ANTIBIOGRAM OF MERCURY RESISTANT *Pseudomonas aeruginosa* FROM SEWAGE SAMPLES OF EASTERN, NEPAL

A
Project Report Submitted to
Department of Microbiology
Central Campus of Technology, Tribhuvan University
In Partial fulfillment for the Requirement of the Award of the Degree of
Bachelor of Science in Microbiology

By
Pankaj Bhattarai
Roll No:-500080012
T. U. Reg No: 5-2-0008-0062-2014
August 2018

RECOMMENDATION

This is to certify that **Mr. Pankaj Bhattarai** has completed this project work entitled **"ANTIBIOGRAM OF MERCURY RESISTANT *Pseudomonas aeruginosa* FROM SEWAGE SAMPLES OF EASTERN NEPAL"** as a part of partial fulfillment of the requirements of Bachelor's degree in Microbiology under my supervision. To my knowledge, this work has not been submitted for any other degrees.

.....
Mr. Hemanta Khanal
Assistant Professor,
Supervisor,
Department of Microbiology,
Central Campus of Technology,
Hattisar, Dharan, Nepal

Date: 13/05/2075

CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Hemanta Khanal**, this project of **Mr. Pankaj Bhattarai** entitled **"ANTIBIOGRAM OF MERCURY RESISTANT *Pseudomonas aeruginosa* FROM SEWAGE SAMPLES OF EASTERN NEPAL"** has been approved for the examination and is submitted to the Tribhuvan University on partial fulfillment of the requirements for B.Sc. degree in Microbiology.

……..……..

Mr. Shiv Nandan Sah
Assistant Professor, Head of Department
Department of Microbiology
Central Campus of Technology
Hattisar, Dharan, Nepal

Date: 13/05/2075

BOARD OF EXAMINERS

Recommended by: **……**

Ast. Prof. Mr. Hemanta Khanal

Supervisor

Approved by: **…….**

Ast. Prof. Mr. Shiv Nandan Shah

Head of Department

Examined by:

…….

(External Examiner)

…….

(Internal Examiner)

Date……/……../……

ACKNOWLEDGEMENTS

It is a great privilege to be able to acknowledge the people without whom this project work wouldn't have been possible. Firstly; I would like to express my gratitude to my respected supervisor Mr. Hemanta Khanal for his guidance throughout my work. His supportive guidance was the base for my work.

I would also like to thank Mr.Shiv Nandan Shah, HOD of microbiology for his help and support. I would like to express my gratitude to Suman Rai, Prince Subba and Man Kumar Tamang for their support and motivations. I am also thankful to my entire teaching and non-teaching staffs of microbiology department for their constant support.

I would also like to thank: Mr. Aindra Bahadur Karki, Kamana Bantawa Rai and Saru Ghale laboratory persons of microbiology department for providing necessary materials and their help on my works.

My loving and respected parents Mr. Bhola Nath Bhattarai and Mrs. Geeta Bhattarai and entire family members for their love support and encouragement to make me stand here today. They not only assisted me financially but also extended their support morally and emotionally.

My senior Mr. Pradip Hangkhim Rai, and dear friends: Bigyan Shrestha, Rubina Katwal, Sarita Khanal, Shraddha Dulal, Bijay Rai, Saraswati Yonghang, Tara Ghimire**,** for their great support and help during the study.

Above, I have gratefully acknowledged people who have been invaluable to me in completing this project work. However, all the opinions expressed in this work, and all errors, are mine.

……..

Pankaj Bhattarai

Date: 13/05/2075

ABSTRACT

Sewage is the combination of wastewater from sources and it consists of all sorts of nutrient for microbial growth. It provides shelter for many microorganisms like *Salmonella, Shigella, Escherichia coli, Streptococcus, Pseudomonas aeruginosa, Mycobacterium, Giardia lamblia.* It may also contain heavy metals. Sometimes sewage contains mercury and its compounds which make the organisms mercury resistant. *Pseudomonas aeruginosa* present in sewage may develop mercury resistance by certain morphological changes or by genetic process. The main objective of this study is to determine the antibiogram of *P. aeruginosa.* In this study, 50 samples were collected from different areas of Sunsari and Morang by using BOD bottles and they were diluted by 10 folds and spread over the Cetrimide agar containing different concentration of mercuric chloride and minimum inhibitory concentration for mercuric chloride was determined. Antibiogram was performed against 13 different recommended antibiotics. *P. aeruginosa* isolates were found resistant to amoxicillin, ampicillin, clindamycin, nalixidic acid and vancomycin and also complete intermediate resistance was shown towards erythromycin. *P aeruginosa* showed complete susceptibility towards tetracyline. Whereas other *P. aeruginosa* isolates were more or less intermediately resistant to rest of the antibiotics.

Keywords: Sewage, mercury resistance, minimum inhibitory concentration, antibiogram

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF ABBREVIATIONS

1. AST- Antibiotic Susceptibility Test
2. MHA- Mueller Hinton Agar
3. MIC- Minimum Inhibitory Concentration
4. QAC- Quaternary Ammonium Compounds

CHAPTER- I

INTRODUCTION

1.1. Background

Environment pollution is the mixture of unwanted substances to the environment and its contents resulting in the deviations from normal conditions causing the effects in health of consumers as well the living beings associated to it . Such unwanted substances may include dirt, dust, smoke, wastewater, dead and debris, ashes excreta etc(Alloway and Ayres 1997). During the condition of pollution environment doesn't know how to react over the substances added to it. For the development of pollution the unwanted substances are added over the environment in unnatural way. Environmental pollution is prevalent in the world since human civilization but its effects are seen well after 19^{th} century after the industrial revolution. Pollution has several negative impacts over the flora and fauna if it's not controlled on time it can also alter the ecosystem. Global warming and ozone layer depletion are some of the major impacts of pollution. The main reason for resulting the pollutions are development of industries, transportation, agricultural activities, and residence and so on. Water, air and soil pollution are the major types of environment pollution(Douglas and Wildavsky 1983).

Water constitutes about 70% of earth's surface. Water is known as the polar solvent and important in domestic as well as industrial activities. As water is an easy solvent any substances can be added easily and contaminate it. Addition of unwanted wastes to the water sources id called water pollution. Water pollution has severe effect on the health of consumers. It's a great problem of today's era and it can lead to the destruction of the world. Some of the causes of water pollution areuse of fertilizers and pesticides, global warming, animal wastes, fuels, urban development, industrial wastes, sewage and so on.. Due to rapid development of technologies and productions of various advanced equipments, industrial wastes and sewage are considered to be the major sources of water pollution(Dugan 2012).

Sewage is the type of wastewater produced from a community which consists of waste water from kitchen, human feces, sanitary wastes, industrial wastewater etc.

Sewage is characterized by its chemical constituents, physical condition and microbial status (organisms and their quantity present on sewage). As sewage contains human feces, it is likely to carry pathogens like bacteria, virus, parasites and protozoa. All categories of sewages are likely to contain and carry pathogens so sewage can act as the medium for carrying the diseases. Mostly prevalent microorganisms in sewage are *Salmonella, Shigella, Escherichia coli, Streptococcus, Pseudomonas aeruginosa, Mycobacterium, Giardia lamblia* and so on (WHO 2006).

Pseudomonas aeruginosa is a Gram-negative, rod shaped bacterium which can be found in man-made as well as inanimate environment. It is citrate, catalase and oxidase positive. It is found in soil, water, normal skin flora, and most man-made environments throughout the world. *P. aeruginosa* is known as a common opportunistic human pathogen as it attacks only the immune-compressed individuals. Some of the species of *P. aeruginosa* are of considerable medical importance as they show multidrug resistance property. So these species are known for their ubiquity, their instrinsically advanced antibiotic resitance mechanisms and their association with serious illness- hospital acquired infections such as ventilator associated pneumonia and various sepsis syndromes (Lahore 2017).

For every microorganisms to grow in suitable conditions they need nutritional factors but sometimes due to adverse environmental conditions such as antimicrobial environment they must show resistant mechanism to grow. This resistant property in a bacterium can be natural as well they can achieve by mutation or by transfer of genetic material from another bacterium. Bacterium contains plasmids (extrachromosomal DNA) for showing resistant mechanism. This property or mechanism of bacterium by which it can resist any agents like antibiotics, heavy metal etc inhibiting the growth is called resistant property (Davies and Davies 2010).

Many microorganisms resist heavy metals like mercury, lead, copper etc to show their growth and survive in adverse conditions too. Mercury is a toxic metal for most living beings which is accumulated in soil, water, etc by daily human activities, urbanization, increasing number of industries, developing technology etc. Therefore mercury pollution has been a global issue and public health problem of concern. Microbes present in environment have developed some resistance mechanism including mercury detoxification process. So these mercury resistant bacteria play

important role not in pollution removal issue but as well in the geochemical cycle of mercury in the environment. Among the microbial mercury resistance mechanisms, the most studied is the enzymatic reduction of ionic mercury to the metallic mercury. This resistance mechanism is encoded by "mer operon" which consists of functional genes responsible for regulation (merR), transport (merC, merT, and merP), decomposition (merB) and reduction (merA) of mercury compounds (Nagajyoti, Lee et al. 2010).

The introduction of various antimicrobials for treating variety of infections showed the necessity of performing antimicrobial susceptibility testing as a routine procedure in all microbiology laboratories. In laboratories it can be made available by using antibiotic disk which will diffuse slowly into the medium where the suspected organism is grown. The basic principle of the antibiotic susceptibility testing has been used in microbiology laboratories over 80 years. Various chemical agents such as antiseptics, disinfectants, and antibiotics are employed to combat with the microbial growth. Among these, antibiotics are generally defined as the substances produced by the microorganism such as *Penicillium*, which has the ability to kill or inhibit the growth of other microorganisms, mainly bacteria (Laxminarayan, Duse et al.2013). Antimicrobial susceptibility tests (ASTs) basically measures the ability of an antibiotic or other antimicrobial agent to inhibit the invitro microbial growth.Generally ASTs are performed in th laboratory by using Mueller Hinton Agar media.It has batch-to-batch reproducibility, low concentration of inhibitors of sulphonamide, trimethoprim and tetracyclines and produce satisfactory results for most of the non-fastidious pathogens. Fastidious organisms which require specific growth supplements need different media to grow for studying the susceptibility patterns (Mahendran, Saravanan et al.).

The Kirby Bauer test is a qualitative assay whereby discs impregnated with a single concentration of different antibiotics or any chemicals that will diffuse from the disk into the agar are used. The selected antibiotic disks are placed on the surface of an agar plate inoculated with test bacteria. During the incubation period, the antibiotics/chemicals diffuse outward from the disks into the agar. This will create a concentration gradient in the agar which depends on the solubility of the chemical and its molecular size. The absence of growth of the organism around the antibiotic disks

indicates that, the respected organism is susceptible to that antibiotic and the presence of growth around the antibiotic disk indicates the organism is resistant to that particular antibiotic. This area of no growth around the disk is known as a zone of inhibition, which is uniformly circular with a confluent lawn of growth in the media.The diameters of the zone of inhibition are measured (including disk) using a metric scale or a sliding caliper. The measured zone diameter can be compared with a standard chart for obtaining the susceptible and resistant values. There are zone of intermediate resistance which means that the antibiotic may not be sufficient enough to eradicate the organism from the body (Piddock 1990).

Minimum Inhibitory Concentrations (MICs) nowadays are being used as the best standard method for determining the susceptibility of organisms to any antimicrobial agents and to monitor the susceptibility tests done by using other methods. MICs are defined as the concentrations of antimicrobial agents that will inhibit the visible growth on overnight incubation and Minimum Bactericidal Concentrations (MBCs) are defined as the lowest concentration of an antimicrobial agent that will prevent the growth of organisms after the subculture on antibiotic free media. MICs are used in laboratory to determine the resistance but not the invitro resistance of new antimicrobial agents (Cos, Vlietinck et al. 2006).

1.2. Statement of Problem

As cited earlier, sewage provides a better nutritional and favourable environment for the growth of *P. aeruginosa.* For the isolation of *P. aeruginosa* from sewage Cetrimide agar can be used as selective medium which inhibits the growth of other bacteria supporting the growth of *P. aeruginosa.* Serial dilutions can be made on sample to reduce the microbial load and those aliquots can be spread over the culture media with mercuric chloride to obtain the microbial growth as well as minimal inhibitory concentrations of mercuric chloride. To preserve the isolated colonies of *P. aeruginosa* for long duration and future use , nutrient broth with glycerol can be used. Various antibiotics can be used to perform ASTs.

1.3. Rationale of Study

P. aeruginosa is normally found on animate and inanimate conditions. It mostly attacks the individuals who have lower immunity i.e. immune-compressed individuals. Due to the adverse environmental conditions and genetic modifications, microorganisms show structural deviation from normal which makes them resistant to some metals as well as antimicrobial agents. When the microorganisms show resistance against antimicrobial agents, they are highly probable in causing infections and diseases. Especially in the case of heavy metals and antibiotic resistant organisms they can grow against some metals and antibiotics which have no further use in their treatment. This might be threatening and critical issue. So by performing the mercury resistant and antibiotic susceptibility tests, treatment of these sorts of bacterial infections and diseases can be made convenient and specific and the treatment process can be more fruitful than before.

1.4. Objectives of the Study

1.4.1. General Objective:

- To carry out antibiogram profiling of mercury resistant *P. aeruginosa* isolated from sewage samples.

1.4.2. Specific Objective:

- To isolate and identify *P. aeruginosa* from sewage samples.
- To screen the mercury resistant *P. aeruginosa*.
- To perform the antibiotic susceptibility test of mercury resistant *P. aeruginosa* isolated from the samples.

1.5. Limitations of study

- Due to time limitations, samples from many areas cannot be covered.
- Only the mercury resistant organisms were screened but not the heavy metal resistant.
- The study of resistant mechanisms was not carried out.
- Only the limited antibiotics were used for antibiogram profiling.

CHAPTER II

LITERATURE REVIEW

2.1. Sewage

For our day to day understanding, Sewage can be defined as dirty or waste water produced after day to day activities of the community. The term sewage is used to indicate the liquid waste from community and it includes the following-Sullage, discharge from latrines, urinals, industrial waste and storm water. Sewage is a complex mixture of chemicals, with many distinctive chemical characteristics. These include high concentrations of ammonium, nitrate, nitrogen, phosphorous, high conductivity, high alkalinity, with pH typically ranging between 7 and 8. Sewage often contains pathogens like bacteria, viruses, parasites, and protozoans. Sewage water is classified into following types namely (Punima, Jain et al 1998)

- **Domestic sewage:** includes sullage and animal discharges.
- **Industrial wastewater:** includes commercial and industrial wastes.
- **Surface run-off:** includes suspended matter from lands and debris from streets(Punmia, Jain et al. 1998).

Sewage water contains the huge diversity of microorganisms: viruses, bacteria, protozoa, parasites and helminths eggs. The presence and concentration of pathogens is mainly determined by two factors, namely, the prevalence of pathogens among the population connected to sewage network and ability of these organisms to survive the sewage and sewage treatment processes. Sewage may become contaminated by approximately 300 species of bacteria and the number in 1 g of a dry weight reaches them from 109 into 1012 cells. The survival time of bacteria in the environment is few months to a several months (Gerardi and Zimmerman 2004). Several pathogenic microorganisms and parasites are commonly found in domestic wastewater, sewage sludge as well as in effluents from wastewater treatment plants. Sewage water bacteria belong to the following groups:

- **Gram-negative facultative anaerobic bacteria:**eg, Aeromonas, Plesiomonas, Vibrio, Enterobacter, Escherichia, Klebsiella, Salmonella and Shigella,
- **Gram-negative aerobic bacteria:**eg,Pseudomonas,Alcaligenes, Flavobacterium, Acinetobacter,
- **Gram-positive spore forming bacteria:**eg, Bacillus spp.,
- **Nonspore-forming Gram-positive bacteria:**eg, Arthrobacter, Corynebacterium, Rhodococcus (Gerba 2009).

2.2. *Pseudomonas aeruginosa*:

Pseudomonas aeruginosa was first recognised in the study "On the blue and green coloration of bandages" in 1882, conducted by Carle Gessard a French pharmacist. In his study he discovered that *P. aeruginosa* was a water-soluble pigment, which under exposure to ultraviolet light, illuminated green-blue. Carle Gessard, back in 1882, concluded that *P. aeruginosa* was of a *pathogenic, infectious* nature, after classifying the strand; due to the similarity between the strand and other similar microbes. Since the discovery of this opportunistic pathogen, breakthroughs have been made, sighting the severity of its power to fester rapidly and oppose treatment. This pathogen is constantly monitored, and its *genome* is continually updated into data bases, due to the potential for it to be used as a biological weapon (Shanmugam 2010).

2.3. Diversity and ecology of *P. aeruginosa* in sewage water:

P. aeruginosa is the major member of a widely distributed genus of bacteria and, under special conditions, may be pathogenic to man. This organism which differs in certain properties from other Pseudomonads is a frequent cause of a wide variety of hospital acquired infections. It is obtained found to be causative agent of fatal septicemia in patients with severe burns or in those debilitated by malignant diseases, by immunosuppressive drugs or by old age. It has been recognized as a major cause of genitourinary tract infections and as one of the most persistent "opportuninstics" invaders (Palleroni and Doudoroff 1972).

P. aeruginosa is ubiquitous in nature and is found in many microenvironments where other bacteria aren't commonly found. It is found in man and animals. In man it is normally found in the lower intestinal tract and studies have shown that 3%-35% of humans tested contain sizeable populations of these organisms in their fecal matter (Donaldson, Lee et al. 2016).

Water is the natural reservoir for *P. aeruginosa*. The presence of *P. aeruginosa* acts as indicator of contamination from surface runoff, domestic and agricultural effluents, or human fecal matter. The major source of *P. aeruginosa* may be domestic sewage and is found in 90% sewage samples. Its concentration in surface waters receiving waste and storm water discharges ranges from 1 to 10,000 cells/100ml. However the intestinal carriage rate for *P. aeruginosa* in human is low suggesting that its presence in water doesn't necessarily result from sewage contamination. Other sources of the organism are thought to be water leaching from agricultural soils, barn yard drainage, and urban runoff (Arnone and Perdek Walling 2007).

2.4. Mercury resistant *P. aeruginosa*:

Frequent discharge of metallic ions containing toxic wastes from industries and urban sectors contain high level of arsenic, mercury, nickel, cadmium, lead and sulphur based byproducts. These wastes appear with vast distribution in urbanized region in various forms and are one of the major causes of metallic pollutions. Accumulation of these heavy metals affects the microbial ecology and growth pattern of microorganisms in aquatic sources. Metallic toxicity and its related antibacterial effects are due to oligodynamic property of metals, which presents obstacle to microbial growth. Despite these antagonistic factors, microbial adaptation system has evolved multiple mechanisms such as volatilization, extracellular precipitation, intake exclusion and extracellular sequestration to counteract adverse effect of these toxic compound (Farstner and Wittman 2012).

Generally, mercury tolerant *P. aeruginosa* are capable of detoxifying the mercuric compounds by two sequentially acting enzymes namely, organomercuriallyase, which cleaves the carbon mercury bond of typical organomercurials and mercuric reductase, which reducing mercury (II) into volatile and non lethal mercury (0). The counteract mechanism can be broad spectrum or specific for typical organometallic compound. Majority of such resistant strains are found to be actively expressing *mer* operon for

mercury detoxification. This not only enables *P. aeruginosa* to survive through toxic environment but also assist in removal of such compounds via detoxification or biosorption (Schaefer 2005).

Pollution of water sources as a result of various metal salt contaminations shown to be related to development of strains capable of tolerating such metal ions. Among these contaminants mercury is one of the leading chemicals in water pollution in industrialized zones. In most cases, the evolutionary pattern of mutagenesis and slow genetic adaptations leads to production of resistant strains. However, unusually high level of tolerance to heavy metal ions suggests a plasmid mediated tolerance strains *P. aeruginosa*. *P. aeruginosa* are presumably more competent to foreign genome for their flexible genetic adaptation. *P. aeruginosa* have been known to carry plasmids and transposable elements for this mode of tolerance (Salt, Blaylock et al.1995).

2.5. Minimum Inhibitory Concentration:

Minimum inhibitory concentration is defined as the lowest concentration of an anti-microbial that will inhibit the visible growth of a organism after overnight incubation .In MIC tests the micro-organisms are subjected to a range of antibiotic concentrations, conventionally two-fold, in solid or liquid medium, in a defined atmosphere, at a defined temperature and for a defined period of time. The macroscopic inhibition of growth is measured as the absence or near absence of growth on a solid medium or as the absence of turbidity in a liquid medium. Thus, the MIC is defined as the lowest concentration which clearly inhibits the growth of the micro-organisms (Lovell Antiaye 2014). An MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. Because a lower MIC value indicates that less of the drug is required in order to inhibit growth of the organism, drugs with lower MIC scores are more effective antimicrobial agents. MIC scores are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. Clinicians use MIC scores to choose which antibiotics to administer to patients with specific infections and to identify an effective dose of antibiotic. This is important because populations of bacteria exposed to an insufficient concentration of a particular drug or to a broad-spectrum antibiotic

can evolve resistance to these drugs. Therefore, MIC scores aid in improving outcomes for patients and preventing evolution of drug-resistant microbial strains (Cos, Vlietinck et al. 2006).

2.6. Antibiotic susceptibility test:

Clinicians use antibiograms to assess local susceptibility rates, as an aid in selecting empiric antibiotic therapy, and in monitoring resistance trends over time within an institution. An antibiogram shows the aggregate number of bacteria tested against antimicrobials and incorporates the extent of bacterial isolates vulnerable to every antimicrobial operator tested. They lend information that can be used to raise awareness about resistance problems, support the use of optimal empiric treatment, and identify opportunities to reduce inappropriate antibiotic usage and to discover success of such efforts (Pakyz 2007).

The most common methods utilized to measure the *in vitro* vulnerability of microorganisms to antimicrobial operators include the disk diffusion method, agar dilution, broth micro-dilution, and testing by antimicrobial gradient agar strips (E-test method)(Nguyen 2018).

The performance of antimicrobial susceptibility testing by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antimicrobial agents, or to detect resistance in individual bacterial isolates.

2.6.1 Commonly used susceptibility methods

There are various methods applicable for antibiotic susceptibility tests and some of them are as follows:

- ***Broth dilution tests:*** One of the earliest antimicrobial susceptibility testing methods was the macrobroth or tube-dilution method. This procedure involved preparing two-fold dilutions of antibiotics in a liquid growth medium dispensed in test tubes. The antibiotic-containing tubes were inoculated with a standardized bacterial suspension of $1–5\times10^5$CFU/ml. Following overnight incubation at 35°C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevented

growth represented the minimal inhibitory concentration (MIC) (Jorgensen and Ferraro 1998).

- ***Antimicrobial gradient method:*** The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. It employs thin plastic test strips that are impregnated on the underside with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. As many as 5 or 6 strips may be placed in a radial fashion on the surface of an appropriate 150-mm agar plate that has been inoculated with a standardized organism suspension like that used for a disk diffusion test. After overnight incubation, the tests are read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip.
- ***Disc diffusion test:*** The disc diffusion susceptibility method is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum of approximately $1–2\times10^{8}$CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) or those included in the US Food and Drug Administration (FDA)-approved product inserts for the disks.
- ***Automated instrument systems:*** Use of instrumentation can standardize the reading of end points and often produce susceptibility test results in a shorter period than manual readings because sensitive optical detection systems allow detection of subtle changes in bacterial growth (Cooper 1963).

CHAPTER III

MATERIALS AND METHODS

3.1. Site of the study

The study was carried out in the laboratory of Department of Microbiology, Central Campus of Technology, Dharan, Sunsari.

3.2. Research method:

The method for this study was qualitative as well as quantitative. This study was based on the culture method.

3.3. Type of study:

The study was of descriptive type.

3.4. Population and Sample:

3.4.1 Sample and sample size

In this study, water (sewage) was used as sample. The total numbers of sewage samples taken for study were 50.

3.4.2 Description of the research site:

For this study the sample was collected aseptically from different places: Sunsari and Morang.

Research Design:

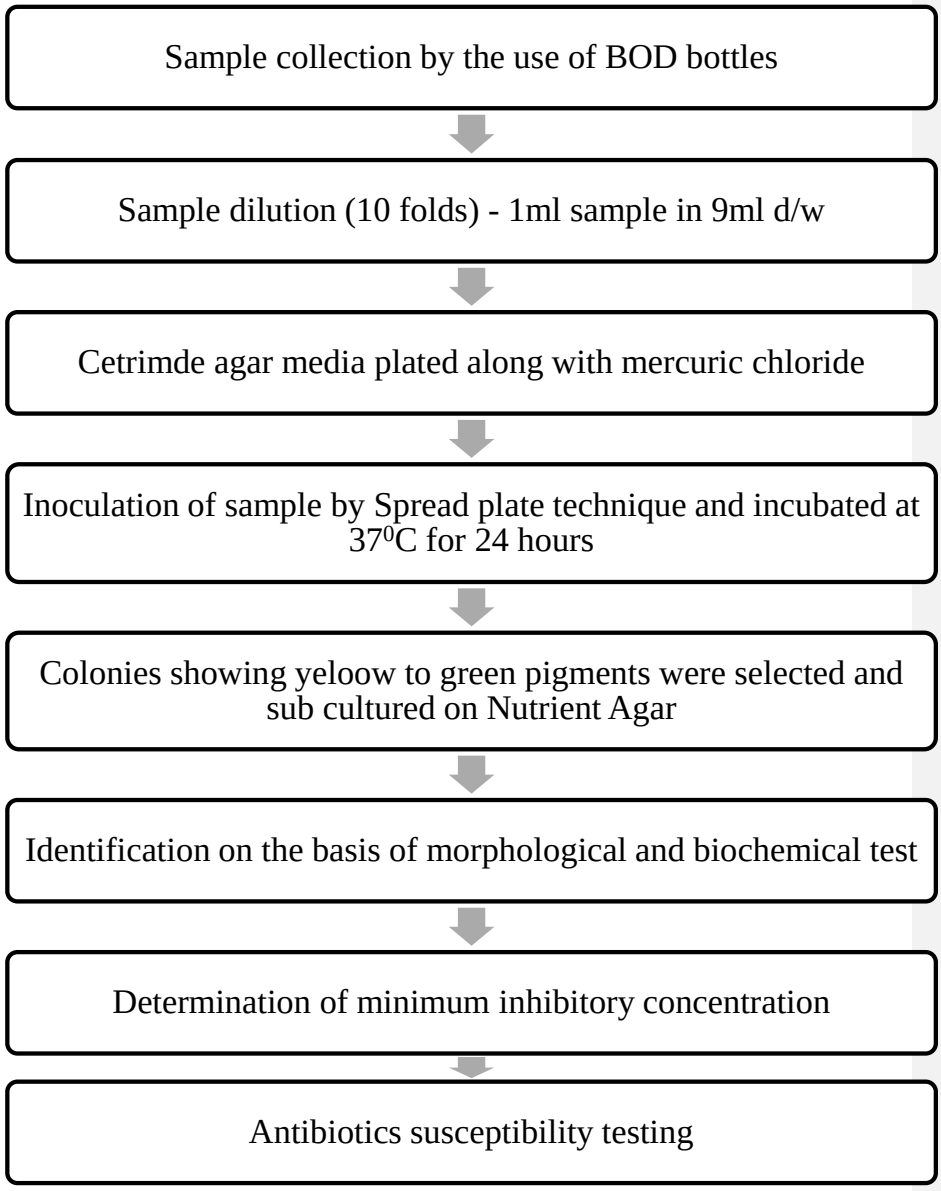


Figure 1: Flow chart for research design

3.4.3 Sample Collection

Sewage samples were aseptically collected into 100ml BOD bottles from different sites of Sunsari and Morang districts. Sewage effluents accumulated in dams were chosen as sample site where the sewage effluents seem to be very stagnant and polluted. During the collection of samples, gloves were used along with face mask for self protection where the bottles were dipped in sewage for collection of samples.

3.4.4 Transportation of sample

The samples were then transported from the site of collection to the laboratory asceptically. The samples were stored in an ice box so as to provide proper conditions for the microorganism.

3.5. Isolation of Mercury resistant bacteria:

The samples were diluted by using distilled water for the isolation of given organism. 10µg/ml to 70µg/ml of mercuric chloride were mixed along with prepared cetrimide agar and plated on sterile petri-plates. Samples were then spread on media and incubated for proper growth of organism. Various biochemical tests were done for the identification of organism. Antibiotic susceptibility test was carried by Kirby Bauer disc diffusion method.

3.5.1 Sample dilution

The collected samples were diluted 10 folds i.e. 1ml of sample in 9ml of sterile distilled water, to acquire more usable concentration of organisms.

3.5.2 Media preparation

For the isolation of mercury resistant *P. aeruginosa*, cetrimide agar media was used. Cetrimide agar was prepared, mercuric chloride was added to it so as to make the concentrations as 10µg/ml to 70µg/ml and autoclaved at 121^0C for 15 minutes. The agar medium along with mercuric chloride was then plated on sterile petri-plates.

3.5.3 Culture

For every sample, 1ml of sample was taken with the help of micro pipette and placed on the cetrimide agar media along with mercuric chloride. The sample was then spread on the media with the help of a sterile dolly rod. The sample was spread well along all the surface of the media. The rod used to spread the sample was allowed to dip in alcohol and flamed for sterile working protocol. The plates were then incubated

at 37^0C for 24 hours. After 24 hours of incubation, colonies giving yellow to green pigments were sub cultured on nutrient agar and incubated at 37^0C for 24 hours.

3.5.4 Storage of isolated organisms

Nutrient broth medium was freshly prepared and 10% glycerol was added on it so as to preserve isolated organisms for a longer duration and future use.

3.5.5 Identification of organism

The organisms were identified by following various biochemical tests and Gram staining was done on organisms for its gram positive or negative test. Likewise various biochemical tests were done, (Catalase test, Oxidase test, IMViC test, and Urease test) for finalizing the identification process.

3.6. Determination of Minimum Inhibitory Concentration:

Minimum inhibitory concentration was determined for the organism by culture of organism in MHA media along with various concentration of mercuric chloride. Table no. 1 depicts the amount of mercuric chloride used along with media for maintaining various concentration of mercuric chloride. The organisms were inoculated on the media with mercuric chloride and incubated at 37^0C for 24 hours. The data obtained was interpreted to determine the minimum inhibitory concentration for mercury resistant *P. aeruginosa.*

Table 1: Maintaining concentration for MIC.

S.N.	Concentration	Mercuric chloride(µg)	MHA (gm)	Water (ml)
1	10 µg	500	1.85	50
2	20 µg	1000	1.85	50
3	30 µg	1500	1.85	50
4	40 µg	2000	1.85	50
5	50 µg	2500	1.85	50
6	60 µg	3000	1.85	50
7	70 µg	3500	1.85	50

3.7. Antibiotic Susceptibility test

Antibiotic susceptibility test was done by Kirby Bauer disc diffusion method. For this procedure, Mueller-Hinton agar was prepared on which the bacterial sample was inoculated. Paper antibiotics discs were then placed on the inoculated agar surface. Plates were then incubated at 37^0C for 24 hours and observations were made.

3.7.1 Media preparation

For antibiotic susceptibility testing by Kirby Bauer disc diffusion method, Mueller Hinton Agar was prepared and autoclaved at 121^0C for 15 minutes. The prepared medium was then plated on sterile petri-plates. Nutrient Broth was prepared and autoclaved at 121^0C for 15 minutes.

3.7.2 Standard McFarland preparation

For the preparation of 0.5 McFarland, 9.95ml of 1% sulfuric acid (H_2SO_4) was prepared along with 0.05ml of 1% barium chloride ($BaCl_2$). Both the solutions were completely mixed to form a turbid suspension and the resulting solution was stored at room temperature. The proportion on which the solutions were mixed was on the basis of McFarland turbidity standard.

3.7.3 Inoculum Preparation

Pure culture was taken with the help of inoculating loop and transferred to 5ml Nutrient Broth. The culture was incubated at 37^0C for 2-8 hours until light to moderate turbidity was observed. The inoculum turbidity was compared with that of standard 0.5 McFarland. Comparative turbidity was maintained by diluting the inoculum to obtain confluent growth on petri-plate.

3.7.4 Test

A sterile non-toxic cotton swab was dipped into the standardized inoculums and the excess fluid was allowed to drip within it by soaking the swab firmly against the upper side of the wall. Entire surface of agar plate was streaked with the swab three times, turning the plate at 60^0 angle between each streaking. The inoculum was then allowed to dry for 5-15 minutes with its lid in place. Antibiotic discs were then applied on the medium using aseptic techniques. The discs were deposited with the aid of flamed forceps to the inoculated medium at least 24mm apart and 15mm away from the wall of plates. The plates were incubated at 37^0C for 24 hours and

observations were made. After 24 hours of incubation, MHA plates were examined and the diameters of zone of complete inhibition for individual antimicrobial agents were then translated into susceptible, intermediate and resistant categories according to the interpretation table of CLSI "Disc Diffusion Supplemental Table". A wide range of antibiotics namely, Amikacin (AK) (30 µg), Amoxicillin (AMC) (10µg), Ampicillin (AMP) (10 µg), Cefotaxime (CTX) (30µg), Clindamycin (CD) (2µg), Co-Trimoxazole (COT) (25µg), Erythromycin (E) (15µg), Kanamycin (K) (30 µg), Nalidixic acid (NA) (30 µg), Nitrofuratoin (NIT) (300 µg), Norfloxacin (NX) (10 µg), Tetracycline (TE) (30 µg) and Vancomycin (VA) (30µg) were used for AST.

CHAPTER IV

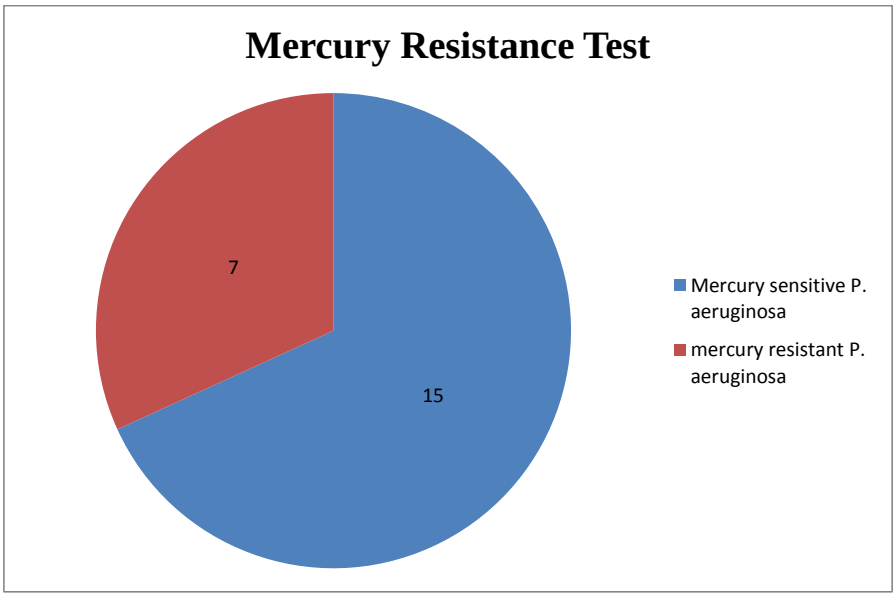
RESULTS

This study was conducted at the Central Campus of Technology, Hattisar, Dharan. Fifty sewage samples were collected from different areas of Sunsari and Morang district.

4.1. Bacterial Isolation:

In this study, 50 sewage samples were collected and studied for the presence of *P.aeruginosa*. All the samples were subjected for the preliminary mercury resistant test by culturing them in media with the concentration of 10µg mercury per ml, clear growth was shown by 22 samples, which were further subjected to determine MIC.

P aeruginosa was confirmed in seven different samples with the minimum inhibitory concentrations ranging from 20µg/ml to 60µg/ml of mercuric chloride. And no growth was seen in the concentration of 70µg/ml.



The isolated organisms were identified on the basis of morphological and biochemical characteristics. The colonial and biochemical characteristics are given in table no. 2 and table no. 3 respectively.

Table 2: Colonial characteristics of*P.aeruginosa*

Organism	Configuration	Margin	Elevation	Color
P.aeruginosa	Rod	Undulate	umbonate	Yellowish to grapish green

Table 3: Biochemical reactions of *P. aeruginosa*

S.N.	**Tests**	**Result**
1	Catalase	Positive
2	Oxidase	Positive
3	Indole	Negative
4	MR (Methyl red)	Negative
5	VP (Voges-Proskauer)	Negative
6	Citrate utilization test	Positive
7	Urease	Negative
8	Gram staining	Negative (Rod)

4.2. Minimum Inhibitory Concentration(MIC):

After incubation for 24 hours at 37^0C temperature, the growth of *P. aeruginosa* on MHA media was shown on 10µg/ml by 22 samples. On increasing the concentration from 20µg/ml to 70µg/ml, seven samples showed their growth on MHA ranging from 20µg/ml to 60µg/ml, whereas no any growth were shown by the samples on the concentration of 70µg/ml.

4.3. Antibiotic Susceptibility Pattern:

P. aeruginosa was subjected to Antibiotic Susceptibility test. AST was performed by using Kirby Bauer Disc Diffusion method. The antibiotics used were Amikacin (AK) (30 µg), Amoxicillin (AMC) (10µg), Ampicillin (AMP) (10 µg), Cefotaxime (CTX) (30µg), Clindamycin (CD) (2µg), Co-Trimoxazole (COT) (25µg), Erythromycin (E)

(15µg), Kanamycin (K) (30 µg), Nalidixic acid (NA) (30 µg), Nitrofuratoin (NIT) (300 µg), Norfloxacin (NX) (10 µg), Tetracycline (TE) (30 µg) and Vancomycin (VA) (30µg).

All *P. aeruginosa* isolates were completely resistant to amoxicillin, ampicillin, clindamycin, nalidixic acid and vancomycin and also complete intermediate resistance was shown towards erythromycin.*P aeruginosa* showed complete susceptibility towards tetracyline. Whereas other *P. aeruginosa* isolates shown more or less intermediate resistant to rest of the antibiotics.

Table 4: Antibiotic susceptibility pattern of *P. aeruginosa*

SN	Strain	AK	AMC	AMP	CTX	CD	COT	E	K	NA	NIT	NX	TE	VA
1	S_1	S	R	R	S	R	S	I	I	R	I	S	S	R
2	S_2	S	R	R	R	R	S	I	S	R	S	S	S	R
3	S_3	I	R	R	R	R	I	I	S	R	S	S	S	R
4	S_4	S	R	R	S	R	S	I	S	R	I	S	S	R
5	S_5	S	R	R	I	R	I	I	R	R	S	I	S	R
6	S_6	S	R	R	S	R	S	I	S	R	S	I	S	R
7	S_7	S	R	R	S	R	I	I	S	R	S	S	S	R

From the data produced from table no. 4, antibiogram profiling was done for the *P. aeruginosa* isolates. The data in table no. 4 gives information about the results of AST conducted on 13 different antibiotics for *P. aeruginosa* isolates on which the results were expressed as R for the resistant organisms, I for the intermediate sensitive organisms and S for the susceptible organisms according to the interpretation table of CLSI “Disc Diffusion Supplemental Table”. Antibiogram was assessed by calculating the percentage of susceptible, resistant and intermediate sensitive organisms on

various antibiotics. Percentages of susceptible, resistant and intermediate organisms were calculated by using the formula:

$$(\mathrm{S})\% = \frac{\text{Total number of susceptible organisms}}{\text{Total number of organisms}} \times 100\%$$

$$(\mathrm{I})\% = \frac{\text{Total number of intermediate sensitive organisms}}{\text{Total number of organisms}} \times 100$$

$$(\mathrm{R})\% = \frac{\text{Total number of resistant organisms}}{\text{Total number of organisms}} \times 100\%$$

Table 5: Antibiogram of *P. aeruginosa*

Antibiotics	***P.aeruginosa*(n=7)**		
	(S) %	**(I) %**	**(R) %**
AK	85.7	14.3	0
AMC			100
AMP			100
CTX	57.14	14.3	28.6
CD			100
COT	57.14	42.86	0
E		100	
K	71.42	14.3	14.3
NA			100
NIT	71.42	28.6	
NX	71.42	28.6	
TE	100		
VA			100

CHAPTER V

DISCUSSIONS

In this study, antibiogram was assessed for the mercury resistant *P. aeruginosa* isolated from sewage water collected from Sunsari and Morang district. (Sewage provides the most nutritive and convenient adaptation for many bacteria, viruses, helminthes and parasites. Among which the most predominant bacteria found in the sewage can be *P. aeruginosa*, which could be added on sewage by domestic sewage as well hospital discharges. Due to the extreme conditions on environment, *P. aeruginosa* showed the genetic as well as morphological changes so that it could be adapted in the changed environment.)

Mercury and mercurial compounds may be added to the sewage by the discharges from industries and factories. Mercury resistant property must be shown by *P. aeruginosa* to adapt in the sewage nearby factories.The survival of bacteria in the presence of mercury by eliminating the potency of mercury and its compounds and undergoing mutation to provide resistance against mercury and its compounds are the results of spread and emergence of mercury resistant bacteria. Frequent exposure of microorganisms to the mercury and its compounds make them mercury resistant. These resistant bacteria eventually find a way into the environment via manure, wastewater and sewage sludge.

In this study, mercuric chloride was used to test the resistance of *P. aeruginosa* against mercury. The sewage samples were collected in sterile B.O.D bottles, labeled and kept in an ice container to preserve the bacteria from getting any changes in it. The samples were safely brought to laboratory for analysis. The selective and differential media cetrimide agar was used for supporting the isolation and differentiation of *P. aeruginosa.* Cetrimide, a QAC is the selective agent inhibits most bacteria except *P. aeruginosa*. The growth of organisms with yellowish to grapish green colonies followed by paper strip oxidase test confirmed *P. aeruginosa.*

Comment [u1]: Look at the font size

Comment [u2]: Discussion delete it

Comment [u3]: PLEASE LOOK AT IT

The sewage sample was diluted 10 folds (1ml sewage and 9ml sterile distilled water) for reducing the microbial loads and spread over cetrimide agar media containing MIC of mercuric chloride to acquire a minimal inhibitory concentration required for the inhibiting the growth of *P. aeruginosa*. After incubation, *P. aeruginosa* had shown yellowish to grapish green colonies on cetrimide agar media with mercuric chloride was taken as mercury resistant *Pseudomonas* spp further confirmation was done by conducting various biochemical tests.

Gram staining of isolated bacteria was done for examining whether it was gram-negative or positive and further biochemical tests like, catalase test, oxidase test, urease test, and IMViC test which includes indole test, methyl red test, Voges-Proskauer test and citrate testwere then conducted. The positive results given by the biochemical test confirmed that the isolated organism was *P. aeruginosa.* Antibiotic susceptibility test was conducted using thirteen different antibiotics. The test organism was first grown on MHA media for conducting AST. The reason for using MHA to conduct AST is because, MHA is a non-selective, non-differential medium i.e. almost all organisms plated on here will grow, MHA contains starch which is known to absorb toxins released from bacteria, so that they cannot interfere with the antibiotics, and MHA being a loose agar, it allows for better diffusion of antibiotics leading to a true zone of inhibition. Before antibiotic susceptibility testing was done, the turbidity of bacterial suspension was compared to standard McFarland used as reference. The turbidity was maintained so that the AST results could not be affected by the concentration of bacteria inoculated on the media.

After incubation for 24 hours, a proper zone of inhibition was produced by antibiotics on the MHA media and the diameter of the zone of inhibition was measured in mm and the data were translated into S, I and R categories. *P. aeruginosa* isolates were observed to be 100% susceptible to tetracycline, followed by 85.7% susceptible to Amikacin and 71.42% to kanamycin, nitrofurantoin and norfloxacin,and 57.14% to cefotaxime and cotrimoxazole respectively. Also *P. aeruginosa* was completely intermediately resistant towards Erythromycin (100%), followed by 42.85% intermediate sensitivity on Cotri-moxazole, 28.6% to Nitrofuratoin and Norfloxacin and 14.3% to Amikacin, Cefotaxime, and Kanamycin respectively.

In our study, resistance was observed highest (100%) to Amoxicillin, Ampicillin, Clindamycin, Nalixidic acid and Vancomycin.

By the knowledge on resistant pattern and properties, proper and effective treatment can be made and application of such organisms can be done in medical and research areas.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1. Conclusion

Mercury resistant *P.aeruginosa* were isolated from fifty sewage samples collected from Sunsari and Morang district. *P. aeruginosa* was identified by gram staining and various other biochemical tests. The test organisms were then subjected for their antibiotic sensitivity against various antibiotics and the results were interpreted.Mercury resistant property might be due to plasmid DNA or by the antimercurial genes contained by microoganisms. Strict quality control measures should be implemented to ensure proper treatment of water and wastewater in these and other treatment plants. This would ensure the discharge of properly treated wastewater into water bodies to prevent the occurrence and spread of water- and food-borne diseases as well as fatal infections.

6.2. Recommendation

- Further research can be carried out to determine the role of resistant genes in providing the organisms to resist mercury.
- Due to limitation of time, only few samples were taken in this study. For more conclusive result, the number of samples can be increased.
- Study of mercury resistant bacteria could be done on molecular level.
- A further study could be done to evaluate the mercury resistant and its impact.

REFERENCES

Alloway B and Ayres DC (1997). Chemical Principles of environment pollution, CRC Press.

Arnone RD and Perdek Walling J (2007). "Waterborne pathogens in urban watersheds." Journal of Water and Health. (**5**)1: 149-162.

Cooper KE (1963). The Theory of antibiotic inhibition zones. Analytical Microbiology, Elsevier: 1-86.

Cos P, Vlietinck J, et al (2006). "Anti-infective potential of natural products : How to develop a stronger in-vitro , a proof of concept". Journal of Ethnopharmacology. **106**(3): 290-302.

Davies J and Davies D (2010). "Origins and evolution of antibiotic resistance" Microbiology and molecular biology reviews **74**(3): 417-733.

Donaldson GP, Lee SM, et al (2016). Gut biogeography of the bacterial microbiota. Nature Reviews Microbiology. **14**(1): 20.

Douglas M and Wildavsky A (1983). Risk and culture: An essay on the selection of technological and environmental dangers, University of California Press.

Dugan P (2012). Biochemical ecology of water pollution, Springer Science & Business Media.

Farstner U and Wittmann GT (2012). Metal pollution in the aquatic environment, Springer Science & Business Media.

Gerardi MH and Zimmerman MC (2004). Wastewater pathogens, John Wiley & Sons.

Gerba CP (2009). Environmentally transmitted pathogens. Environmental Microbiology (Second Edition), Elsevier**:** 445-484.

Jorgensen JH and Ferraro MJ (1998). Antimicrobial susceptibility testing: general principles and contemporary practices. Clinical Infectious Diseases: 973-980.

Lahore CI (2017).World Journal Of Pharmaceutical Reasearch

Laxminarayan R, Duse A et al. "Antibiotic resistanceâ€"the need for global solutions." The Lancet infectious diseases**13**(12): 1057-1098.

Lovell-Antiaye P (2014). Antimicrobial Activity of Hibiscus Sabdariffa Against Clinical Isolates of Bacteria, University of Ghana.

Mahendran S, Saravanan S et al. Antibacterial and in vitro antioxidant activity of sulfated polysaccharide from green seaweed Ulva reticulata.

Nagajyoti PC, Lee KD et al. (2010). "Heavy metals, occurrence and toxicity for plants: a review." Environmental chemistry letters. **8**(3): 199-216.

Nguyen PH (2018). Defining the Aetiology and Antimicrobial Susceptibility Patterns of the Predominant Bacteria Associated with Bloodstream Infections at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, The Open University.

Pakyz AL (2007). The utility of hospital antibiograms as tools for guiding empiric therapy and tracking resistance: insights from the Society of Infectious Diseases Pharmacists. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy. **27**(9): 1306-1312.

Palleroni NJ and Doudoroff M (1972). Some properties and taxonomic sub-divisions of the genus Pseudomonas. Annual Review of Phytopathology. **10**(1): 73-100.

Piddock LJ (1990). Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. Journal of Applied Bacteriology. **68**(4): 307-318.

Punmia BC, Jain AK et al (1998). Waste water engineering, Firewall Media.

Salt DE, Blaylock M et al. (1995). Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Nature biotechnology. **13**(5): 468.

Schaefer JK (2005). The Role Of Mercury Resistance Genes In The Environment, University of Medicine and Dentistry of New Jersey.

Shanmugam M (2010). Detection of metallo-beta-lactamases producing pseudomonas aeruginosa in hospitalized patients at vims, bellary.

World Health O (2006). Guidelines for the safe use of wastewater, excreta and greywater, World Health Organization.

APPENDICES

APPENDIX A: MATERIALS USED

Glasswares:

1. B.O.D bottles
2. Pipettes
3. Petri plates
4. Conical flask
5. Beakers
6. Glass rods
7. Slides
8. Cover slips

Equipments:

1. Autoclave
2. Hot air oven
3. Microscope
4. Incubator
5. Ice box

Microbiological and Biochemical media:

1. Cetrimide Agar
2. Mueller Hinton agar
3. Nutrient broth
4. Simmons citrate media
5. MR-VP broth
6. Urease media

Chemicals and reagents:

1. Alcohol
2. Lysol
3. Glycerol
4. Sodium chloride
5. Sulfuric acid
6. Barium chloride
7. Catalase reagent (3% H_2O_2)
8. Alpha-napthol (5%)
9. Crystal violet
10. Gram's Iodine
11. Safranin
12. Oxidase reagent
13. Kovacs reagent
14. Methyl red
15. Potassium hydroxide

Materials:

1. Test tube rack
2. Wash bottle
3. Burner

Others:

1. Cotton swab
2. Inoculating loop

3. Labelling tape

Sample:

Water

APPENDIX B: COMPOSITION OF MEDIA USED

1. Cetrimide Agar:

Ingredients	Gms / Litre
Pancreatic digest of gelatin	20
Potassium sulphate	10
Magnesium Chloride	1.4
Cetrimethyl Ammonium Bromide	0.3
Glycerine	10ml
Agar	13.6

2. Nutrient Broth:

Ingredients	Gms / Litre
Beef extract	1
Yeast extract	2
Peptone	5
Sodium chloride	5

3. Mueller Hinton Agar:

Ingredients	Gms / Litre
Beef extract	2
Acid hydrolysate of casein	17.5
Starch	1.5
Agar	17

4. MR-VP media:

Ingredients	Gms/ Litre
Peptone	7
Dextrose	5
Dipotassium phosphate	5

5. Simmons citrate:

Ingredients	Gms/ Litre
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15

6. Christensen's urea agar (urease test):

Ingredients	Gms/ Litre
Urea	20
Sodium chloride	5
Monopotassium phosphate	2
Peptone	1
Dextrose	1
Phenol red	0.012
Agar	15

APPENDIX C: STAINS AND REAGENTS USED

1. Crystal violet:

Crystal violet	20g
Ethyl alcohol	95ml
Ammonium oxalate	9g
Distilled water	905ml

2. Gram's iodine:

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

3. 95% ethyl alcohol:

Ethyl alcohol	95ml
Distilled water	5ml

4. Safranin:

Safranin	10ml
(2.5% safranin in 95% ethyl alcohol)	
Distilled water	100ml

5. Oxidase reagent:

Tetramethyl-p-phynylenediamine	0.1g
Dihydrochloride	
Distilled water	10ml

6. Kovacs reagent:

Dimethyl amino benzaldehyde	5g
Amyl alcohol	75ml
Conc. Hydrochloric acid	25ml

7. Methyl red solution:

Methyl red	0.05g
Ethyl alcohol	28ml
Distilled water	22ml

8. **VP reagent:**

VP reagent –I

α- Napthol	5g
ethyl alcohol	100ml

VP reagent –II

Potassium hydroxide	40g
Distilled water	100ml

APPENDIX D: PROCEDURE OF BIOCHEMICAL TESTS

1. Indole test:

The bacterial colony was inoculated on tryptone broth and then incubated at 37^0C for 24 hours. After 24 hours of incubation, 1ml of Kovac's reagent was added. Appearance of red color (red ring) on the top of media indicates positive indole test.

Principle:

This test is used to determine the ability of bacteria to oxidize the tryptophan by producing tryptophynase enzyme.

2. MR-VP test:

The bacterial colonies were inoculated into MR and VP broth and incubated at 37^0C for 24 hours. After incubation, 5 drops of methyl red indicator were added to MR broth and mixed well for MR test. The positive test was indicated by the development of red color, and negative with yellow color. For VP test, 5 drops of Barritt's reagent was added to VP broth and shaken well. Positive test is indicated by the development of pink red color.

Principle of MR test:

The principle of this test is to detect the ability of bacteria to produce and maintain sufficient stable acid from glucose fermentation which is indicated by MR indicator.

Principle of VP test:

This test detects the ability of bacteria to produce a neutral end product, acetyl methyl carbinol (acetoin) from glucose by fermentation.

3. Citrate utilization test:

A bacterial colony was stabbed on the butt of the Simmons citrate agar and then streaked on slant by a sterile inoculating needle. Then the inoculated media were incubated at 37^0C for 24 hours. A positive test was indicated by the growth of organism and change of color of media from green to blue. Bromothymol blue is green acidic (pH 6.8 and below) and blue when alkaline (pH 7.6 and higher).

4. Catalase test:

3% H_2O_2 was taken in a clean and dry test tube (3ml). A small amount of culture from nutrient agar plate was added and mixed with the help of glass rod. Positive test is indicated by the formation of bubbles of oxygen gas.

Principle:

Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. Bubbles of oxygen are released if the organism is catalase producer.

5. Oxidase test:

The test organism was spread on the filter paper soaked by the oxidase reagent with the help of glass rod. The organism producing oxidase enzyme gives purple color on the paper.

Principle:

This test used for determine the presence of cytochrome oxidase in bacteria that catalyse the transport of electrons between electron donor and redox dye. Tetramethyl p-phenylene diamine dihydrochloride in reagent is reduced to deep purple color.

6. Urease test:

The test organism was streaked on the Urease agar slant with a loopful of pure culture. The tube was incubated at 37^0C for 24 hours.

Principle:

Many organisms have an urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

APPENDIX E: TABLES

Table 6: Biochemical test result:

S.N.	Tests	Result
1	Catalase	Positive
2	Oxidase	Positive
3	Indole	Negative
4	MR (Methyl red)	Negative
5	VP (Voges-Proskauer)	Negative
6	Citrate utilization test	Positive
7	Urease	Negative
8	Gram staining	Negative

Table 7: McFarland standard:

S.N.	McFarland turbidity standard no.	0.5	1	2	3	4
1	1% Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
2	1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
3	Approx. cell density (1×1^8 CFU/ml)	1.5	3	6	9	12

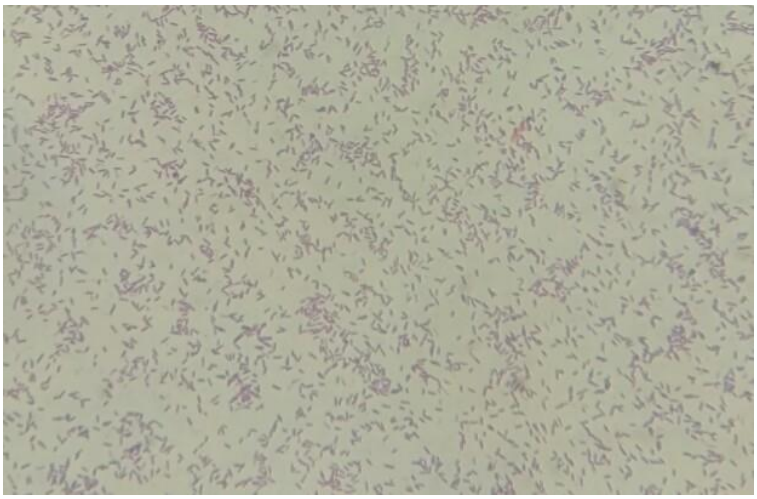
Table 8: Zone interpretation chart of antibiotic testing:

Antibiotics Used	Symbol	Disc content	Diameter of zone of inhibition (mm)		
			Resistant	Intermediate	Susceptible
Amikacin	AK	30 µg	<14	15-16	>17
Amoxicillin	AMC	10 µg	<13	14-17	>18
Ampicillin	AMP	10 µg	<13	14-16	>17
Cefotaxime	CTX	30 µg	<22	23-25	>26
Clindamycin	CD	2 µg	<14	15-20	>21
Co-Trimoxazole	COT	25 µg	<10	11-15	>16
Erythromycin	E	15 µg	<13	14-22	>23
Kanamycin	K	30 µg	<14-17	18	_
Nalidixic acid	NA	30 µg	<13	14-18	>19
Nitrofuratoin	NIT	300 µg	<14	15-16	>17
Norfloxacin	NX	10 µg	<12	13-16	>17
Tetracycline	TE	30 µg	<11	12-14	>15
Vancomycin	VA	30 µg	<14	15-16	>17

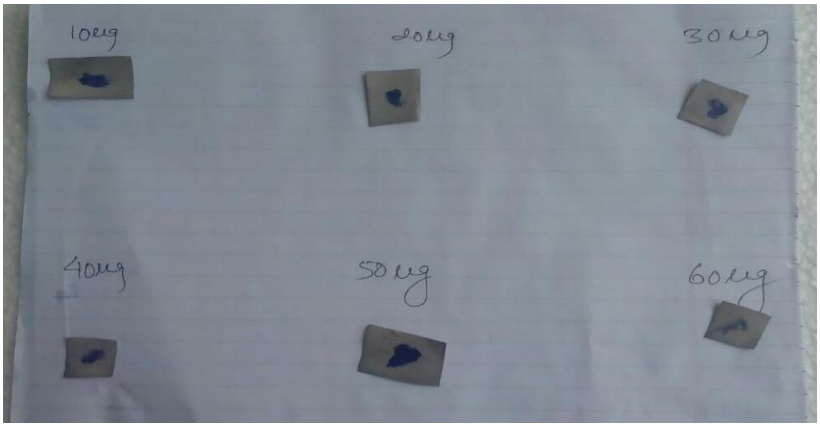
Source: CLSI document M100-S23 (M02-11): Disc diffusion supplemental table"

Photograph 1: Sample collection process

Photograph 2: Samples brought to laboratory by using B.O.D bottles

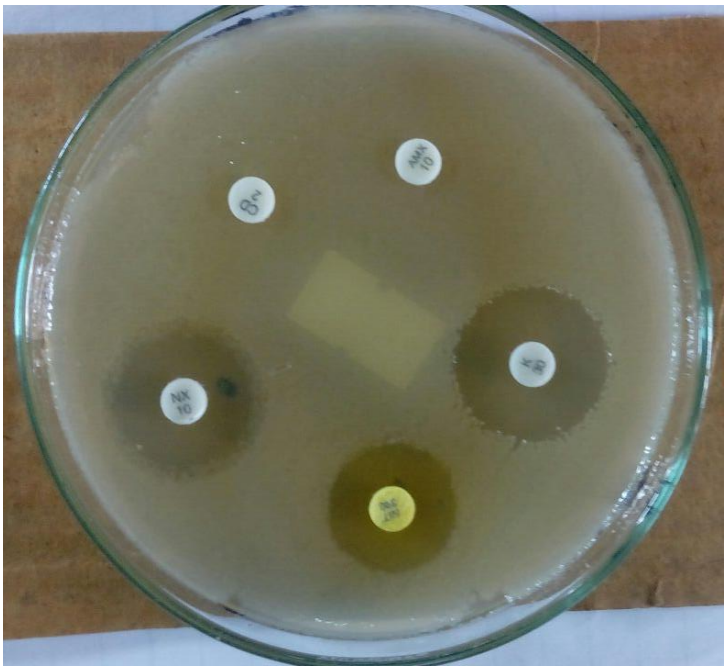


Photograph 3: *P. aeruginosa* under 100X power (Gram staining)



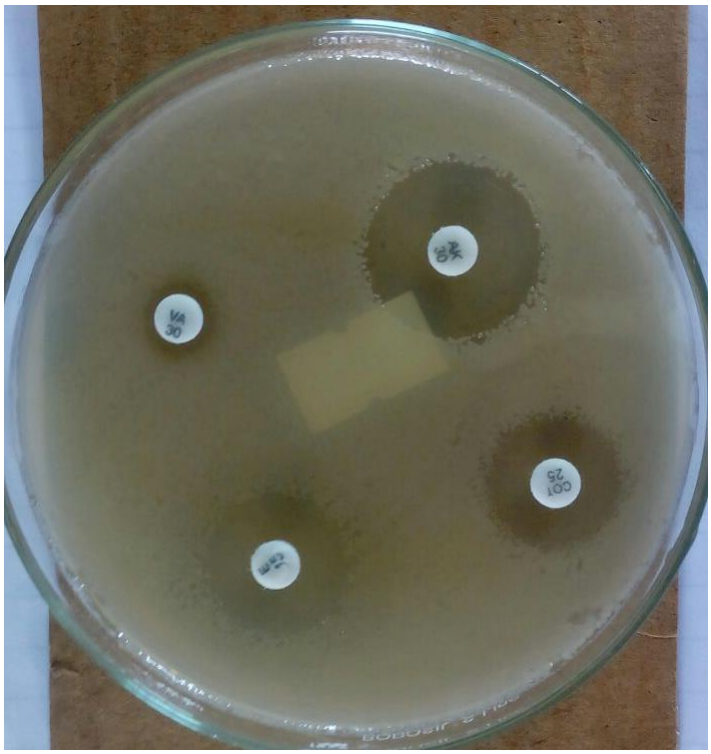
Photograph 4: Oxidase test of isolated organisms

Photograph 5: citrate test



Photograph 6: Antibiotic susceptibility test

Antibiotics used are: Clindamycin (CD), Amoxicillin (AMX), Kanamycin (K), Nitrofurantoin (NIT) and Norfloxacin (NX)



Photograph 7: Antibiotic susceptibility test

Antibiotics used are: Cotrimoxazole (COT), Amikacin (AK), Vancomycin (VA) and Erythromycin (E)