spp.*** ***Enterococcus spp.***	P	10 µg	≤28	—	≥29
			≤14	—	≥15

Source: CLSI- 2015 [Note: S=Sensitive, R=Resistant, I=Intermediate]

3.2.10 Maintenance of stock culture by sterile buffered glycerin method

After the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose, pure culture of at least one organism per isolates were stored in sterilized 80% glycerin and used as stock culture. The equal volume of 80% glycerin and bacterial culture were mixed and sealed with paraffin wax and stored at 37° c. The isolated organisms were given code name for convenience. (Buxton and Fraser, 1977)

CHAPTER 4

RESULTS

The present study was conducted to assess the isolation and characterization of multiple drug resistant bacteria from waste water of hospital and non-hospital environment.

4.1 Results of total viable counts

The result of total viable count revealed that maximum number of countable bacteria (2.20×10^{10}) CFUs/ml was present in the sample that was collected from Abdur Rahim Medical College Hospital (MARMCH) Site-2, and minimum number of bacteria (1.0×10^{10}) CFUs/ml was present in sample that was collected from Kalitola. The number of total viable count was shown in table 4.

Table 4: Results of total viable counts of samples from each sampling point (dilution 10^{-7})

Sampling Site	Number of colony	Result CFUs/ml
MARMCH Site-2	110	2.20×10^{10}
SHD Site-1	More than 300	TNTC
IBCHS Site-3	79	1.58×10^{10}
HSTU campus Site-1	51	1.02×10^{10}
Baser Hat Bazar Site-1	More than 300	TNTC
Bahadur Bazar Site-1	More than 300	TNTC
Lilir Mor Site-2	55	1.10×10^{10}
Kalitola	50	1.0×10^{10}

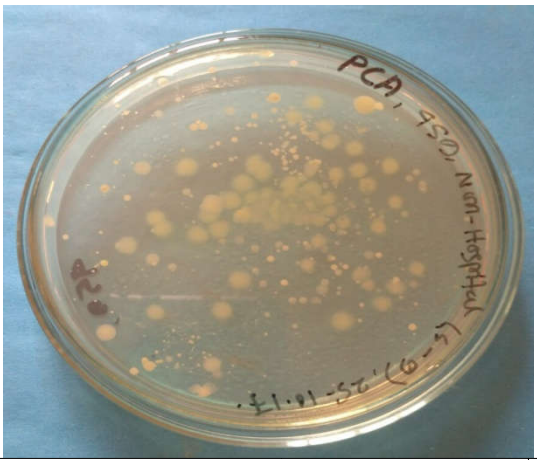


Plate 1: Gray white or yellowish colonies on plate count agar

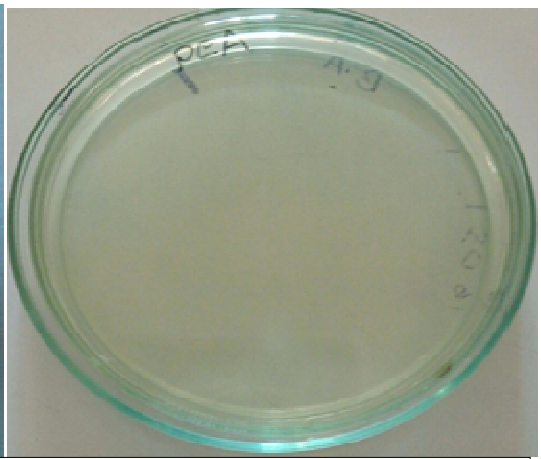


Plate 2: Fresh Plate Count Agar plate

4.2 Distribution of Samples Taken From Hospital Environments of Dinajpur

Hospital samples are taken from 3 different hospital of Dinajpur, Total 10 samples were collected from different sits of these three hospital and a total 32 bacterial isolates were collected. 13(40.6) bacterial isolates were collected from M. Abdur Rahim Medical college Hospital, Dinajpur (MARMCH), 10(31.2%) from Sadar Hospital Dinajpur (SHD), 9(28.1%) from Islami Bank Community Hospital (IBCH). Distribution of all hospital samples and isolated organisms from these samples were shown in table 5.

Table 5: Distribution of samples taken from hospital environments of Dinajpur

Sample sites	Total Samples N (%)	Sample Positive N (%)	Total number of bacterial isolates N (%)
M. Abdur Rahim Medical College Hospital, Dinajpur (MARMCH)	4(40)	4(100)	13(40.6)
Sadar Hospital Dinajpur (SHD)	3(30)	3(100)	10(31.2)
Islami Bank Community Hospital, Dinajpur (IBCH)	3(30)	3(100)	9(28.1)
Sub total	10	10(100)	**32(100)**

4.3 Distribution of Samples Taken From Non-Hospital Environments of Dinajpur

Total 10 samples were collected from 5 different non-hospital sits. Total 23 bacterial isolates were collected. 8(34.8%) from HSTU campus, equally 4(17.4%) isolates were collected from Baser Hat Bazar, Bahadur Bazar; Lilir Mor. 3(13.0%) bacterial isolates were collected from Kalitola. Distribution of all non-hospital samples and isolated organisms from these samples were shown in table 6.

Table 6: Distribution of samples taken from non-hospital environments of Dinajpur

Sample sites	Total Samples N (%)	Sample Positive N (%)	Total bacterial isolates recovered N (%)
HSTU campus	3 (30%)	3 (100%)	8(34.8)
Baser Hat Bazar	2 (20%)	2 (100%)	4 (17.4)
Bahadur Bazar	2 (20%)	2 (100%)	4 (17.4)
Lilir Mor	2 (20%)	2 (100%)	4 (17.4)
Kalitola	1 (10%)	1 (100%)	3 (13.0)
Sub total	**10 (100%)**	**10 (100%**	**23(100)**

4.4 Number of Bacteria Isolated from Each Sampling Points

A total of 20 waste water samples were processed for the presence of drug resistance bacterial pathogens. Of these samples 100% of the samples were positive to one or more isolates. Among the total samples 55 bacterial isolates were recovered. Among them 32 (58.2%) were from hospital environment and 23 (42.1%) were from non-hospital environment which was shown in table 7.

Table 7: Number of bacteria isolated at each sampling points from hospital and non-hospital environments of Dinajpur

Bacterial isolates	Hospital environment No. (%)	Non - Hospital environment No. (%)	Total No. (%)
E. coli	10 (31.2)	6 (26.0)	16 (29)
Pseudomonas spp	7 (21.9)	5 (21.7)	12 (21.8)
Klebsiella spp	5 (15.6)	4 (17.4)	9 (16.4)
Salmonella spp	5 (15.6)	3 (13.0)	8 (14.5)
Staphylococcus spp	3 (9.4)	2 (8.7)	5 (9)
Vibrio spp	2 (6.3)	3 (13.0)	5 (9)
Total	**32 (100)**	**23 (100)**	**55 (100)**

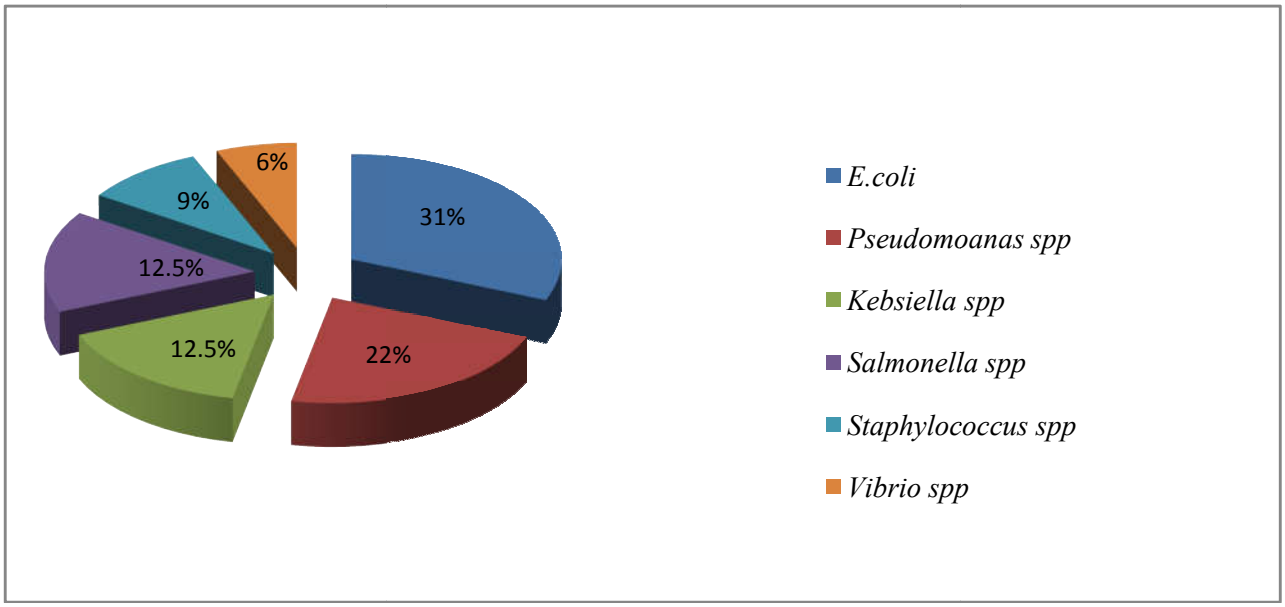


Figure 7: Percentages of all bacterial isolates from hospital waste water

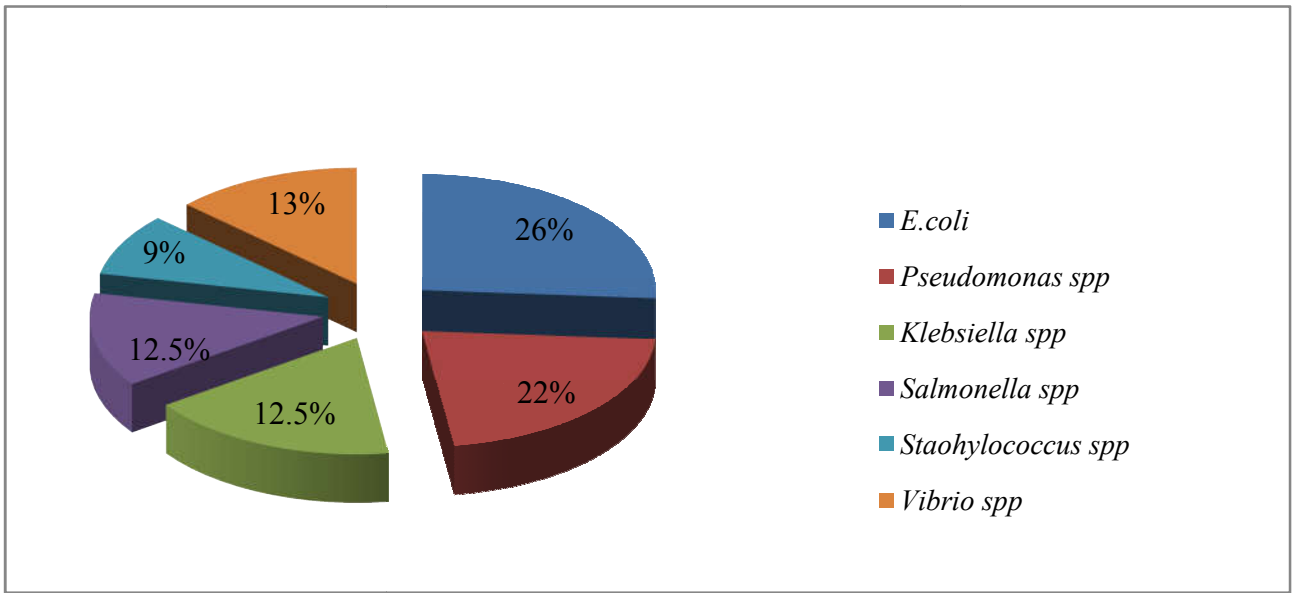


Figure 8: Percentages of all bacterial isolates from non-hospital waste water

4.5 Identification of bacteria by different bacteriological methods

4.5.1 Results of Cultural Examination

The cultural characteristics of *E. coli, Klebsiella spp, Salmonella* spp, *Vibrio spp, Pseudomonas spp, and Staphylococcus spp*, on various media are presented in table (8)

Table 8: The result of cultural characteristics of the bacteria isolated from different hospital & non-hospital environments.

Name of bacteria	Staining characteristic	Name of media	Colony characteristics
1. E. coli	Gram negative large rod shaped pink color.	Nutrient Agar	Large, mucoid, white colony.
		Mac-Conkey's Agar	Produce large mucoid rose-pink colony.
		EMB agar	Transmitted light blue-black center with a narrow, clear edge. Blue-green metallic sheen with reflected light.
2. Pseudomonas spp.	Gram negative small rod shaped pink colour	Nutrient agar	Large, smooth, low convex and greenish pigment with fruity odor.
		Mac-Conkey agar	Pale colour flat non-lactose fermenting Colonies
		Cetrimide agar	Colonies are greenish in color
3. *Klebsiella* spp	Gram negative rod shaped pink color.	Nutrient Agar	Large colony.
		Mac-Conkey's Agar	Large, red, mucoid
		EMB agar	Mucoid, no metallic sheen. With transmitted light, gray brown centers and pink color with clear edges.
4. *Salmonella* spp.	Gram negative small rod shaped pink color.	Nutrient agar	Smooth. Opaque, translucent colonies.

		Mac-Conkey agar	Small, white, translucent dewdrop like colonies.
		S.S agar	Opaque, smooth, round with black centered colonies.
5. *Staphylococcus spp.*	Gram positive cluster liked violet color.	Nutrient Agar	Black color/ non-colour smooth, glistening colonies.
		Manitol Salt Agar	Yellow colonies.
		Staphylococcus Agar No.110	Yellow colonies.
6. *Vibrio spp.*	*Vibrio* species are straight or curved Gram negative non-spore forming rods.	MacConkey Agar	Colorless colonies
		TCBS Agar	Colonies are large yellow or green

4.5.3 Nutrient Agar

Nutrient agar plates spread with the samples revealed the growth of bacteria after 24 hours of incubation at 37◦ C aerobically.

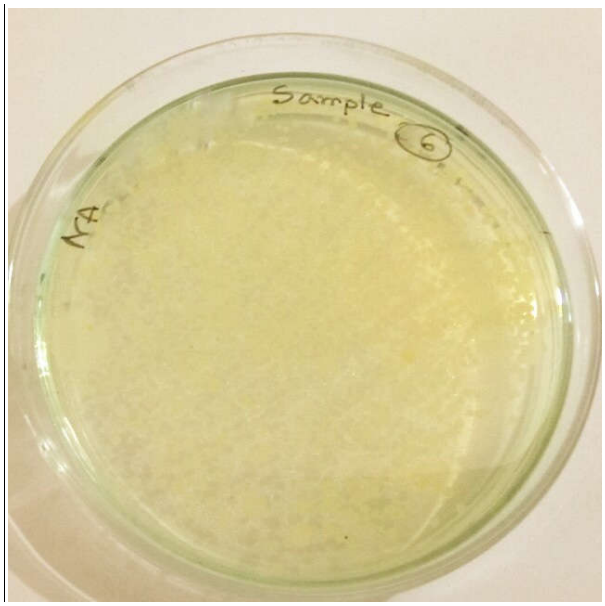


Plate 3: Gray white or yellowish colonies on Nutrient agar.

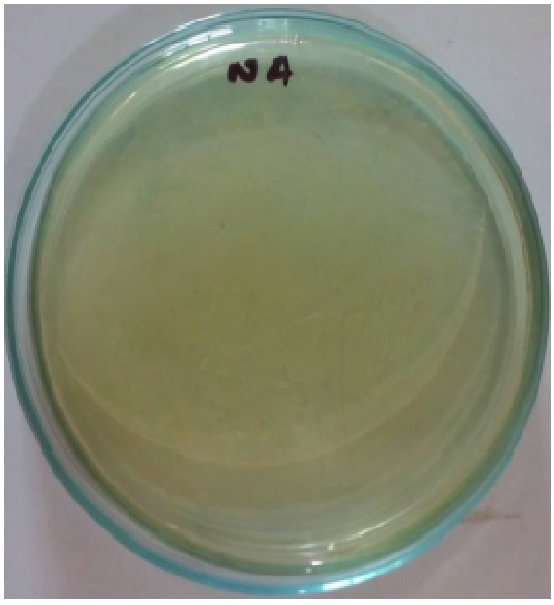


Plate 4: Fresh Nutrient agar plate.

4.5.4 Mac-Conkey Agar

Mac-conkey agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37° C aerobically.

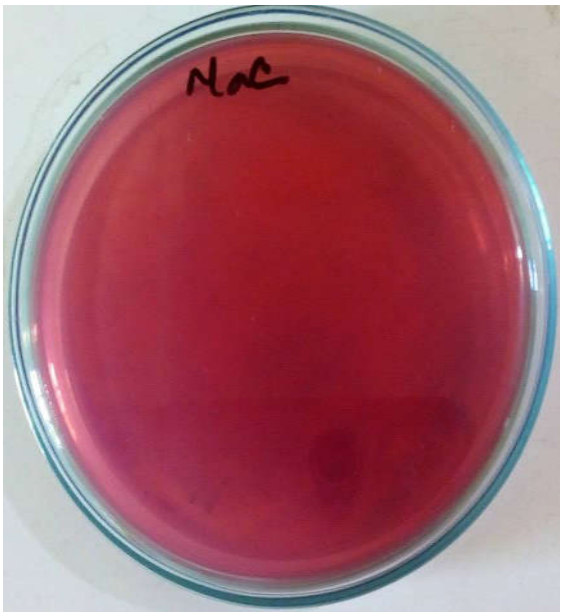


Plate 5: Fresh Mac-Conkey Agar plate

Plate 6: Rose pink colony of ***E. coli*** on Mac-Conkey Agar

Plate 7: Pinkish-off white ***Klebsiella* spp**. on Mac-Conkey Agar

4.5.5 Eosin Methylene Blue agar

Eosin methylene blue agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37° C aerobically.

Plate 8: Fresh Eosin Methylene Blue agar plate

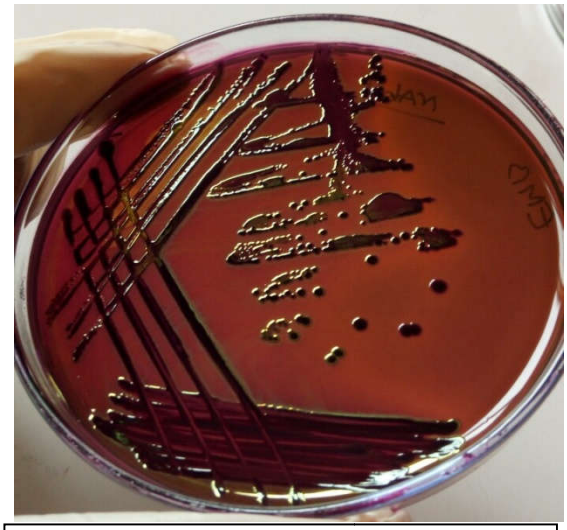


Plate 9: Blue-green metallic sheen and gray-brown center ***E.coli*** on Eosin Methylene Blue

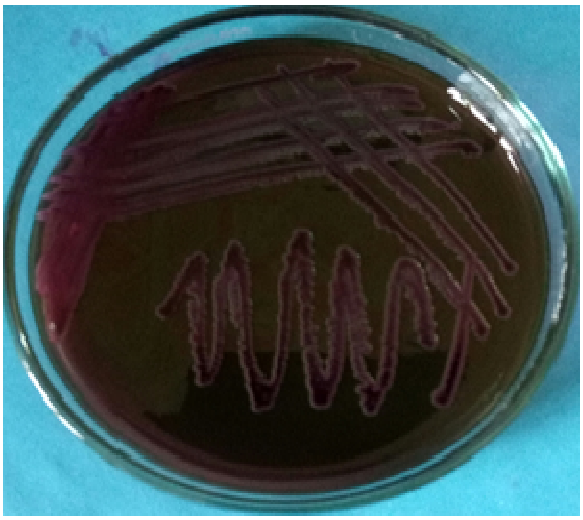


Plate 10: Gray brown centers and pink color ***Klebsiella spp*** on Eosin Methylene Blue agar.

4.5.6 Salmonella-Shigella Agar

Salmonella-shigella agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically.

Plate 11: Fresh Salmonella-Shigella Agar plate

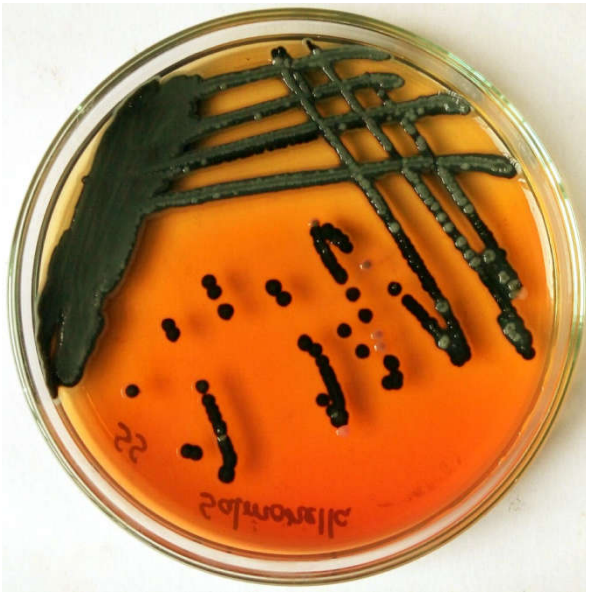


Plate 12: Black center, transparent colonies of ***Salmonella spp*** on SS agar

4.5.7 Cetrimide Agar

Cetrimide agar streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically.

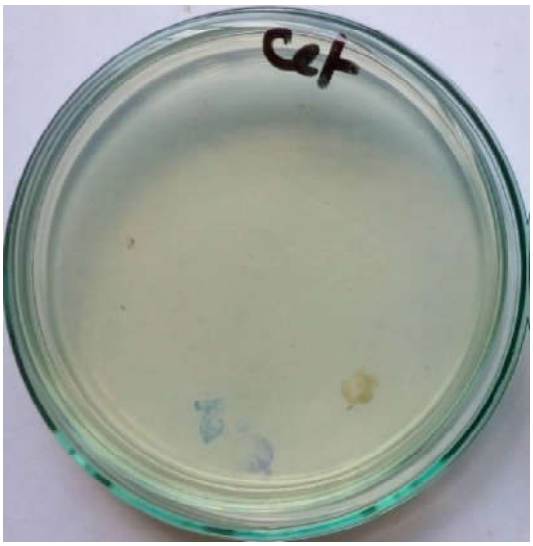


Plate 13: Fresh Cetrimide Agar plate.

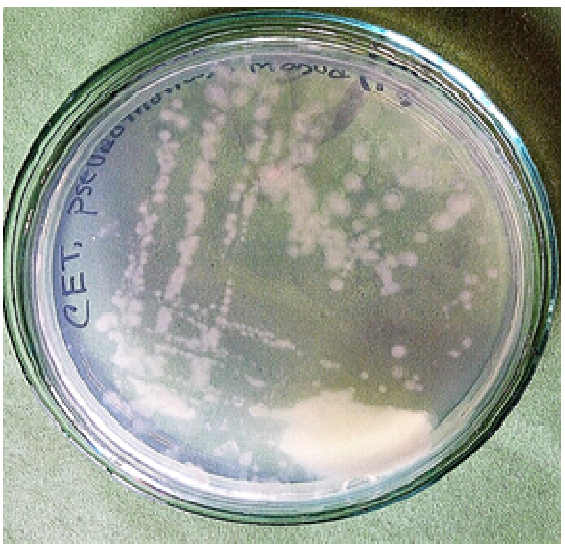


Plate 14: Greenish white color colonies of ***Pseudomonas spp*** on Cetrimide Agar.

4.5.8 Manitol Salt Agar

Manitol salt agar plates streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically.

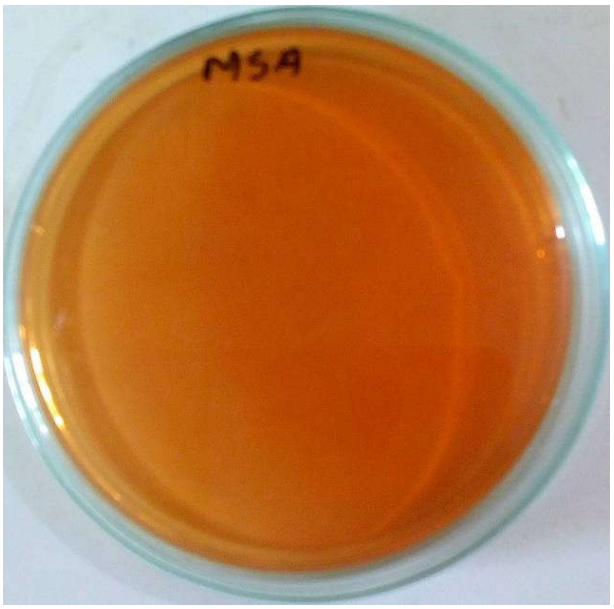


Plate15: Fresh Manitol SaltAgar.

Plate 16: Yellow color colonies of ***Staphylococcus spp*** on Manitol Salt Agar

4.5.9 Blood Agar

Blood agar plates streaked separately with the *Staphylococcus spp* & *Streptococcus spp* and incubated at 37°C aerobically for 24 hours.

Plate 17: Fresh Blood Agar plate.

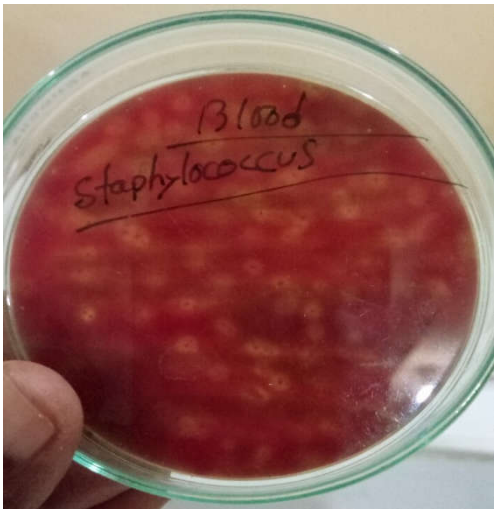


Plate 18: β-hemolytic colonies of ***Staphylococcus spp*** on Blood agar

4.5.10 TCBS Agar

TCBS agar streaked separately with the organisms revealed the growth of bacteria after 24 hours of incubation at 37° C aerobically.

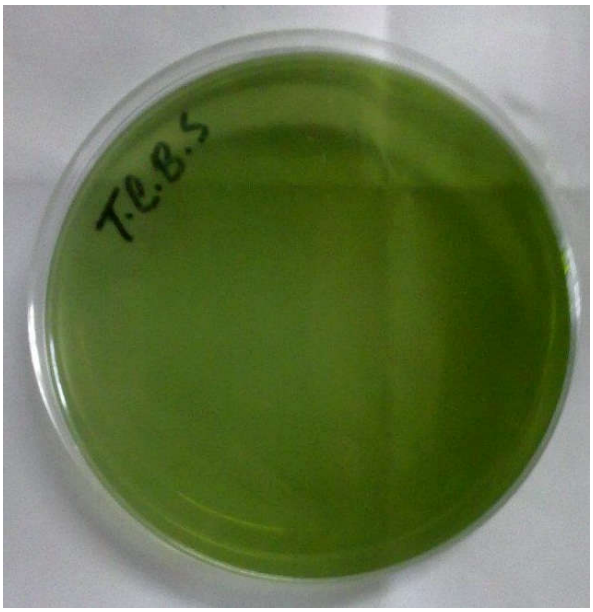


Plate19: Fresh TCBS Agar plate.

Plate 20: Yellow colonies of ***Vibrio cholerae*** on TCBS agar

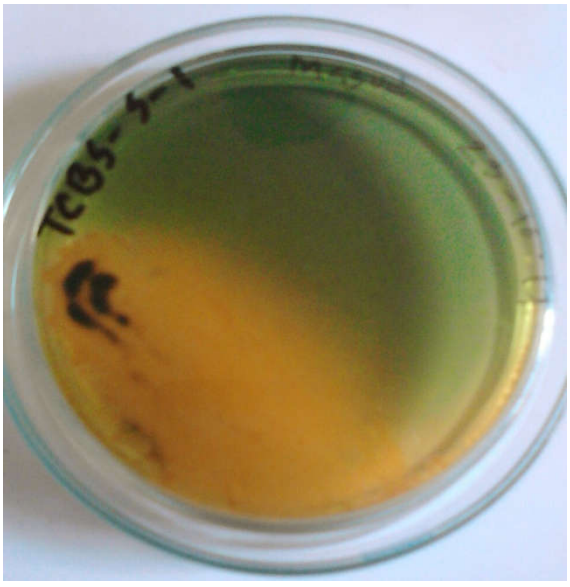


Plate 21: Greenish Colonies of ***Vibrio parahaemolyticus*** and yellowish colonies of ***Vibrio cholera*** on TCBS agar

4.5.11 Microscopic Examination

Microscopic observation was performed to observe shape and gram reaction of the isolates. Under microscopy both gram positive and negative isolates were found. Gram negative isolates were curved, slender and rod shape. Gram positive isolates were grape like cluster.

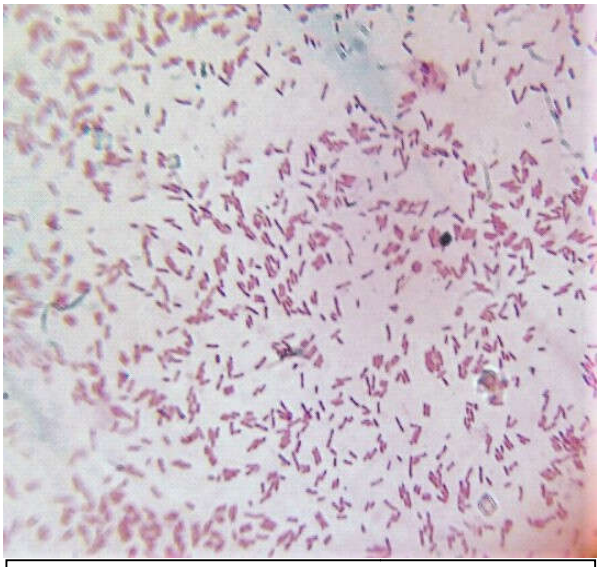


Plate 22: Gram negative large rod shaped pink colour ***E. coli*** under 100x microscopy.

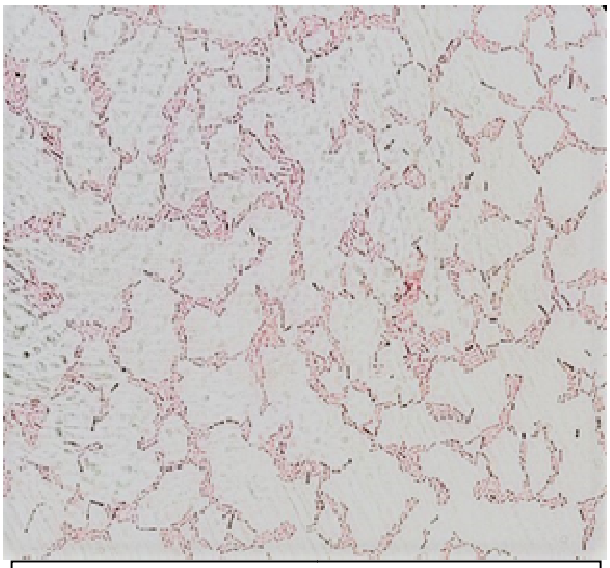


Plate 23: Gram negative rod shaped pink colour ***Klebsiella*** spp under 100x microscopy.

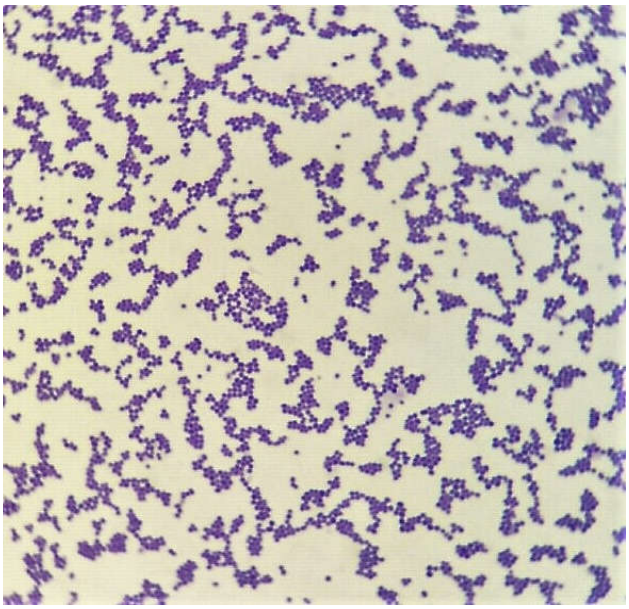


Plate 24: Gram positive cluster like violet colour ***Staphylococcus*** *spp* under 100x

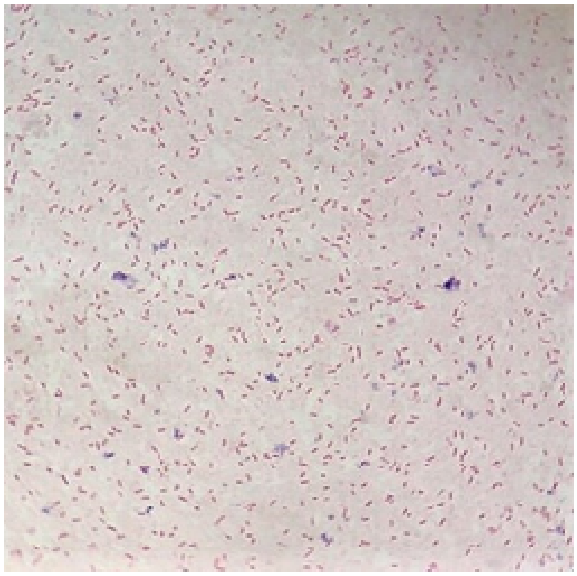


Plate 25: Gram negative small rod shaped pink color ***Pseudomonas spp*** under 100x microscopy.

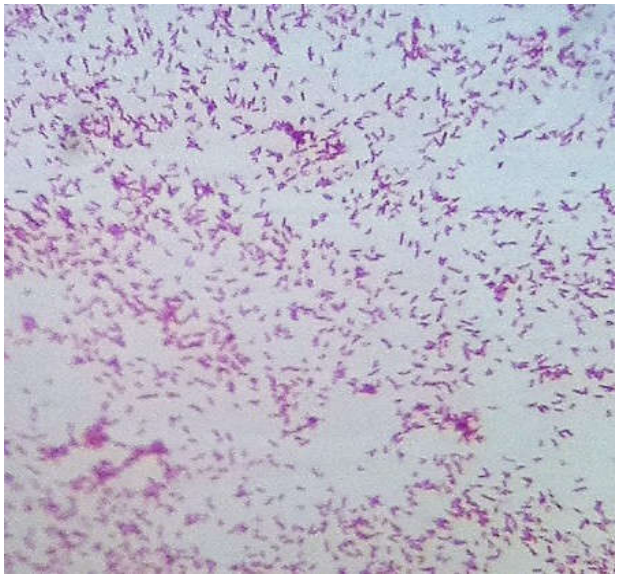


Plate 26: Gram negative small rod shaped pink colour ***Salmonella spp*** under 100x microscopy

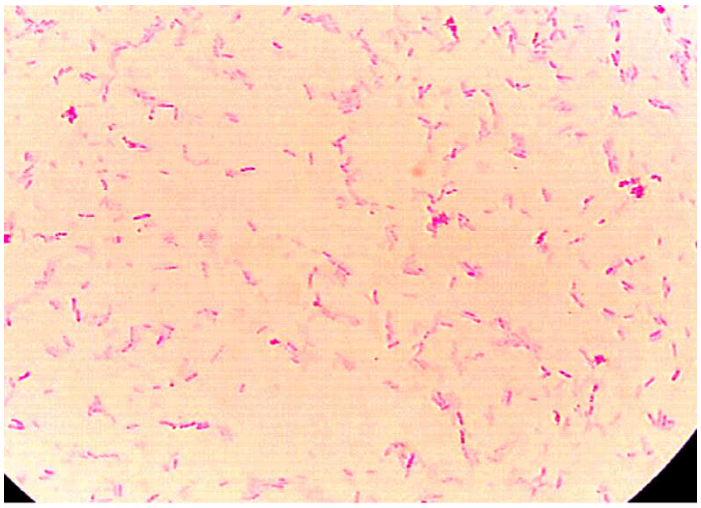


Plate 27: Gram negative small rod shaped pink cooler ***Vibrio spp*** under 100x microscopy

4.6 Result of Biochemical tests

Table 9: Result of Biochemical tests

Name of the Test → / Name of the Organisms ↓	Catalase	MR	VP	Indole	Citrate Utilization	MIU	TSI		
							Slant	Butt	H_2S
E. coli	+	+	-	+	-	+	**Y**	**Y**	**-**
Pseudomonas spp.	+	-	-	-	+	+	**R**	**R**	**-**
Klebsiella Spp	+	-	+	-	+	-	**Y**	**Y**	**-**
Salmonella spp.	+	+	+	+/-	+	+	**Y**	**R**	+
Staphylococcus spp.	+	+	+	-	+	-	**Y**	**R**	**-**
Vibrio spp.	+	+	+/-	+	+	+	**Y**	**Y**	**-**

[Y= Yellow; R= Red]

4.6.1 Catalase test

All isolates were positive for catalase test with gas bubble formation.

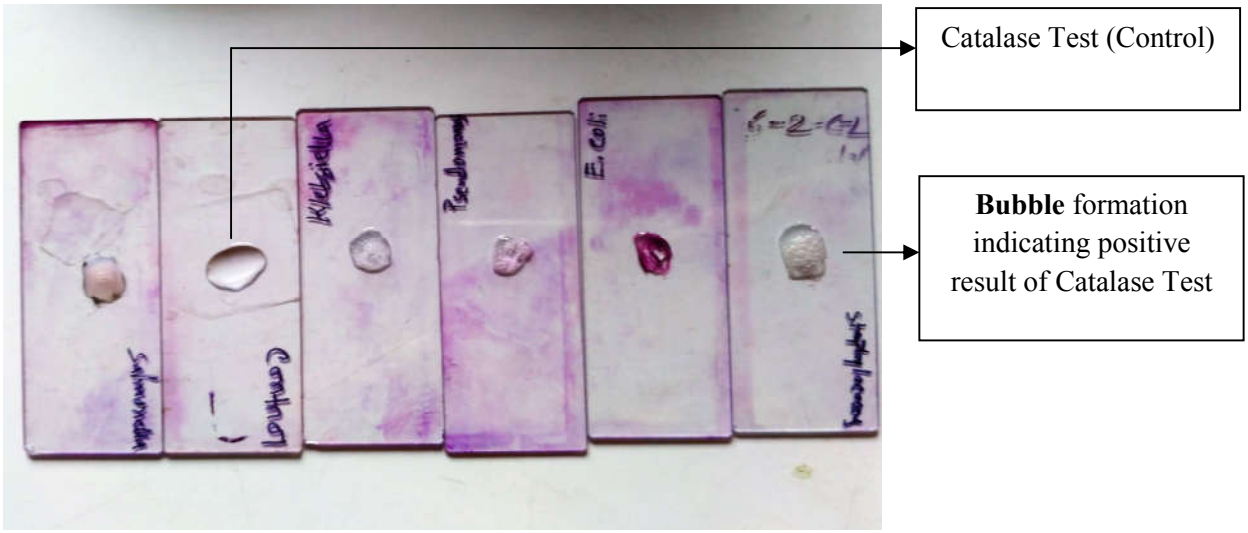


Plate 28: Catalase Test

4.6.2 Methyl Red test

The *E.coli, Salmonella, Staphylococcus* were positive. *Klebsiella, pseudomonas* and *Vibrio sp* were negative for methyl red test.

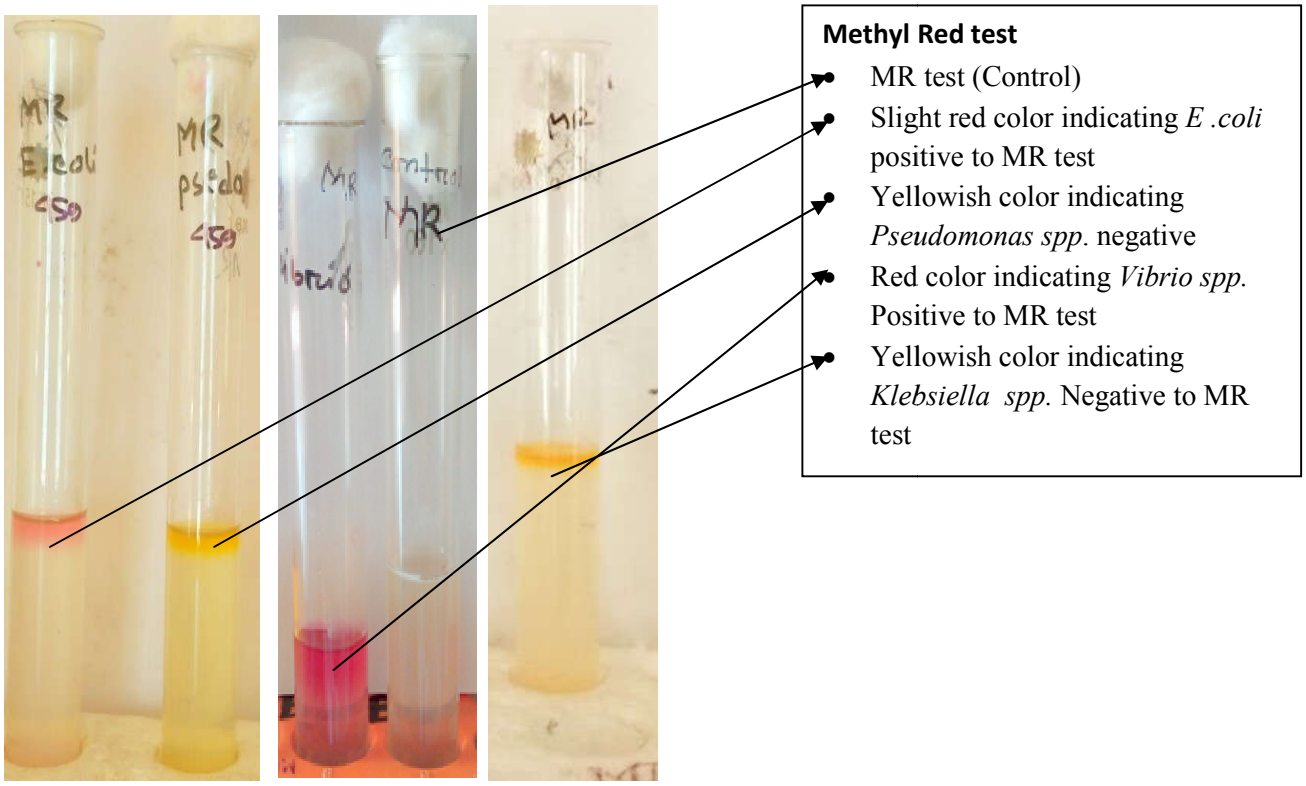


Plate 29:: Methyl Red test

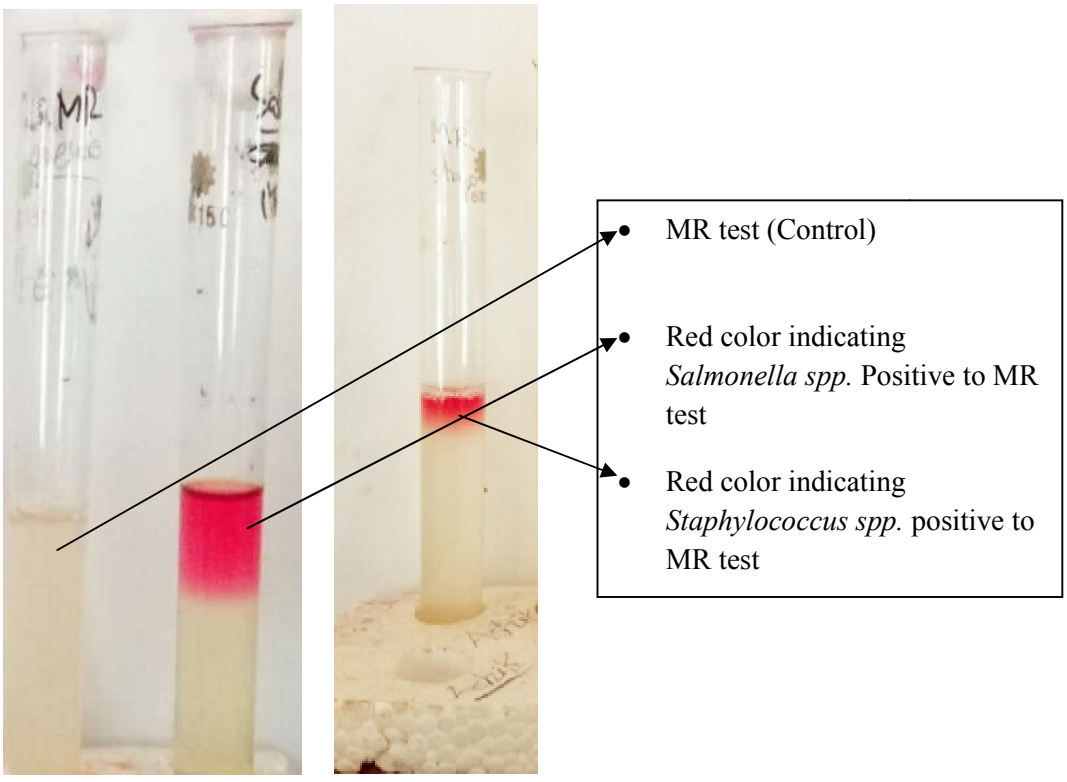


Plate 30: Methyl Red test

4.6.3 Voges-Proskauer (VP) Test

*Pseudomonas spp and E.*coli was negative, other organisms were positive to VP test.

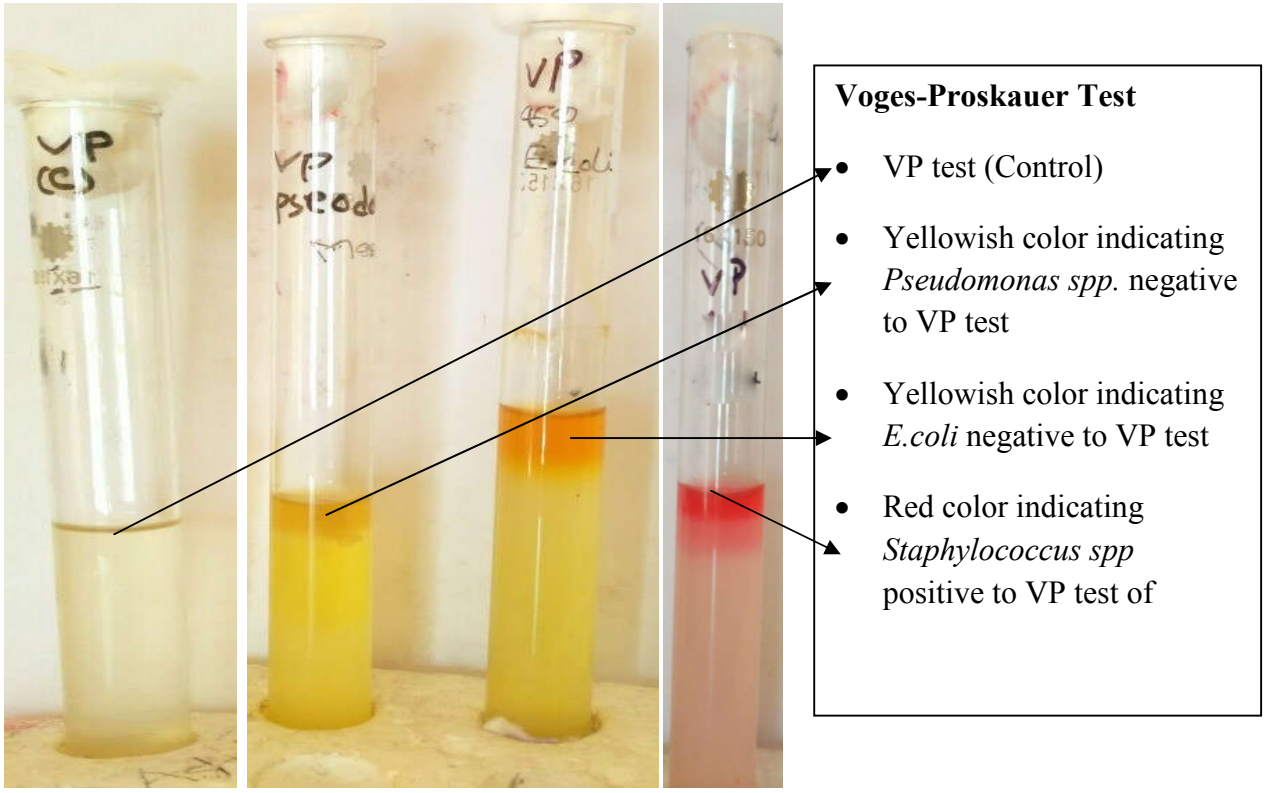


Plate 31: Voges-Proskauer (VP) Test

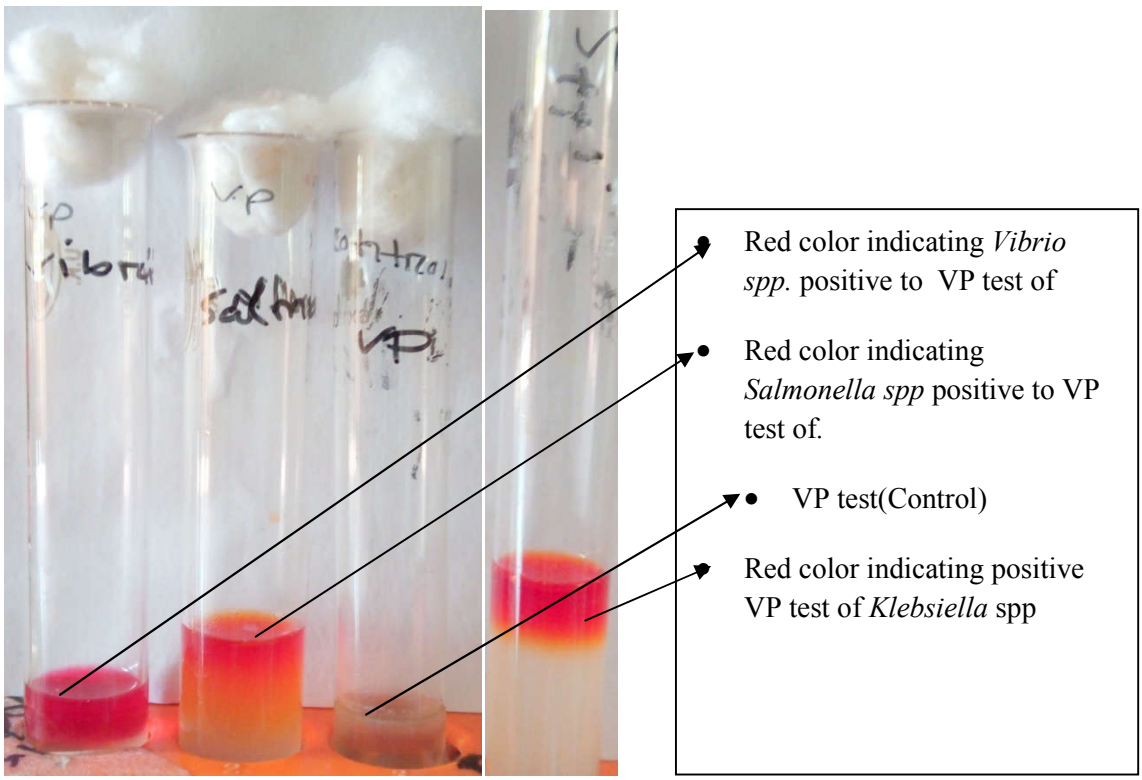
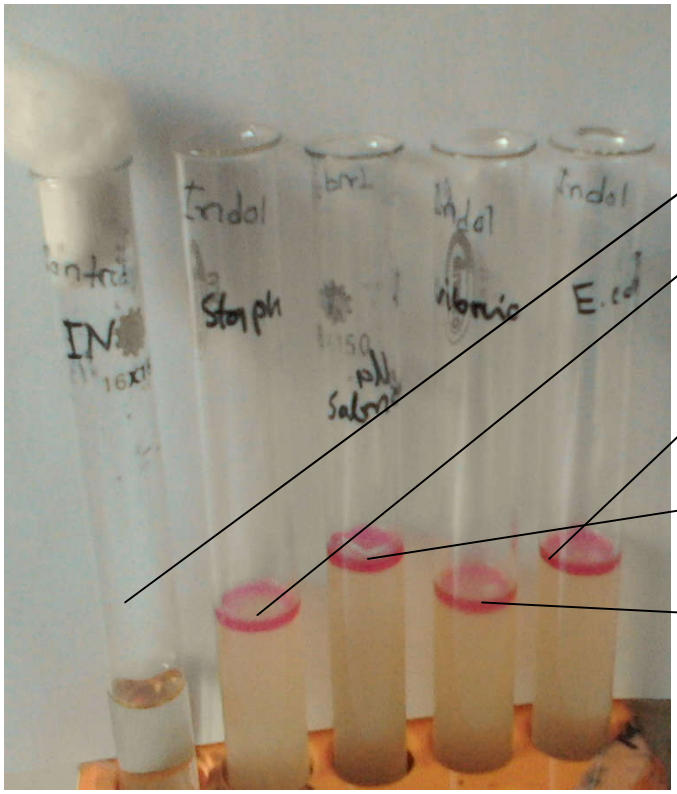


Plate 32: Voges-Proskauer (VP) Test

4.6.4 Indole Test

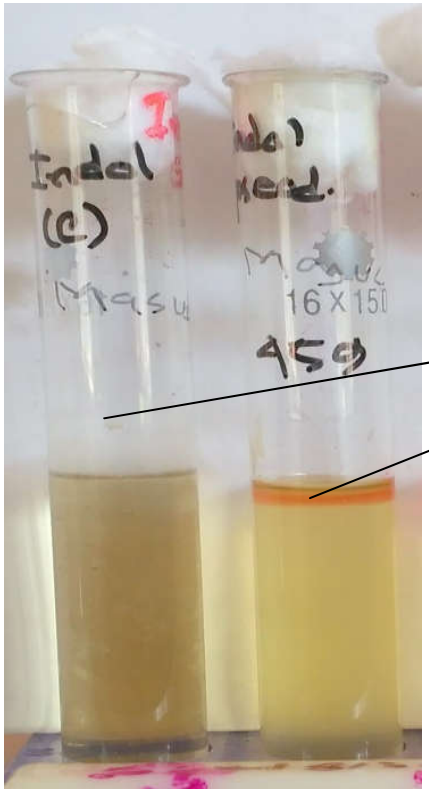
Without *Pseudomonas spp.* all isolates was positive to indole test



Indole Test

- Indole test (Control)
- Red color indicating *Staphylococcus spp.* Positive to Indole test
- Red color indicating *E.coli.* Positive to Indole test
- Red color indicating *Salmonella spp.* Positive on Indole test
- Red color indicating *Vibrio spp.* Positive to Indole test

Plate 33: Indole Test



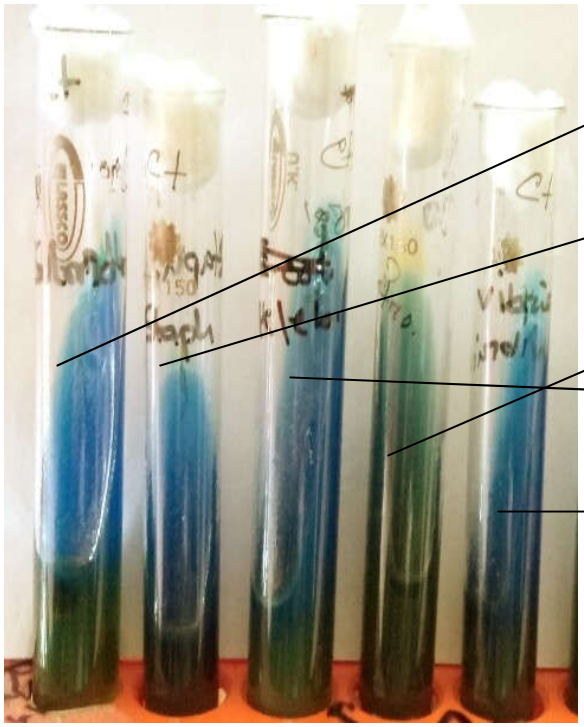
Indole Test

- Indole test (Control)
- No red color in the tube indicating *Pseudomonas spp.* Negative to Indole test

Plate 34:: Indole Test

4.6.5 Simmon's Citrate test

Only *E.coli* were negative, but all other organisms were positive to Simmon's Citrate test



Simmon's Citrate test

- Blue color in the tube indicating *Salmonella spp.* Positive to SC test
- Blue color in the tube indicating *Staphylococcus* spp Positive to SC test
- SC test (Control)
- Blue color in the tube indicating *Klebsiella spp.* Positive to SC test
- Blue color in the tube indicating *Vibrio spp.* Positive to SC test

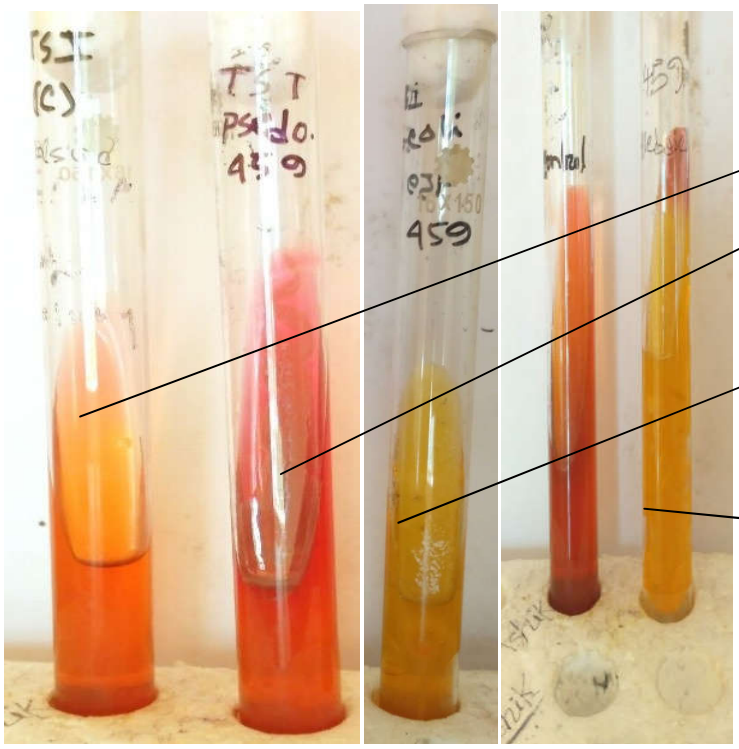
Plate 35: Simmon's Citrate test

- SC test (Control)
- Greenish color in the tube indicating *E.coli* Negative to SC test
- Blue color in the tube indicating *Pseudomonas spp.* Positive to SC test

Plate 36: Simmon's Citrate test

4.6.6 Triple Sugar Iron (TSI) Test

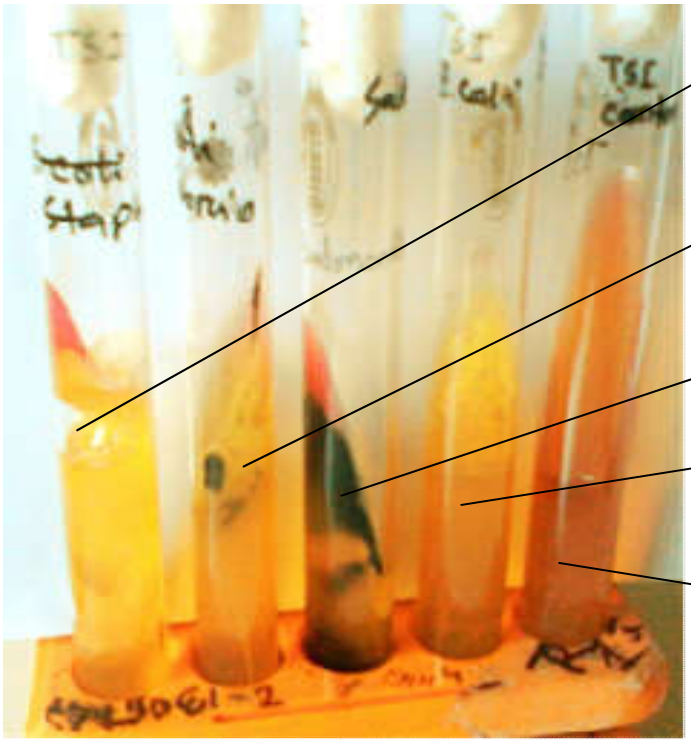
Only *Pseudomonas ssp* was negative, but all other organisms were positive to TSI test.



Triple sugar Iron (TSI) test

- TSI test (Control)
- Red color in the butt and slant indicating *Pseudomonas spp.* Negative to TSI test
- Yellow color butt and slant indicating *E.coli* Positive to TSI test
- Yellow color butt and slant indicating *Klebsiella spp.* Positive to TSI test

Plate 37: Triple sugar Iron (TSI) test



- Yellow color butt and slant with gas formation at butt indicating *Staphylococcus spp* Positive to TSI test.
- Yellow color butt and slant indicating *Vibrio spp.* Positive to TSI test
- *Salmonella spp.* Positive with H_2S Production
- Yellow color butt and slant indicating *E.coli* Positive TSI test
- TSI test (Control)

Plate 38: Triple sugar Iron (TSI) test

4.7 Result of PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Pseudomonas aeruginosa*

Out of 12 (14.3) *Pseudomonas* isolates 1 of the isolate was resistant to 8 antibiotics. Here is the molecular characterization of that *Pseudomonas spp.* Molecular characterization confirmed that this isolates is ***Pseudomonas aeruginosa.***16S rRNA gene region was amplified with the universal primers, Forward primer- 27F(5'AGAGTTTGATCCTGGCT CAG3') Reverse primer-1492R(5'TACCTTGTTACGA CTT 3').PCR Amplification band was found at 1399 bp.

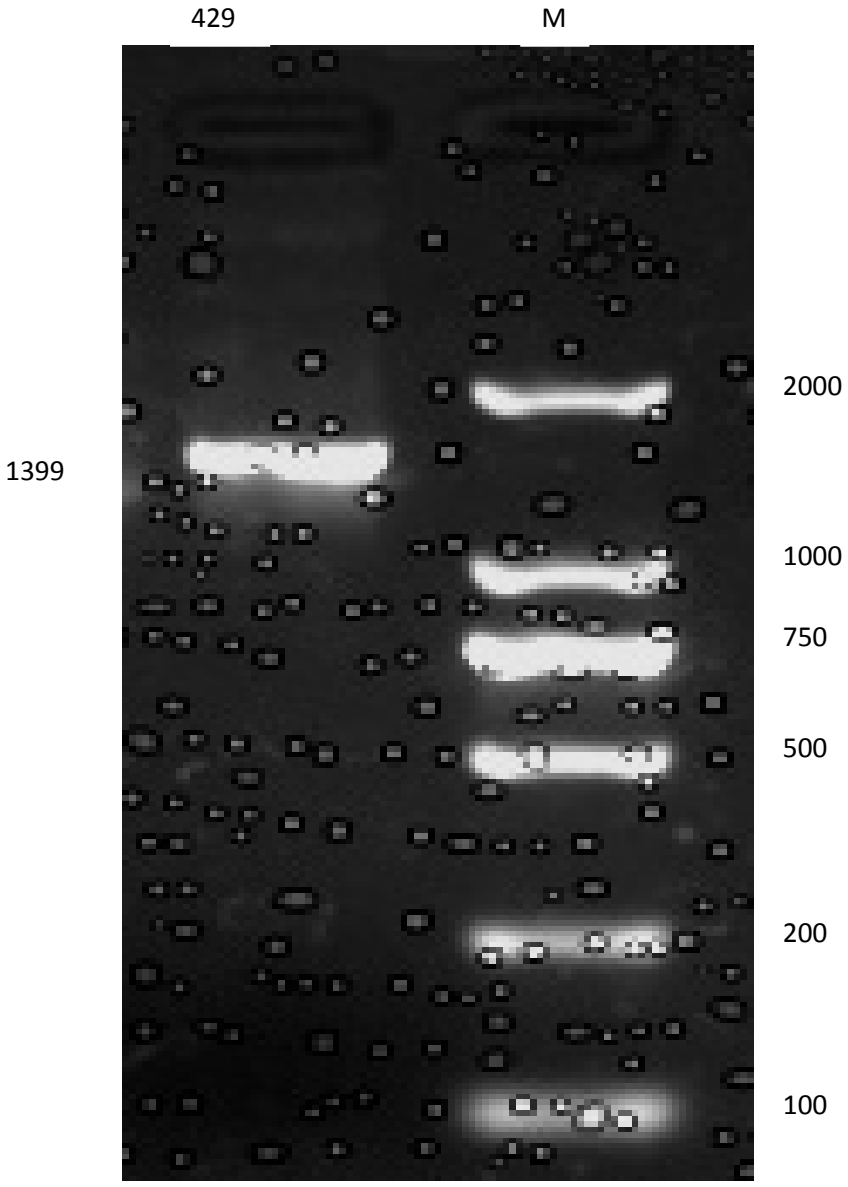


Figure 9: Result of amplification of 16S rRNA gene region of *Pseudomonas aeruginosa* by PCR.
Note: PCR= Polymerase Chain Reaction, kb= kilo base.

4.7.1 Electropherogram

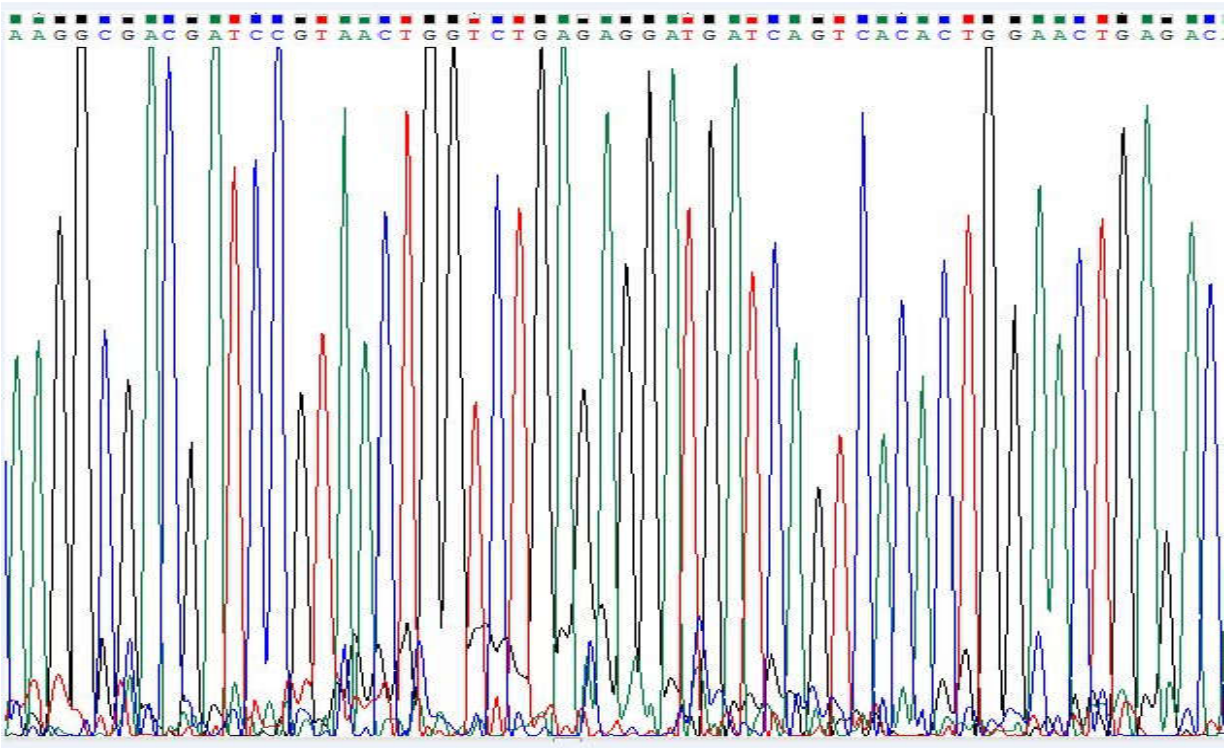


Figure 10: Electropherogram

4.7.2 Contig Sequence of *Pseudomonas aeruginosa*

Contig 429: 1399 bp, Blast: 97% similar

Identified strain: *Pseudomonas aeruginosa* strain DSM 50071

4.7.3 Contig Sequence of *Pseudomonas aeruginosa*

Contig 429

TGGTTGTGCTGCTTAATTCATAAAAAGGGGCCGCCCTCCGTTTTTTTTTGTTGGTT
AATGCTTAGGAATCTGCCTGGCCCGGGGGATTTGTTTGTTTTCGGTTGCTAATACC
GCATACGTCCTGAGGGATAAAGTGGTTTTTCTTCGGACCTCACGCTATCAGATGA
GCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCC
GTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGA
GGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCAC
CGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCCAGTTGGATGTGAAATC
CCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGA
GGGTGGTGGAATTTCCTGTGTAGCGGTGAAAATGCGTAGATATAGGAAGGAACA
CCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGAC
TAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCG
CCTGGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGGCCCG
CACAAGCGGTGGGAGCATGTGGTTTAATTCGAAAGCAACGCGAAGAACCTTACC
TGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTC
AGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTC
TAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATC
ATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTT
GCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCA
GTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGT
CACGGTGAATACGTTTCCGGTCCTTGTCCTCTCAAGAAGTCACAAACATGGGACC
CGGTTGTATTTTATCCACCCTCACTAAGTACAAGCAA

4.7.4 Phylogenic tree analysis of *Pseudomonas aeruginosa*

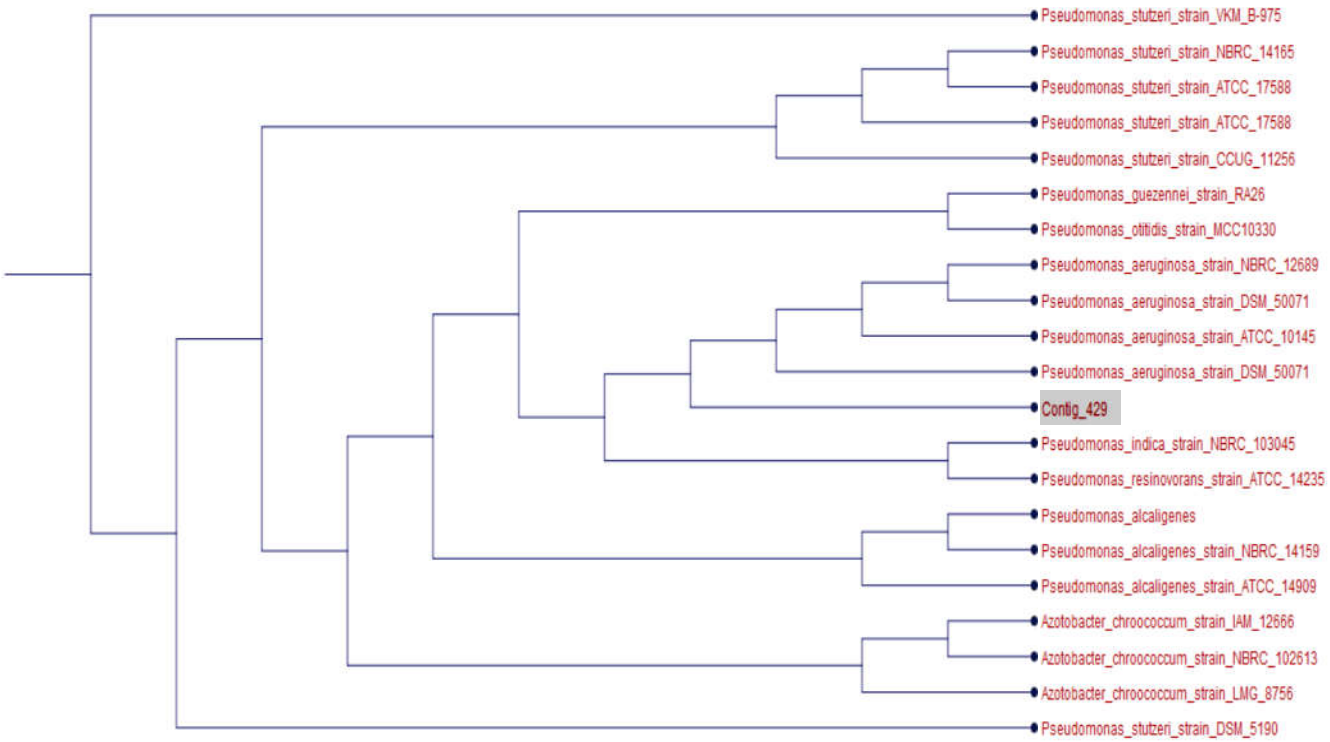


Figure 11 : Phylogenic tree analysis of *Pseudomonas aeruginosa.*

4.8 Results of antibiotic susceptibility test

4.8.1 Antibiotic Susceptibility Pattern of Isolated Organisms from Hospital Waste water

All these isolates were collected from waste water of different hospital environments of Dinajpur. The table below showing only the average zone of inhibition for those organisms was tested against at least 10 different antibiotics that are available in the market. The hospital isolates were selected for antibiotic susceptibility test on the basis of their sampling site importance. Hospital isolates was from MARMCH, SHD, and IBCH. Antibiotic susceptibility pattern of isolated organisms from hospital waste water are shown in table 10;

Table 10: Antibiotic susceptibility pattern of isolated organisms from hospital waste water

Name of the antibiotic and their disc concentration (µg/disc)	Zone of inhibition & interpretation of the test organism					
	E.coli **(mm)**	***Pseudomonas spp.*** **(mm)**	***Klebsiella spp.*** **(mm)**	***Salmonella spp.*** **(mm)**	***Staphylococcus spp.*** **(mm)**	***Vibrio spp.*** **(mm)**
Ampicillin (25)	0 (R)	0 (R)	0 (R)	0 (R)	14 (I)	0 (R)
Amoxicillin (30)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)
Amikacin (30)	0 (R)	0 (R)	12 (R)	16 (S)	12 (R)	0(R)
Chloramphenicol (30)	22 (S)	NT	12 (I)	24 (S)	18(S)	15(R)
Ciprofloxacin (5)	17 (I)	12 (R)	14 (R)	16 (I)	15(I)	18 (S)
Gentamycin (10)	20 (S)	21 (S)	14(I)	15 (S)	12(R)	15(S)
Kanamycin (30)	0 (R)	0 (R)	12(R)	7 (R)	13(R)	16 (I)
Penicillin(10)	0 (R)	0 (R)	8(R)	0 (R)	17(S)	NT
Tetracycline (30)	15 (S)	16(S)	10 (R)	14(I)	13(R)	12 (R)
Vancomycin (30)	6 (R)	0 (R)	NT	16 (I)	8(R)	13(I)

Source: CLSI- 2015 [Note: S=Sensitive, R=Resistant, I=Intermediate]

4.8.2 Antibiotic Susceptibility Pattern of Isolated Organisms from Non-Hospital Waste water

All these isolates were collected from waste water of different non-hospital environments of Dinajpur. The table below showing only the average zone of inhibition for those organisms was tested against at least 10 different antibiotics that are available in the market. The non-hospital isolates were selected for antibiotic susceptibility test on the basis of their sampling site importance. Hospital isolates was from HSTU campus, Baser Hat Bazar, Bahadur Bazar, Lilir Mor, Kalitola. Antibiotic susceptibility pattern of isolated organisms from non-hospital waste water are shown in table 11;

Table 11: Antibiotic susceptibility pattern of isolated organisms from non-hospital waste water

Name of the antibiotic and their disc concentration (µg/disc)	Zone of inhibition & interpretation of the test organism					
	E.coli **(mm)**	***Pseudomonas spp.*** **(mm)**	***Klebsiella spp.*** **(mm)**	***Salmonella spp.*** **(mm)**	***Staphylococcus spp.*** **(mm)**	***Vibrio spp.*** **(mm)**
Ampicillin (25)	6 (R)	14 (I)	13(R)	15(I)	12(R)	15(I)
Amoxicillin (30)	12 (R)	9(R)	12 (R)	18(S)	14(R)	17(S)
Amikacin (30)	17 (S)	0 (R)	16 (I)	15(I)	14(I)	15(S)
Chloramphenicol (30)	10(R)	21(S)	12(R)	17(S)	18(S)	17(S)
Ciprofloxacin (5)	12 (R)	16(I)	14(R)	16(I)	18 (I)	21(S)
Gentamycin (10)	14 (S)	20(S)	18 (S)	12(R)	7(R)	17(S)
Kanamycin (30)	13 (S)	8(R)	12(R)	0 (R)	8 (R)	0 (R)
Penicillin(10)	6 (R)	14(S)	12 (I)	8(R)	10 (R)	10 (R)
Tetracycline (30)	14 (I)	11 (R)	7 (R)	14 (I)	11 (R)	14 (I)
Vancomycin (30)	10 (R)	14(S)	8 (R)	16 (I)	8(R)	18 (S)

Source: CLSI- 2015 [Note: S=Sensitive, R=Resistant, I=Intermediate]

4.8.3 Drug resistance pattern of hospital isolates (Percentages)

The antibiotic study revealed that all tested Hospital *E.coli* isolates was 100% resistant to Ampicillin, followed by Amikacin, Penicillin and vancomycin was 80% resistant. All tested Hospital *Pseudomonas spp* was100% resistant to Ampicillin, Amikacin and Penicillin, followed by Amoxicillin, Ciprofloxacin, Kanamycin, Tetracycline, and Vancomycin and was 75 resistant. None of the tested Hospital *Klebsiella spp was* 100% resistant to any of the antibiotics, but Ampicillin, Amoxicillin, Kanamycin and penicillin was 75% resistant to *Klebsiella spp.* From *Salmonella spp;* 100% was resistant to Ampicillin and Kanamycin. Among tested hospital *Staphylococcus spp* amoxicillin, Amikacin and Kanamycin was 100% resistant. *Vibrio spp* was 100% resistant against Ampicillin, Amikacin, Kanamycin and Tetracycline. The antibiotic study also revealed that among the tested hospital isolates; about (83.3%), was resistant against Ampicillin, followed by Amikacin, Kanamycin and Penicillin (77.8%) which are shown in table 12;

Table 12: Drug resistance pattern of hospital isolates

Name of the antibiotic and their disc concentration (µg/disc)	Percentages N (%)						
	E.coli **n=5**	***Pseudomonas spp.*** **n=4**	***Klebsiella spp.*** **n=4**	***Salmonella spp.*** **n=2**	***Staphylococcus spp.*** **n=2**	***Vibrio spp.*** **n=1**	**Total N=18**
Ampicillin (25)	5(100)	4(100)	3(75)	2(100)	1(50)	1(100)	15(83.3)
Amoxicillin (30)	3(60)	3(75)	3(75)	1(50)	2(100)	-	12(66.7)
Amikacin (30)	4(80)	4(100)	2(50)	1(50)	2(100)	1(100)	14(77.8)
Chloramphenicol (30)	2(40)	2(50)	2(50)	-	1(50)	-	7(38.9)
Ciprofloxacin (5)	2(40)	3(75)	2(50)	1(50)	1(50)	-	9(50)
Gentamycin (10)	1(20)	2(50)	1(25)	-	1(50)	-	5(27.8)
Kanamycin (30)	3(60)	3(75)	3(75)	2(100)	2(100)	1(100)	14(77.8)
Penicillin(10)	4(80)	4(100)	3(75)	1(50)	1(50)	1(100)	14(77.8)
Tetracycline (30)	1(20)	3(75)	2(50)	1(50)	1(50)	1(100)	9(50)
Vancomycin (30)	4(80)	3(75)	2(50)	1(50)	1(50)	-	11(61.1)

[Note; (-) =Not Resistant]

4.8.4 Drug resistance pattern of non- hospital isolates (percentages)

The antibiotic study revealed that the non hospital *E.coli* isolates was 100% resistant to Ampicillin, Ciprofloxacin and Penicillin, other antibiotics was either sensitive or 50% resistant. Only Amoxicillin and Amikacin was 100% resistant to *Pseudomonas Spp* from non hospital source. *Klebsiella spp* was 100% resistant against Ampicillin and Chloramphenicol. Non hospital *Salmonella spp*. isolates was 100% resistant against Amikacin and Gentamycin, other antibiotics was sensitive. Amoxicillin, Vancomycin and penicillin was 100% resistant against the non hospital *Staphylococcus spp*, other rest of the antibiotics was either 50% resistant or sensitive. Only Kanamycin was 100% resistant against the tested non-hospital *Vibrio spp.* The antibiotic study also revealed that among the tested non- hospital isolates was mostly resistant against amoxicillin and Penicillin (66.7%) followed by Ampicillin and Vancomycin (58.3%) which are shown in table 13;

Table 13: Drug resistance pattern of non-hospital isolates

Name of the antibiotic and their disc concentration (µg/disc)	Percentages N (%)						
	E.coli **n=2**	***Pseudomonas spp.*** **n=2**	***Klebsiella spp.*** **n=2**	***Salmonella spp.*** **n=2**	***Staphylococcus spp.*** **n=2**	***Vibrio spp.*** **n=2**	**Total N=12**
Ampicillin (25)	2(100)	1 (50)	2(100)	-	1(50)	1(50)	7(58.3)
Amoxicillin (30)	1 (50)	2(100)	1 (50)	1(50)	2(100)	1(50)	8(66.7)
Amikacin (30)	1(50)	2(100)	-	2(100)	-	-	5(41.7)
Chloramphenicol (30)	1(50)	-	2 (100)	1(50)	1(50)	1(50)	6(50)
Ciprofloxacin (5)	2(100)	1(50)	1(50)	-	1(50)	-	5(41.7)
Gentamycin (10)	-	-	-	2(100)	1(50)	-	3(25)
Kanamycin (30)	1(50)	1(50)	1(50)	-	1(50)	2(100)	6(50)
Penicillin(10)	2(100)	1(50)	1(50)	1(50)	2(100)	1(50)	8(66.7)
Tetracycline (30)	1(50)	1(50)	1 (150)	-	1(50)	1(50)	5(41.7)
Vancomycin (30)	1 (50)	1(50)	1 (50)	1(50)	2(100)	1(50)	7(58.3)

[Note; (-) =Not Resistant]

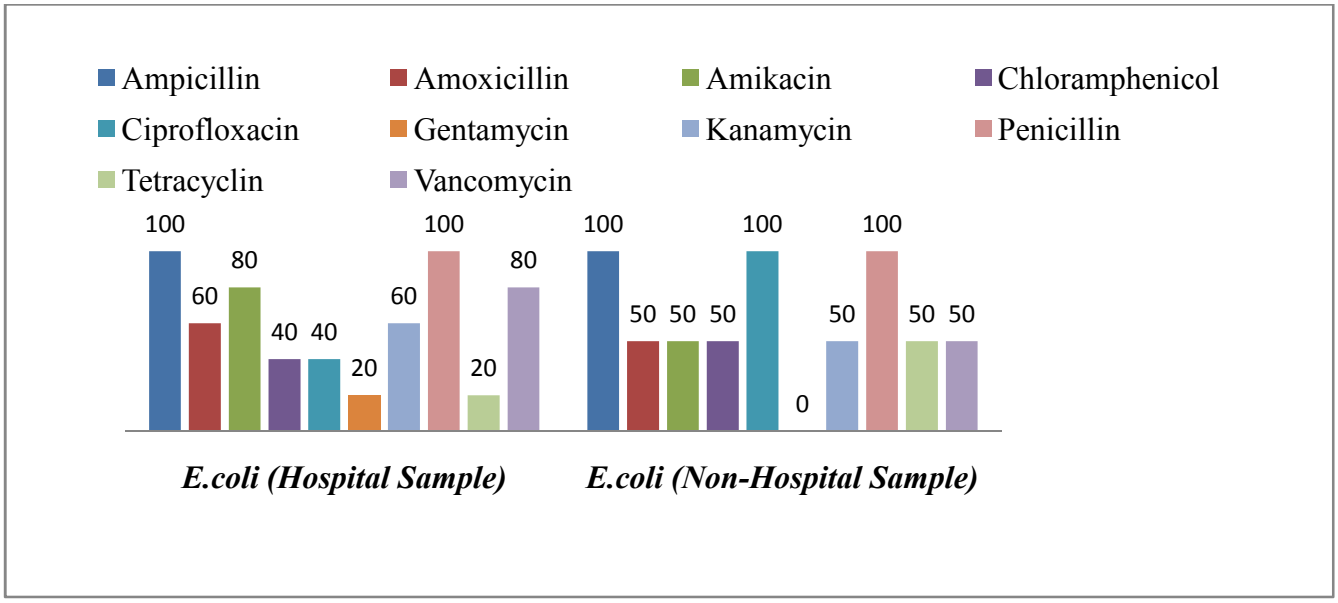


Figure 12: Antibiotic resistant pattern of ***E.coli*** from hospital and non-hospital waste water

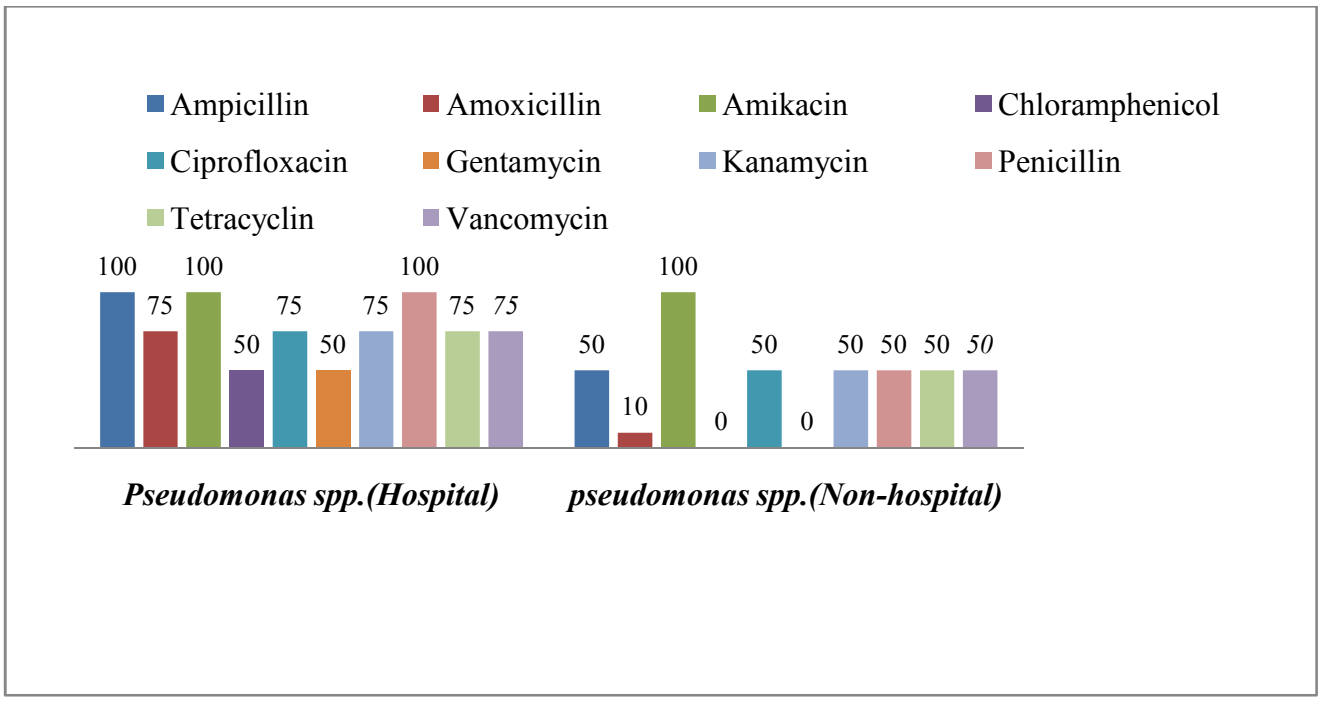


Figure 13: Antibiotic resistant pattern of ***Pseudomonas spp.*** from hospital and non-hospital waste water

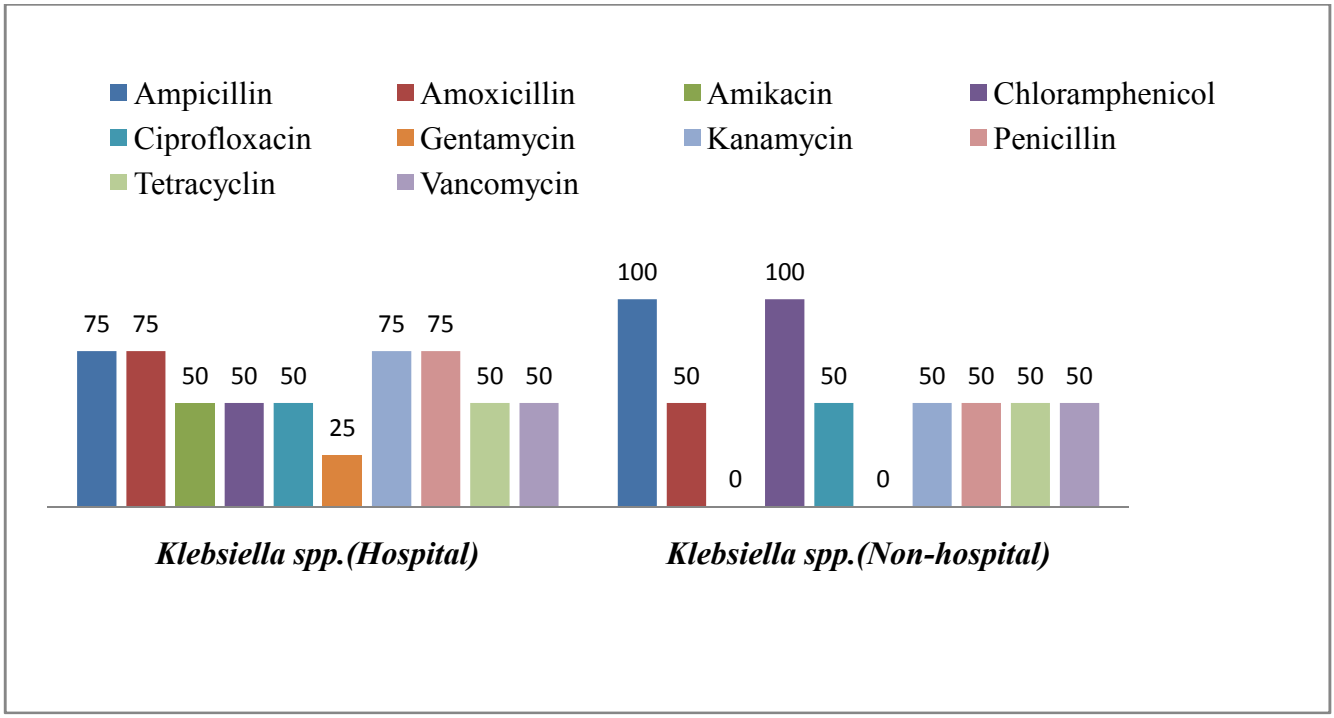


Figure 14: Antibiotic resistant pattern of ***Klebsiella spp.*** from hospital and non-hospital waste water

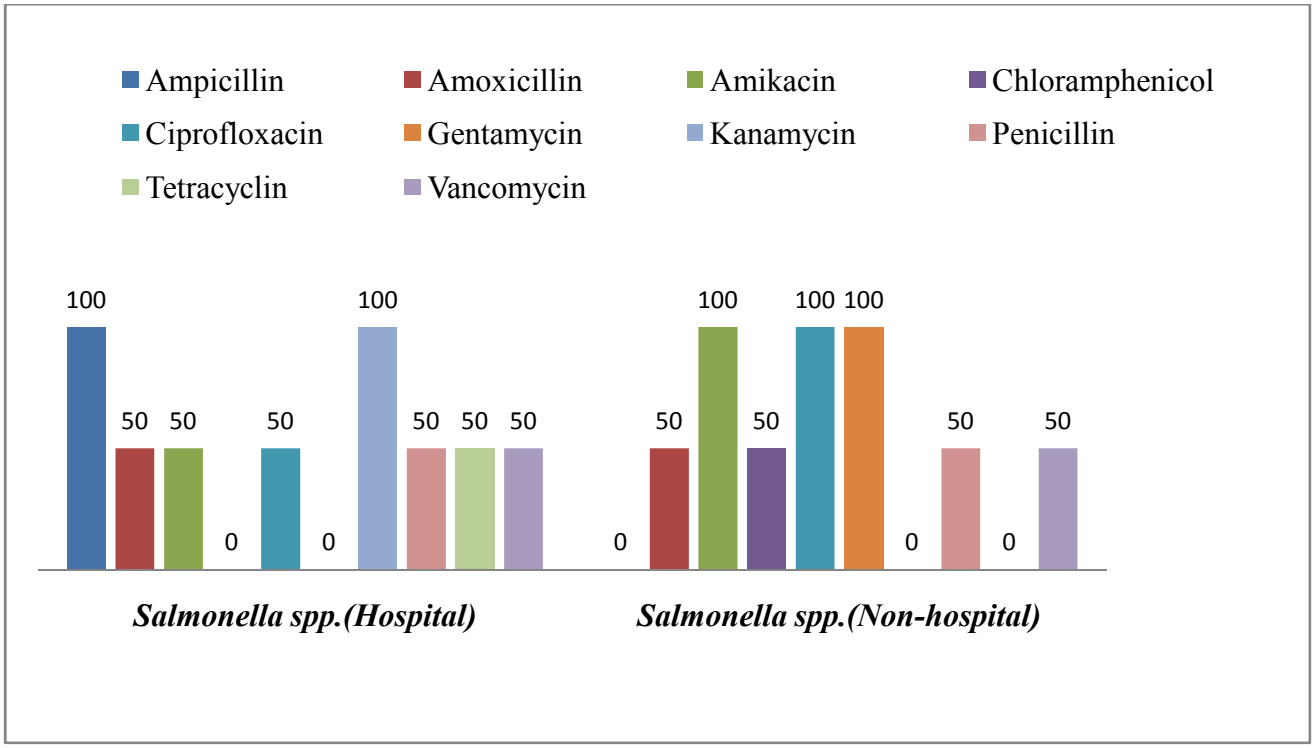


Figure 15: Antibiotic resistant pattern of ***Salmonella spp.*** from hospital and non-hospital waste water

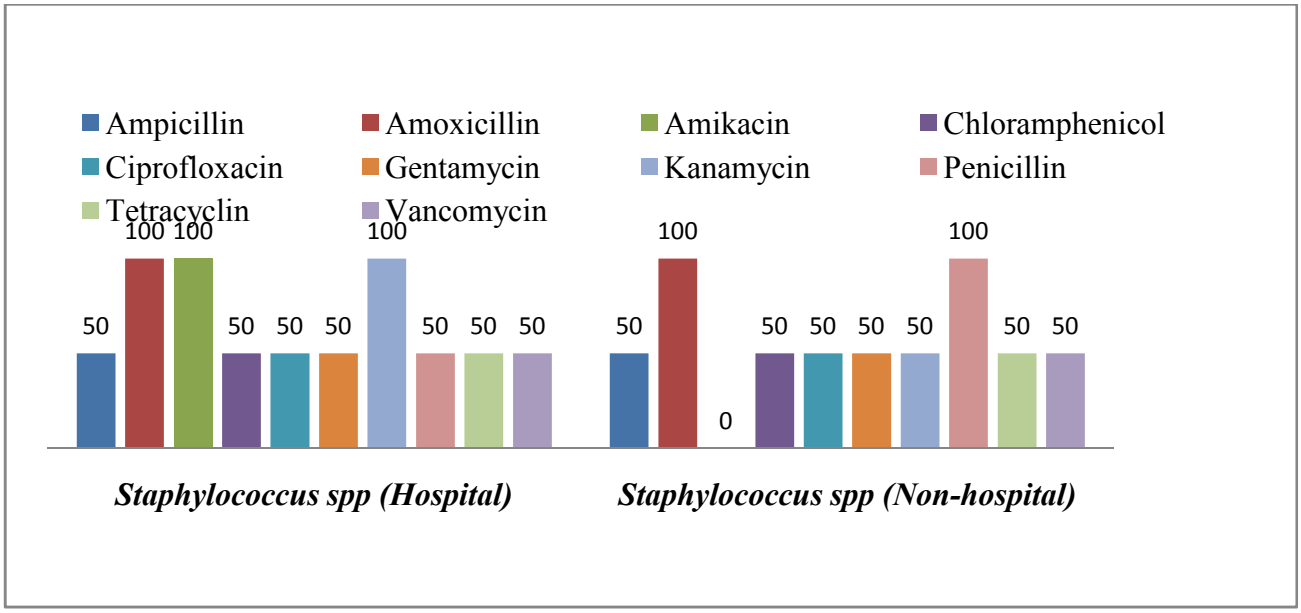


Figure 16: Antibiotic resistant pattern of *Staphylococcus spp*

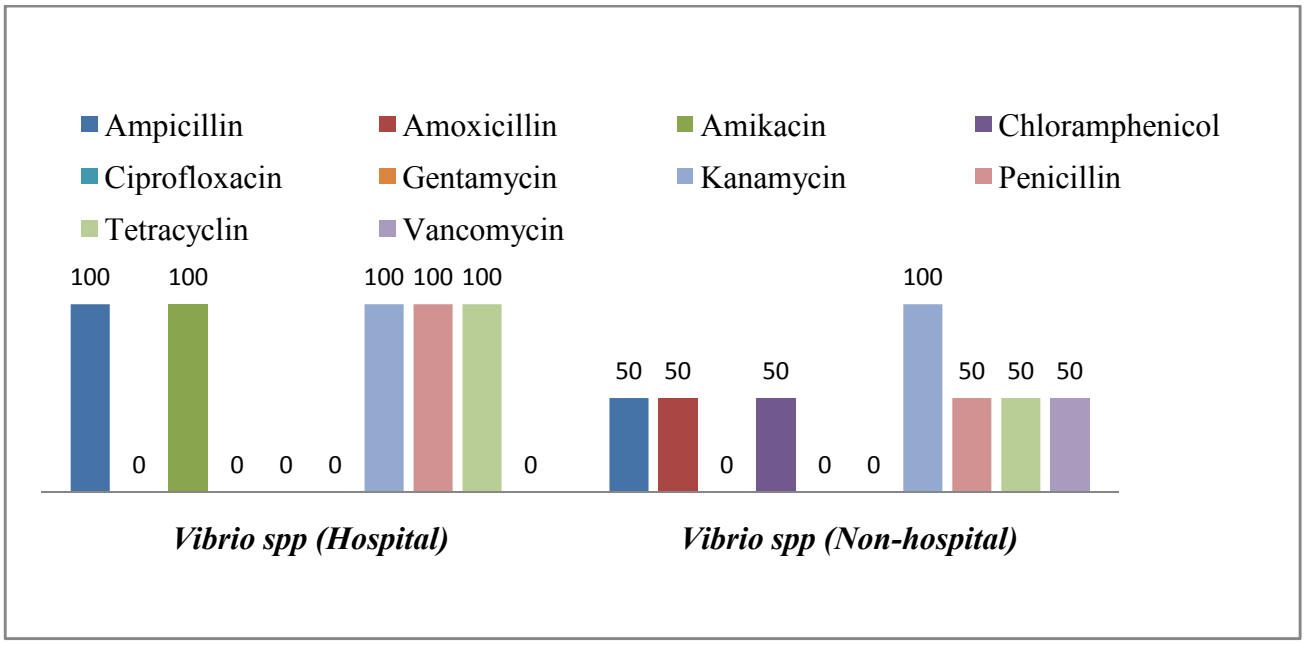


Figure 17: Antibiotic resistant pattern of *Vibrio spp*

4.7.5 Antibiogram test of *E.coli* against 10 different antibiotics

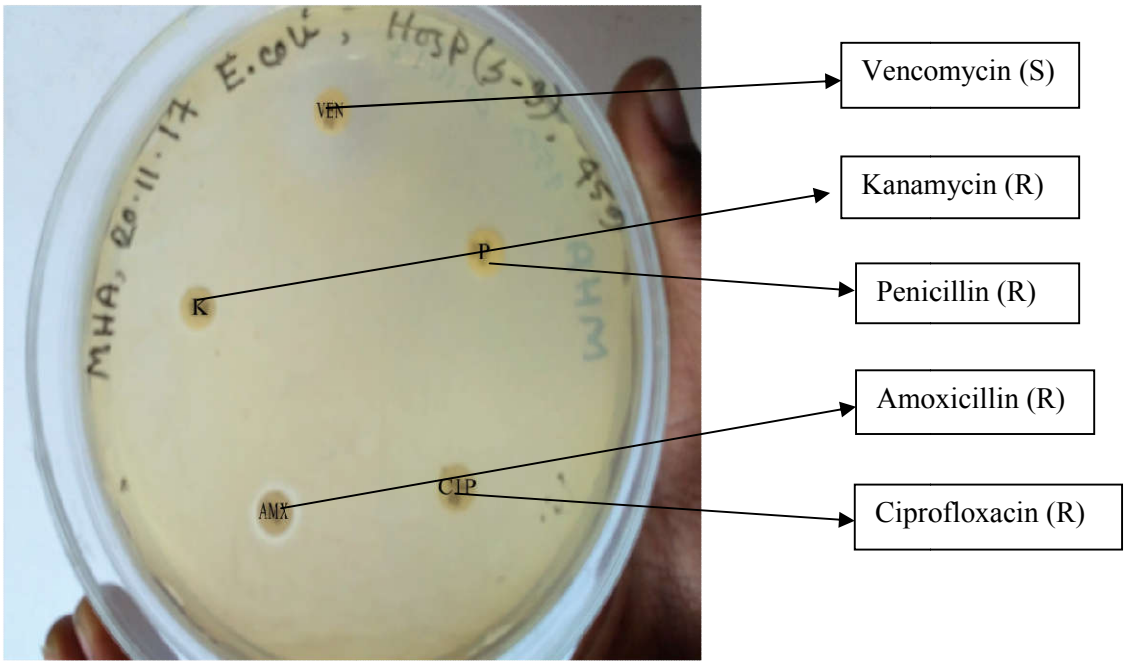


Plate 39: Antibiogram test of *E.coli*

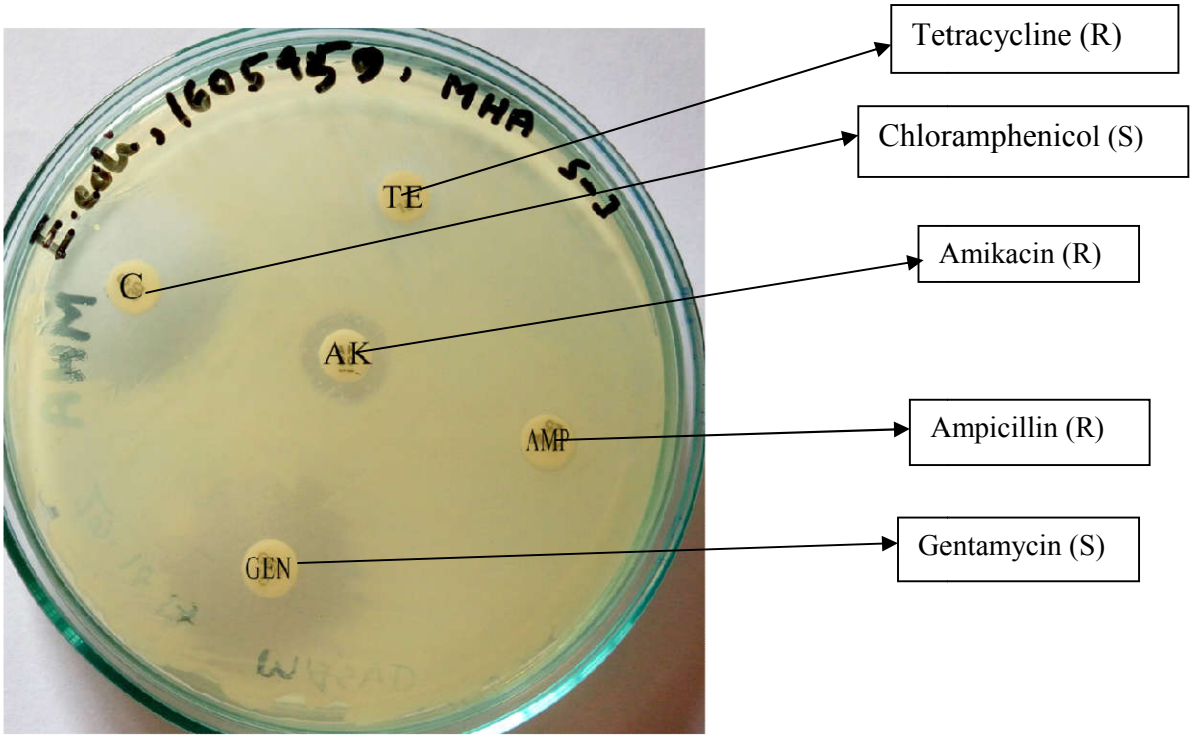


Plate 40: Antibiogram test of *E.coli*

4.7.6 Antibiogram test of *Pseudomonas spp* against 10 different antibiotics

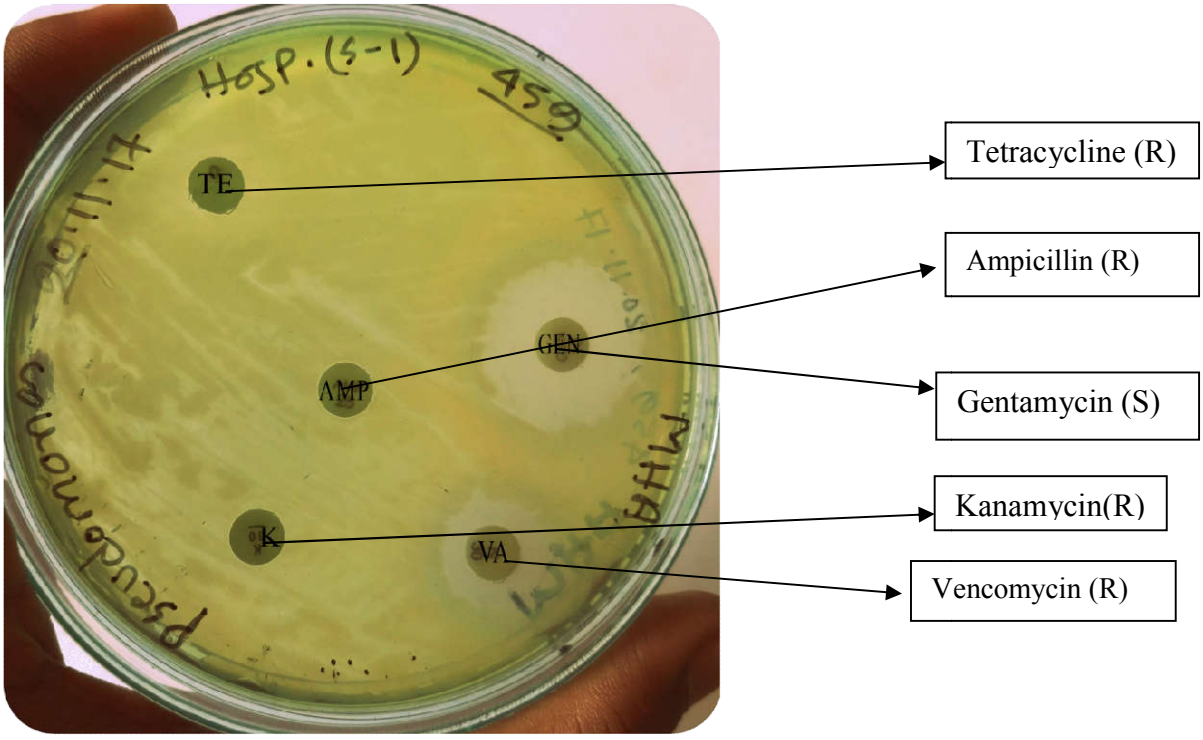


Plate 41: Antibiogram test of *Pseudomonas spp*

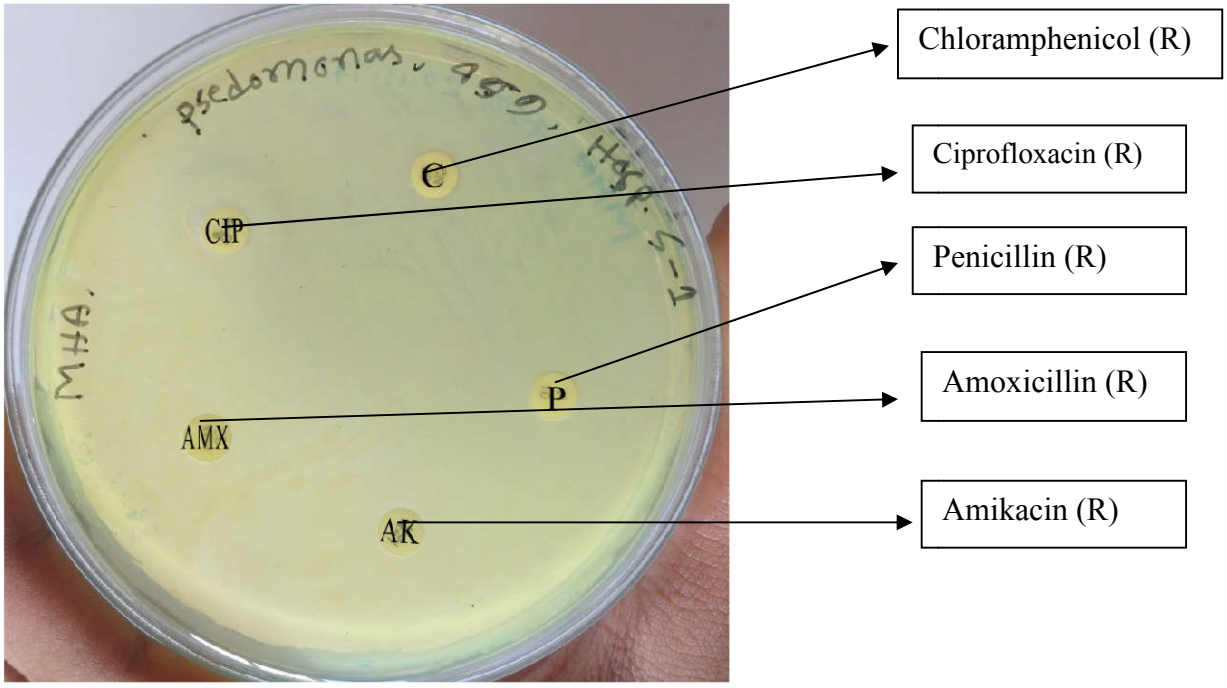


Plate 42: Antibiogram test of *Pseudomonas spp*

4.7.7 Antibiogram test of *Klebsiella spp* against 10 different antibiotics

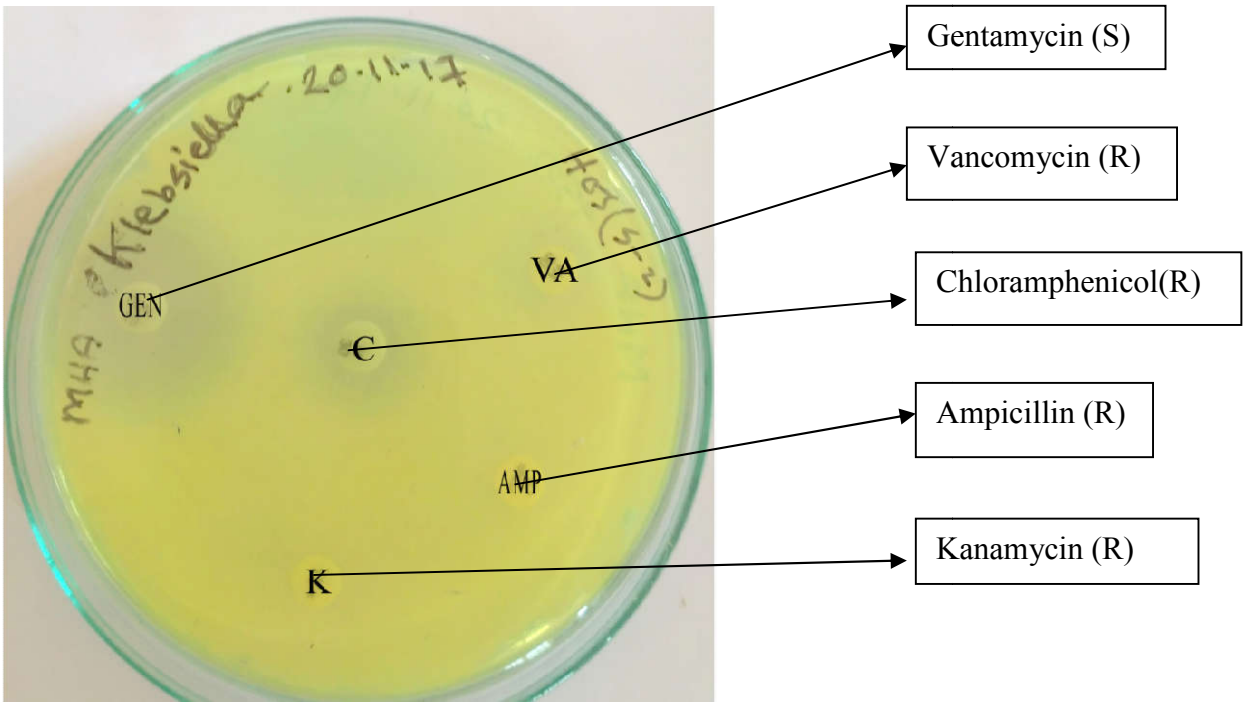


Plate 43: Antibiogram test of *Klebsiella spp*

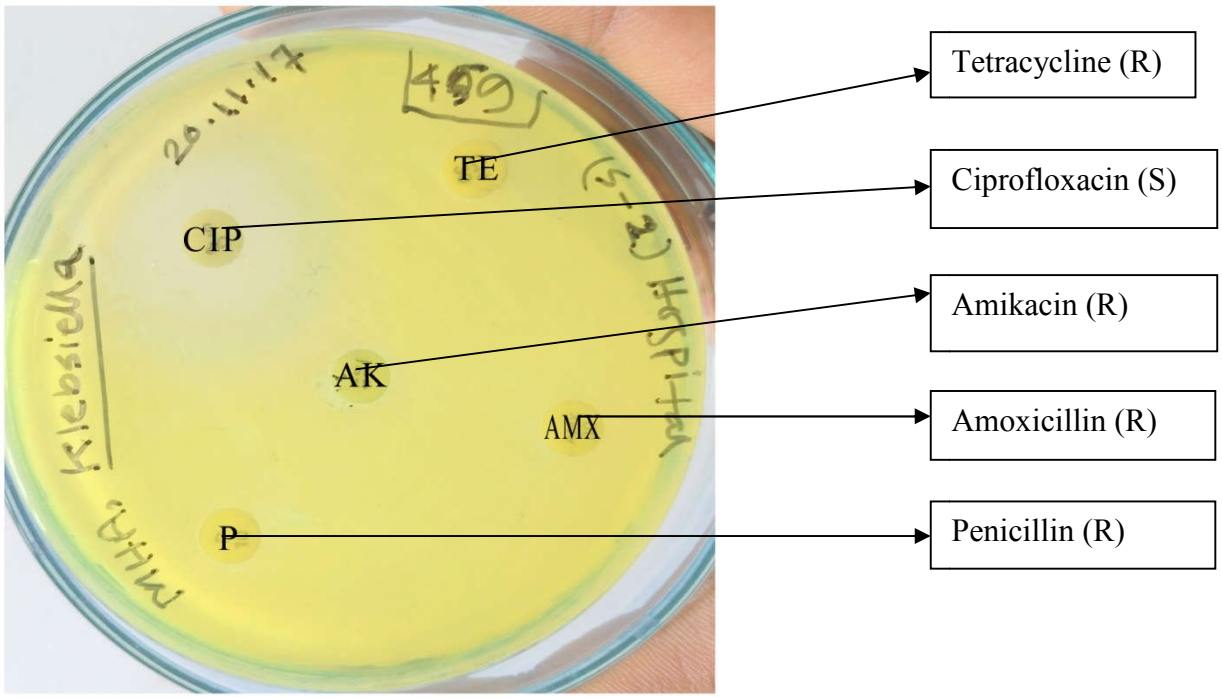


Plate 44: Antibiogram test of *Klebsiella spp*

4.7.8 Antibiogram test of *Vibrio spp* against 11 different antibiotics

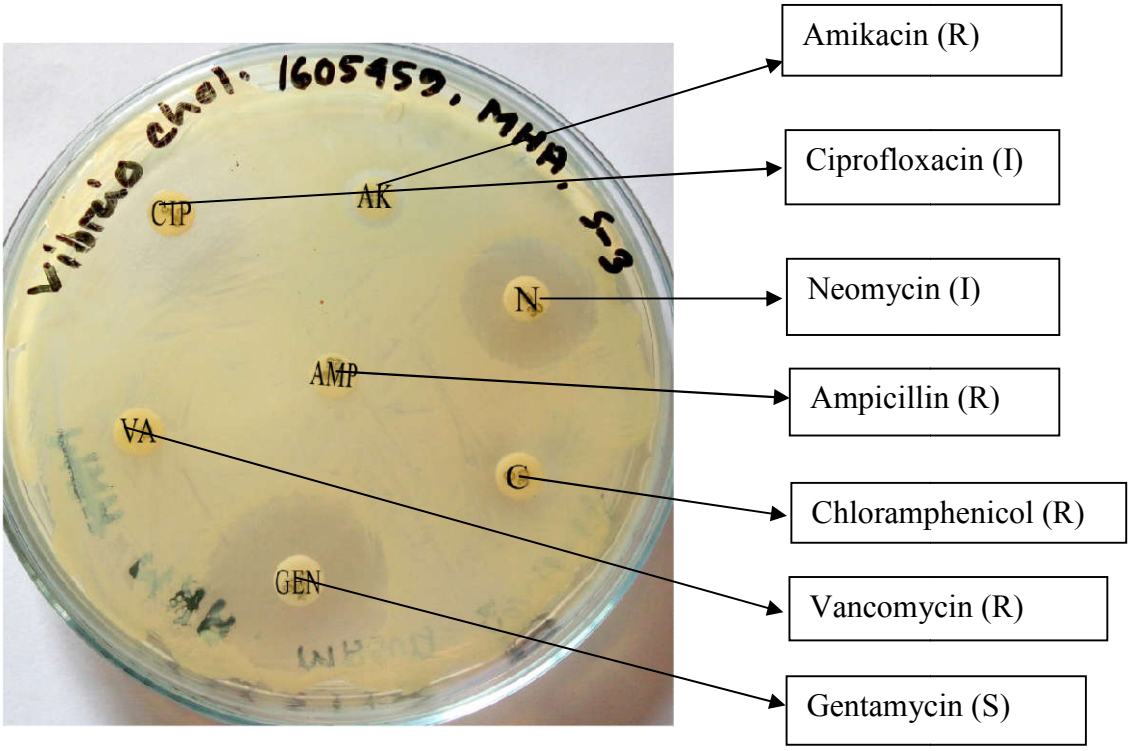


Plate 45: Antibiogram test of *Vibrio spp*

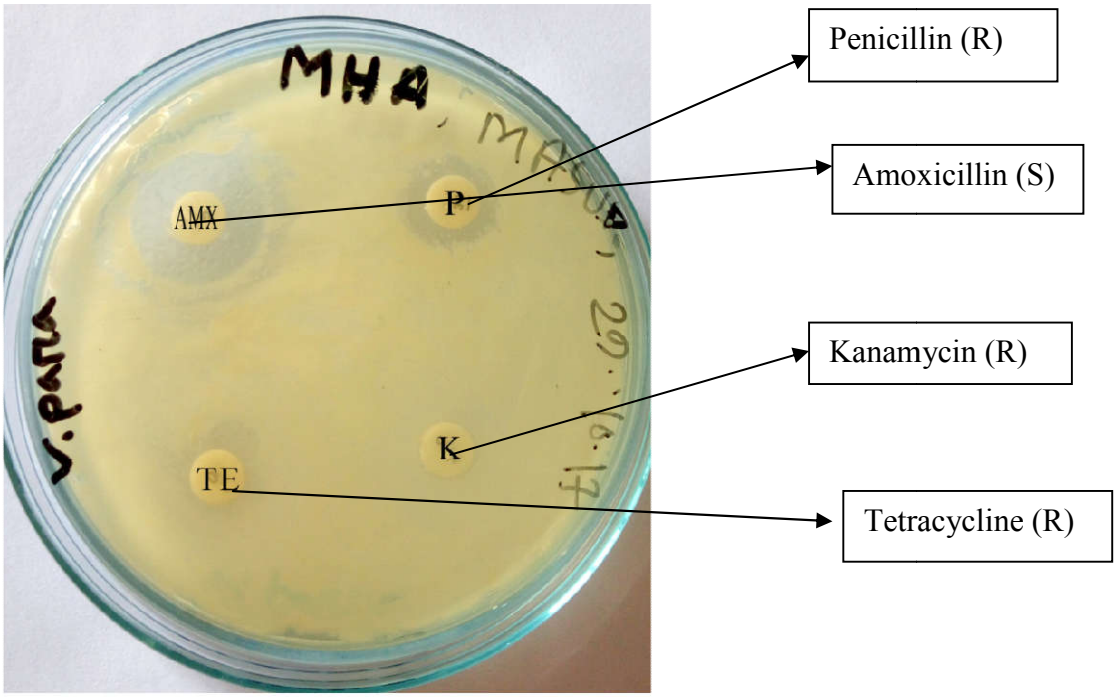


Plate 46: Antibiogram test of *Vibrio spp*

4.7.9 Antibiogram test of *Salmonella spp* against 9 different antibiotics

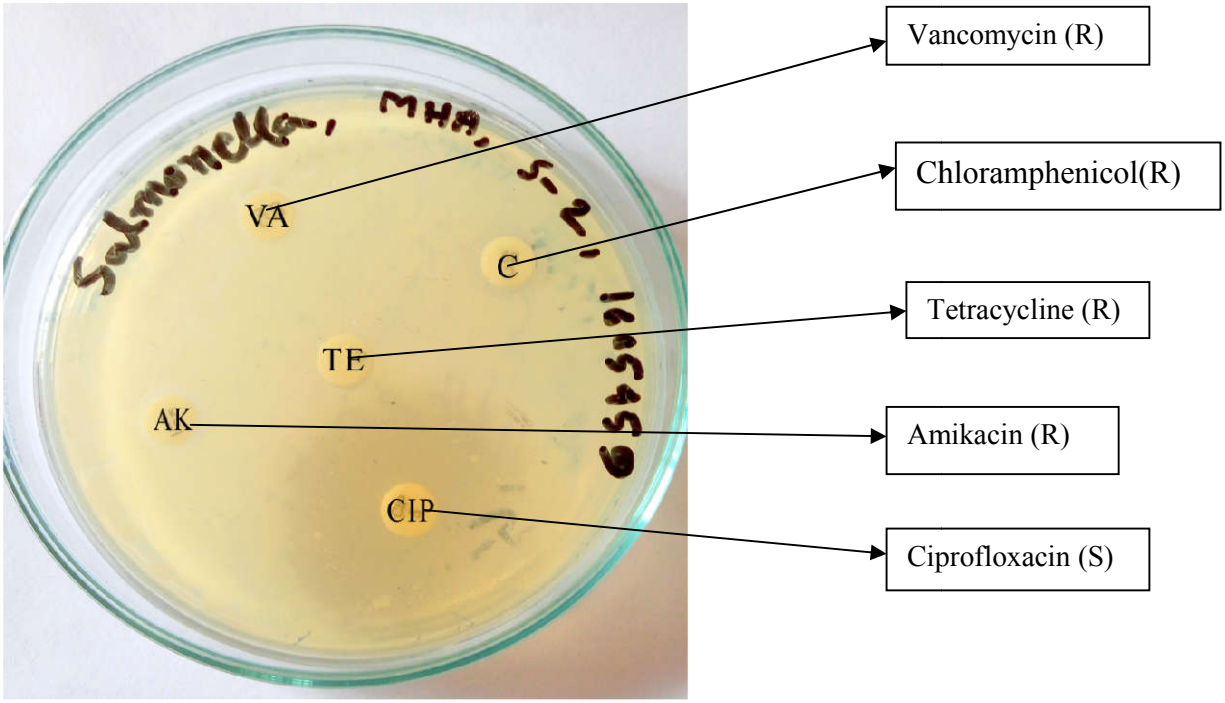


Plate 47: Antibiogram test of *Salmonella spp*

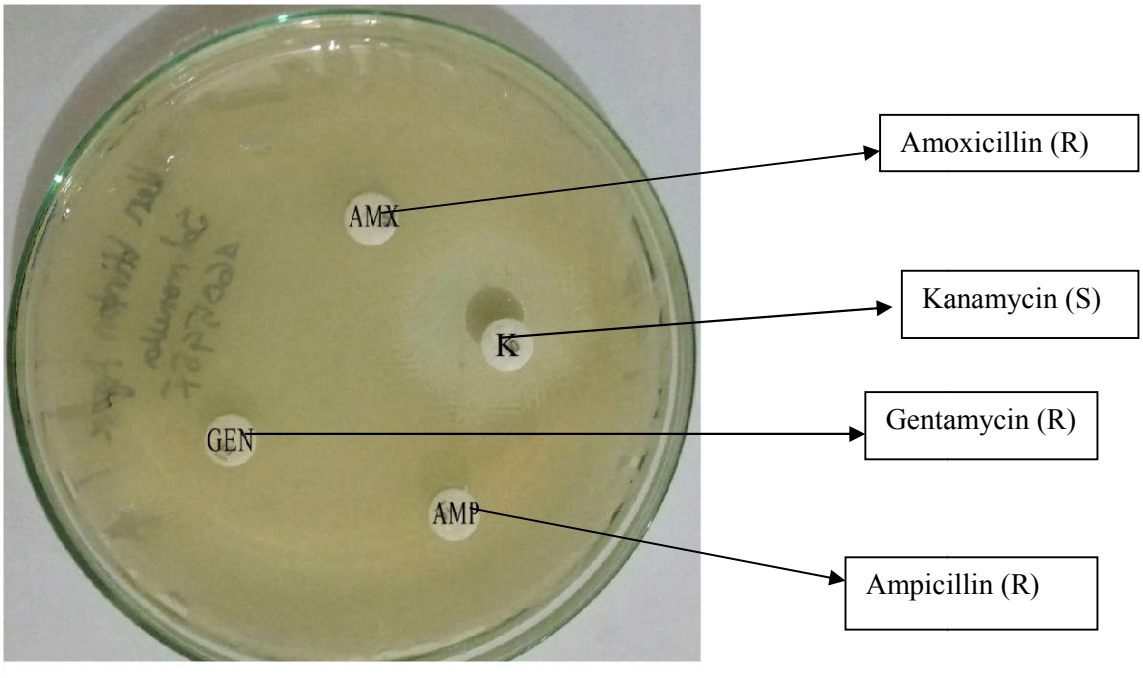


Plate 48: Antibiogram test of *Salmonella spp*

4.7.10 Antibiogram test of *Staphylococcus spp* against 12 different antibiotics

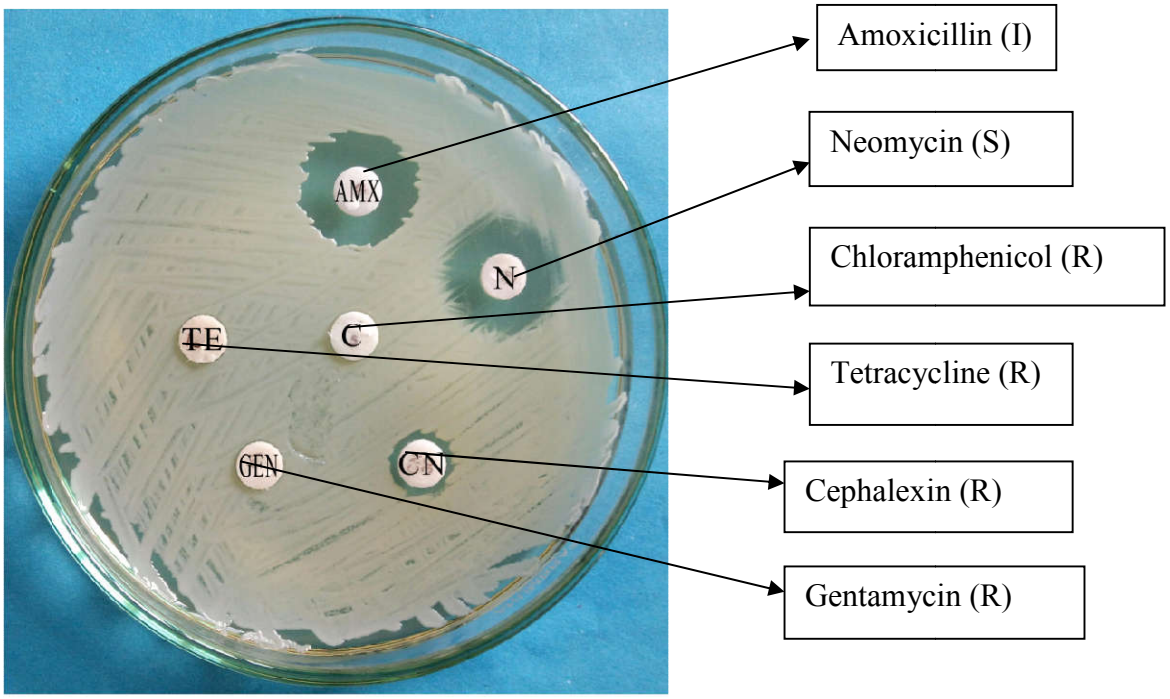


Plate 49: Antibiogram test of *Staphylococcus spp*

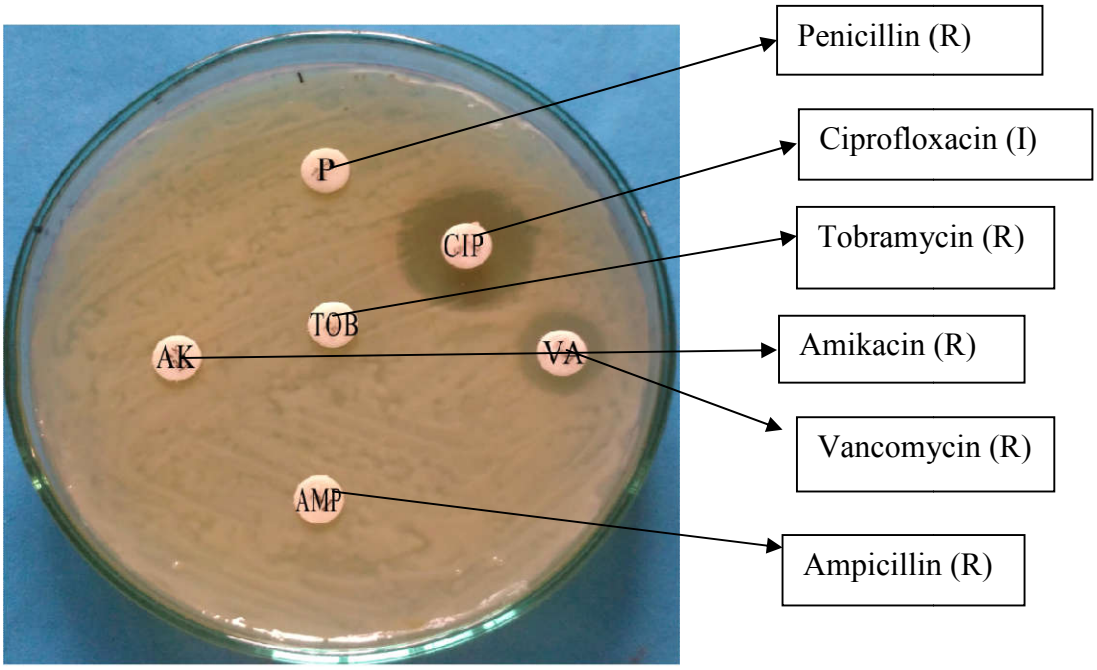


Plate 50: Antibiogram test of *Staphylococcus spp*

CHAPTER 5

DISCUSSION

Drug resistance in bacteria is widespread problem throughout the world and is increasing day by day. The current study was carried out for the isolation and characterization of multiple drug resistant bacteria pathogens from waste water of hospital and non-hospital environments of Dinajpur district of Bangladesh. This study was conducted to see the drug resistance pattern of this area of Bangladesh. For this study, a total of 20 waste water samples were collected from different hospital and non-hospital area. A series of tests were conducted for isolation, identification and antibiotics sensitivity of identified bacteria. In this study, 6 different types of bacteria were isolated and identified. Isolated bacteria were identified on the basis of colony morphology, Gram reaction, microscopic character, biochemical characteristics and molecular characteristics of 1 selected isolate. Molecular charactirization was done to identify *Pseudomonas species* by 16S rRNA Gene Sequencing.

Result of total viable count showed that maximum countable bacteria (2.20×10^{11}) CFUs that were from MARMCH Site-2 and minimum number of countable bacteria (1.0×10^{11}) was isolated from sample of Kalitola. Maximum number of total bacterial isolates was 13 and minimum number of bacterial isolates was 3 that were collected respectively from MARMCH and Kalitola.

In current study, a total 55 bacterial isolates were isolated. Among them 32 (58.2%) were from hospital environment and 23 (42.1%) were from non-hospital environment. The rate of isolation of bacterial pathogens in the hospital environment was higher than the non-hospital environment. The finding of this study is almost similar to Moges *et al*., (2014) where he found 65 (57.5%) isolates from hospital environment and 48 (42.5%) were from non-hospital environments. 6 different bacterial isolates were *E.coli, Pseudomonas spp., Klebsiella spp., Salmonella spp., Staphylococcus spp. and Vibrio spp*. Most frequently isolated bacteria was *E.coli* 16 (29) followed by *Pseudomonas spp*. 12(21.8%) and *Klebsiella spp.* 9(16.4%). Similar result was showed by Onuoha *et al*., (2017) and Elmanama *et al*., (2006). Similar study in Dhaka City, Bangladesh reported that frequently isolated bacteria were *Escherichia coli* and *Klebsiella pneumonia* isolates from two renowned hospital of Dhaka city **(**Rabbani *et al.,*2017). Guessennd *et al.*, (2013) also reported that they mostly isolated *E. coli, K.*

pneumoniae, P. aeruginosa, and *Staphylococcus spp.* from hospital waste water. Yang *et al.,* (2008) also reported that *E. coli* were the leading bacterial isolates in both clinical and sweage samples.

In current study the cultural characteristics of *E.coli* isolates was greenish black colony with metallic sheen in EMB, rose pink color smooth transparent colony in Mac-Conkey agar and smooth glistening and opalescent colony in nutrient agar (Table 4.5) which were similar to the findings of other authors (Carter, 1979; Buxton and Fraser, 1977) In biochemical test *E.coli* isolates fermented different sugar with the production of acid and gas after incubation. The isolates also revealed positive reaction in MR and negative reaction in VP test. (Table 4.6). In Gram staining the morphology of the *E.coli* showed gram negative large rod arranged in single or paired and after motility test it founded positive. The cultural characteristics of *Pseudomonas spp* isolates were greenish or yellowish green in color that is similar to the findings of (Cheesbrough, 1985). In grams staining the *Pseudomonas* isolates were gram negative pink color short rods. In biochemical test *Pseudomonas* spp were positive to catalase, citrate and motility test positive, and were negative to MR, VP and indole test that was similar to the findings of Cappuccino and Sherman. The cultural characteristics of *Klebsiella* isolates were pinkish-off white color on Mac-Conkey agar and gray brown centers and pink color on Eosin Methylene Blue agar. In Grams staining the *Klebsiella* isolates were gram negative pink color short rods. In biochemical test *Klebsiella* spp were positive to catalase, citrate and VP test positive and negative to MR, indole and MIU test. The cultural characteristics of *Salmonella* isolates were black center, transparent colonies on SS agar and were Gram negative small rod shaped pink color colonies under microscope. In biochemical test *Salmonella* isolates were positive to catalase, MIU, MR and VP test and variable to indole test. H_2S production was positive with the blackening of test tube. In present study colony characteristics of the *Staphylococcus spp* were observed on different media were similar to the findings of Buxton and Fraser, 1977. In Grams staining the morphology of the *Staphylococcus spp* exhibited gram positive, cocci shape grape like clusters which was supported by several authors (Freeman, 1985; Marchant and Packer 1967) Colony characteristics of *Vibrio* isolates of this study were yellow color and some greenish colonies were also present that possibly the colonies of *Vibrio parahaemolyticus.* In Grams staining the morphology of the *Vibrio spp* were Gram negative small rod-shaped pink. In biochemical test *Vibrio* isolates were positive to almost all tests without VP test where a variable result was observed.

Multiple drug resistance was common in gram negative isolates to commonly used antibiotics in the study area. *E. coli*, *Pseudomonas spp, Salmonella spp* and *Vibrio* spp were 100% resistant to Ampicillin. This finding is inconsistent from reports in Brazil that the overall resistance rates were low in the isolates of *E. coli, P. aeruginosa, K. pneumoniae* and *A. baumannii* and the susceptibility pattern of *E.coli* and *Klebsiella* for ampicillin was found 40% and 70%, respectively (Resende *et al*, 2009). Among all isolates (83.3%) of the isolates were resistant to Ampicillin, followed by Amikacin, Kanamycin and Penicillin all were (77.8%) resistant. (Table:4.9). This finding agreed with the result of Moges *et al*., (2014). Similar study reported that most isolates was resistant to Ampicillin (73.9 %) (Zubair *et al*., 2013), similarly Krcme´ry *et al.,* (1989) showed as high as 80% Ampicillin- resistant *E.coli* from municipal waste water. One of the *Pseudomonas spp* isolates was resistant to 8 out 10 antibiotics that were used in current study. Another study showed that *Pseudomonas spp*. was resistant to 10 out of 12 antibiotics (Moges *et al*., 2014). The resistant pattern of Gram-negative isolates for Ciprofloxacin was moderate (50%) in the present study, this was different from other study done in Bangladesh where of 100 % was resistant (Islam et al., 2008). Gentamycin was the most effective antibiotic to all of the isolates as it was 72.5% and 75% sensitive to hospital and non-hospital isolates respectively, this result is similar to Ibrahim *et al*., (2010) where he also found Gentamycin as most effective antibiotic.

One of the goals of this current study was to compare between drug resistant bacterial isolates from hospital and non-hospital waste water; in this case the result of this study showed that hospital isolate was more resistant to most of the antibiotic which were used. As the antibiotic study revealed that among the tested hospital isolates; about 83.3%, was resistant against Ampicillin, followed by Amikacin, Kanamycin and Penicillin, all were 77.8% resistant. On the other hand, antibiotic study result revealed that among the tested non- hospital isolates were mostly resistant against Amoxicillin and Penicillin (66.7%) followed by Ampicillin and Vancomycin (58.3%).

Result of molecular characterization revealed that isolated multi drug resistant *Pseudomonas spp* are the ***Pseudomonas aeruginosa.*** Similar kind of multi drug resistant *Pseudomonas aeruginosa* was identified from hospital waste water by Tumeo *et al.,*2008, but he said that there was a difference between *Pseudomonas aeruginosa* that were collected from hospitalized patients and waste water. So current study result suggests that multi drug resistant *Pseudomonas aeruginosa* is predominant in hospital waste water. One study carried

out in Bangladesh in 2008 found out that the resistance development was directly related to the use of antibiotics (Islam *et al.,* 2008) The results further suggested that the multi-drug resistant bacteria & plasmid containing multi drug resistant genes present in the hospital waste might act as a possible source of transfer of these highly resistant genes to the bacterial population.

The bacterial isolates from hospital environments were less resistant to Gentamycin (27.8% resistant) and Chloramphenicol (38.9% resistant) but resistant to other antibiotics must not have been grown. The number of multi drug resistant (MDR) bacteria was still alarmingly high for the effluent samples from hospitals. More distressing was the pattern of MDR. Simultaneous resistance for most of the antibiotics including Penicillin (77.8%), Kanamycin (77.8%), Vancomycin (61.1%) MDR pattern for hospital isolates. This pattern of antimicrobial resistance in bacteria is highly consistent with the results of the study carried out in India (Chitnis *et al.,* 2000). The pattern was almost the same for the various genera grown from the effluent samples. The MDR pattern seen in the bacterial isolates from hospital effluent samples included many of the antibiotics being currently used in the treatment of infectious diseases.

There were some limitations in the current study that can be sort out in future study, some of these limitations were (1) More sample could be collected to find out more strong evidence of MDR bacteria in the hospital waste water (2) Plasmid level characterization of MDR bacteria should have been done (3) all isolates should have been tested with more antibiotics to analyze MDR pattern more precisely.

From the results it is clear that hospital waste water is full of drug resistant pathogens those are mainly resistant against commonly used antibiotics, which suggested a selection pressure is present that induces the organisms to become resistant. Untreated hospital waste in the study area may be a possible cause to increase drug resistance in the common waste water isolates to become pathogenic bacteria.

CHAPTER 6

CONCLUSION

The study result suggests that hospital and non-hospital waste water of the study area are mainly contaminated with Enterobacteriaceae, *Pseudomonas spp, Vibrio spp* and gram-positive *Staphylococcus spp.* The high frequency of detection of pathogenic bacteria in current study may be due to the admission of cases with these bacterial infections. The isolated bacteria are fully resistant against commonly used antibiotics like Ampicillin and Penicillin. The present study demonstrated that untreated hospital waste disposal could contribute to the development of antibiotic resistance in environmental organisms. Resistance pattern varied from isolates to isolates but maximum resistance was observed in one *Pseudomonas spp.* isolates which was resistant up to 8 antibiotics out of 10 antibiotics tested and molecular characterization revealed that it was *Pseudomonas aeruginosa*.The amounts of antibiotics used in hospital and private households released into hospital and municipal waste water sewage induces a selection pressure on bacteria. So, there is a need to take proper steps to reduce the risk using so many antibiotics and their residues both in hospital and households.

From the research work, it can be concluded that there is an urgent need to raising awareness and education on medical waste issues. Proper waste management strategy is needed to ensure health and environmental safety. It is therefore, advised that all stakeholders and the health sector authorities should look after this issue seriously and takes effective ways to control the spreading of resistant gene in the environment.

❖ Future Study

- ✓ Plasmid level characterization of multi drug resistant bacterial pathogens forms hospital waste.
- ✓ Statistical analysis to find out a co-relation between multi drug resistance and commonly used antibiotics in selected hospital
- ✓ Study on Physicochemical parameters of hospital sewage sludge.
- ✓ Drug resistant pathogens in the environmental lakes beside hospital.

CHAPTER 7

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APPENDIX

APPENDIX 1

Composition of Media

1. Nutrient broth

Ingredients per litter of deionized water	**g/L**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH(at25°C)	7.4±0.2

2. Nutrient agar

Ingredients per litter of deionized water	**g/L**
Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	20.0
Final pH	7.1±0.1

3. Salmonella Shigella agar

Ingredients per litter of deionized water	5.00 gm
Peptic digest of animal tissue	
Beef extract	5.00 gm
lactose	10.00
Bile salts mixture	8.50 gm
Sodium citrate	10.00
Sodium thiosulphate	8.50 gm
Ferric citrate	1.00 gm
Brilliant green	0.00033 gm
Neutral red	0.025
Agar	15.00 gm
Distilled water	1000 ml

	Final pH(at25°C)	7.0±0.2 gm
5.	**Thiosulfate-Citrate-Bie Salts-Sucrose (TCBS) agar**	
	Ingredients per litter of deionized water	gtL
	Sucrose	20.0 gm
	Dipepton	10.0 gm
	Sodium Citrate	10.0 gm
	Sodium Thiosaulfate	10.0 gm
	Sodium Chloride	10.0 gm
	Yeast extract	5.0 gm
	Sodium cholate	3.0 gm
	Ferric citrate	1 gm
	Bromothymol blue	0.04 gm
	Thymol blue	0.04 gm
	Agar	15.gm
6.	**MacConkey Agar**	
	Ingredients	**g/L**
	peptone	17.0
	Protease peptone	3.0
	Lactose	10
	Bile salt	1.5
	Sodium cholride	5.0
	Agar	13.5
	Neutral Red	0.03
	Crystal violet	0.001
	Final pH	7.1±0.2
7.	**Eosine methylene blue agar**	
	Ingredients	**g/L**
	Peptone	100
	Lactose	10.0

	K2HP04	2.0
	Eosin	0.4
	Methylene blue	0.065
	Agar	20.0
	Final pH	6.8±0.2

8. Blood agar

Ingredients	**g/L**
Agar	15.0
Beef extract	10.0
Peptone	10.5
Sodium chloride	5.0
Final pH	7.3±0.2

9. MR VP medium (Himedium, India)

Composition	7.0
Buffered peptone	
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH(at 25°CO	6.9±0.2

11. Simmon's citrare agar

Ingredients	**g/L**
Magnessium sulphate	0.20
Ammunium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothvmol blue	0.08
Agar	15.0

12. TSI Agar slant

Ingredients	3.00 gm
Lab Lamco Powder	

Yeast extract	3.00 gm
Peptone	20.00 gm
Sodium chloride	5.00 gm
Lactose	10.00 gm
Sucrose	10.00 gm
Glucose	1.00 gm
Ferric citrate	0.3 gm
Sodium thiosulphate	0.3 gm
Phenol red	0.3 gm
Agar	12.00 gm
Distilled water	1000

APPENDIX 2

Preparation of reagents

1. Peptone water

peptone	1 gm
Distilled water	1000 ml

2. Kovacs reagent for indole preparation 5 gm

P- dimethyl aminobenzal dehyde

Amyl alcohol	75 gm
Conc. HCL	25 ml

3. V-P reagent-1

5% alpha- naphthanol in absolute ethyl alcohol

4. V-P reagent-2

40% potassium hydroxide containing 0.3% creatine. The ingredient was dissolved by heating gently over a steam bath. When in solution, added 0.052 gm of cotton blue dye.

5. Methyl red Solution

Methyl red	0.05 gm
Ethanol(absolute)	28 ml
Distilled water	22 ml

6. Phenol red solution

0.2^0o aqueous solution of phenol red

7. Gram stain solutions

a. **Stock crystal violet**

crystal violet	10 gm
Ethyl alcohol	1000m1

b. Stock oxalate

Ammonium oxalate	1 gm
Distilled water	1000 ml

Crystal violet working solution: 20 ml of solution no. I mixed with 80 ml of solution no. 2. Additional dilution was made when desired.

c. Lugol's Iodine solution

Iodine crystal	1 gm
Potassium iodide	2gm

Dissolved completely in 10 ml of distilled water, then added to distilled water to make 300 ml. stored in ambar bottle.

d. Ethyl alcohol 250 ml

e.	**Acetone**	250 ml
f.	**Counterstain**	2.5 ml
	Safranine	
	Ethyl alcohol (95%)	100 ml

Safranine working solution:

The stock safranine is usually diluted as 1:4 with distilled water.