Comparative analysis of resistance profile of *Pseudomonas aeruginosa* against clinically relevant antibiotics

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May 2015

Submitted in partial fulfillment of the

5 Year Dual Degree Programme B.Tech-M.Tech

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology,

Waknaghat

Contents

Certificate

This is to certify that project report entitled **"Comparative analysis of resistance profile of *Pseudomonas aeruginosa* against clinically relevant antibiotics",** submitted by **Ms.Akanksha Tomar** in partial fulfillment for the award of degree of Bachelor of Technology in biotechnology to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma.

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Acknowledgement

First and foremost I would thank the omnipresent ‘GOD’ for bestowing up on me his choicest blessings without which this work would have not been accomplished.

It was a great pleasure and privilege to work under the guidance of Dr. Jitendraa Vashistt as my project guide and Dr. Rahul Shrivastava who have been incessantly motivating and encouraging me since the inception of the project to work hard and put in every possible effort to make the project a success.

I would like to pay most sincere thanks to Dr. R.S.Chauhan, Head of Department, Department of Biotechnology and Bioinformatics, for providing me with opportunities and facilities to carry out the project.

I am also thankful and indebted to PhD scholar Miss. Nutan Thakur who has been supervising me day and night and has been helping me at every odd hour acquainting me with the basics and subtleties of Microbiology and Molecular biology. I would like to acknowledge Venus medical research centre for providing us amniotic which we used in experiment.

I feel short of words while rendering my thanks to my friend Shilpa for her immense support, love, care and timely help she has given me. I would like to thank the lab technician staff Mr. Baleshwar Shukla, Mr. Kamlesh.

Lastly I fold my hands to thank my parents Mr. Shailendra Singh Tomar and Mrs. Bina Singh for all their love, care, support and motivation.

Date: **Name of the student**

Summary

Antibiotic resistance is an increasing concern worldwide, especially in Gram negative bacilli where there is a paucity of new and effective antimicrobial agents. *Pseudomonas aeruginosa* is inherently resistant to various antimicrobial agents like beta-lactams, aminoglycosides and fluoroquinolone. This makes *Pseudomonas aeruginosa* a potent pathogen which is responsible for increased rates of mortality and morbidity in infected person. It is the fourth most common isolated nosocomial pathogen accounting for approximately 10% of all hospital acquired infections. Case mortality rate for patients infected with *P.aeruginosa* approaches 50%.

The aim of the study was to find the prevalence of *P. aeruginosa* in different clinical samples and their resistance pattern against different classes of antibiotics. Biochemical characterization of *P. aeruginosa* was done by the following tests: Indole test, Methyl Red test, Vogues Proskauer test, Citrate test, Triple Sugar Iron (TSI) test and Catalase test. Antibiotic sensitivity testing (AST) was performed by the Kirby‑Bauer disc diffusion method in accordance with the CLSI 2014 guidelines. Minimum inhibitory concentrations of single conventional drug and combined drugs for each isolate were also determined.

Percentage prevalence of *Pseudomonas aeruginosa* was found to be 5%. These isolates were completely susceptible to netilitin, tobramycin, streptomycin, ofloaxcin and levofloaxcin. High rate of sensitivity was found in case aminoglycosides amikacin (75%), beta-lactam cetriaxone (75%) and fuoroqounolone norfloaxcin (75%). Increased susceptibility of 50% was found in case of cefepime, ceftazidime and ciprofloxacin. Total resistance to drugs vancomycin and nalidixic acid was also observed. No synergistic effect was found in case of combination drugs. The evaluation of MIC studies showed antibiotic breakpoints at elevated concentration.

Signature of student **Signature of Supervisor**

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Date

List of symbols and acronyms

mg	-	Milli gram
° C	-	Degree centigrade
ml	-	Milli liter
µl	-	Micro-liter
µg	-	Microgram
A.S.T	-	Antibiotic Susceptibility Test
M.I.C	-	Minimum Inhibitory Concentration
X.D.R	-	Extreme Drug Resistant
P.D.R	-	Pan Drug Resistant
M.D.R	-	Multi Drug Resistant
cAMP	-	cyclic Adenosine Mono-Phosphate
E.S.B.L	-	Extended Spectrum Beta Lactamases
M.B.L	-	Metallo Beta Lactamases

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Chapter 1

Introduction

P. aeruginosa septicemia incidence on the rise among hospitalized adults

September 8, 2014

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WASHINGTON, D.C. — The incidence of *Pseudomonas aeruginosa* septicemia has increased among hospitalized adults within recent years and there has been no evident improvement in mortality or length of hospital stay, according to data presented here at ICAAC 2014.

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"Data from a recent study demonstrated that the incidence of septicemia overall increased 32.4% from 2005-2010, but in the same time period, we found that *P. aeruginosa* septicemia increased by 51.2%," **Brian Werth, PharmD**, assistant professor in the University of Washington School of Pharmacy, told *Infectious Disease News*. "It's outpacing the general trend of septicemia as a whole, which may be an indicator that the increase in septicemia might be related to multidrug-resistant pathogens."

Brian Werth

Werth and colleagues conducted a retrospective study using data from the CDC's National Hospital Discharge Surveys. They identified adult patients who had a *P. aeruginosa* septicemia discharge from 1996 to 2010. They also described the all-cause, in-hospital mortality rates and the hospital lengths of stay among the patients.

Within the 15-year period, there were 213,553 patients who had a *P. aeruginosa* septicemia discharge. The incidence in 1996 was 6.5 per 10,000 discharges, which declined to 3.1 per 10,000 discharges in 2001. However, the incidence more than doubled from 2001 to 2010, back to 6.5 per 10,000 discharges.

"The incidence has increased to the same rate it was in 1996, which is really troubling," Werth said. "We know from other studies that the number of isolates resistant to antibiotics is increasing, and if the incidence is also

Pseudomonas aeruginosa is an aerobic, non spore forming Gram negative straight or slightly curved rod about 1-3 µm long and 0.5-1.0 µm wide and has polar flagella. It is also a non fomenter of lactose, glucose and sucrose [3]. Carle Gessard first discovered *P.aeruginosa* in 1882[2]. The genome of this microbe is among the largest in the bacterial kingdom allowing for great genetic capacity and high

adaptability to environmental changes. In fact *Pseudomonas aeruginosa* has 55567 genes in 6.26 Mbp of DNA [4].

Pseudomonas aeruginosa naturally occurs in soil and water reservoirs. It is frequently found in aerators and taps of sink, in respiratory therapy equipments and shower heads. It also contaminates bronchoscopes and lead to outbreak of infection. Finally, *P.aeruginosa* may be found on the surface of many types of raw fruits and vegetables [2].

Pseudomonas aeruginosa is an opportunistic nosocomial pathogen responsible for a wide range of infections that may present high rates of antimicrobial resistance [5, 6, 7]. It is the fourth most common isolated nosocomial pathogen accounting for approximately 10% of all hospital acquired infections. Case mortality rate for patients infected with *P.aeruginosa* approaches 50%. Severe immunodeficiency and medical devices predispose the patients to *P.aeruginosa* infections [6].

Pseudomonas aeruginosa is becoming more resistant to conventional drug therapy due to indiscriminate use of antibiotics. There is a need to frame rational drug therapy to prevent the misuse of antibiotics. Prior AST (antibiotic susceptibility test) and MIC determination is required to reduce the incidences of morbidity and mortality due to *Pseudomonas aeruginosa* infections.

Chapter 2

Review of literature

P.aeruginosa was first discovered by Carle Gessard, a chemist and bacteriologist from France, in 1882, through an experiment that identified this microbe by its water soluble pigment that turned blue-green when exposed to ultra violet light. This experiment was the focus point of his paper on "The Blue and Green Coloration that Appears on Bandages". Along with the findings from his experiment he went to properly name the strain *P.aeruginosa*, determine its pigment derivative, and developed theory to its pathogenic nature and its infectious similarities found in similar microbes [2].

Taxonomical classification of *P.aeruginosa* is as follows:

Kingdom: Bacteria
Phylum: *Proteobacteria*
Class: *Gamma Proteobacteria*
Order: *Pseudomonadales*
Family: *Pseudomonadaceae*
Genus: *Pseudomonas*
Species: *Pseudomonas aeruginosa* [1]

Mechanism of Pathogenesis

Break in the first line of defense, such as cuts in skin, burns, wounds or compromised immune system are usually required by *P.aeruginosa* to initiate infection in a patient. The bacteria have a wide range of secreted virulence factors which enables it to cause widespread and often overwhelming infections. Cell-to-cell signaling system controls the production of these factors in a co-ordinate and cell-density-dependent manner [8].

According to Lark *et al P.aeruginosa* has been shown to possess a type III secretion system which enables it to secrete proteins without cleavage of a signal peptide and transfer the virulence factors into eukaryotic cells. The type III secretion systems are activated by host cell contact. The information of secretion is located in the amino-terminal portion of the protein. The translocated factors from *P.aeruginosa* identified so far are Exoenzyme Y (Exo Y), Exoenzyme U (Exo U), Exoenzyme T (Exo T) and Exoenzyme S (Exo S). Clinical isolates contain either the exoS or exoU gene, while almost all

forms of *P.aeruginosa* have exoT and exoY, which indicates that ExoT and ExoY are important for the pathogenesis [9].

ExoY is an adenylated cyclase and the cells intoxicated with ExoY show a round morphology that correlates with increased cAMP levels [11].

ExoU (PepA) expression is correlated with acute cytotoxicity and bacterial-mediated epthilial cell damage in a mouse model of acute pneumonia. Recently, it has been reported that ExoU poissesses lipase activity and disrupts the membranes of the infected host cells [12].

ExoS causes decrease in host cell DNA. Recently has been reported thet the amino-terminal (AA 96-232) is a Secr./Transl.GAP ADP-ribotranssferase E381 GTPase Activating Protein (GAP) for the members of the Rho family. The carboxyterminal has been shown to be cytotoxic to eukaryotic cells. It contains an ADPribosylatransferase activity (AA 233-453), which covalently transfer ADP-ribose from NAD to eukaryotic target proteins [13]. It has previously been suggested that this enzymatic activity induces programmed cell death in the infected cell. The first target found for ExoS *in vitro* was Ras, which shown to be ADP-ribosylated on Arg41 and Arg 128 by ExoS [14]. The ADP- ribosyltransferase activity of ExoS Has been shown to be dependent on eukaryotic co-factor named FAS, for Factor Activating exoenzyme S. FAS has been identified as a member of the eukaryotic family 14-3-3 family, which is involved in many eukaryotic signal transduction pathways. Amino acids 51-72 of ExoS harbor a membrane localization domain (MLD), which localizes the toxin to membrane region inside the eukaryotic cell [15].

ExoT like ExoS, has been reported to contain a carboxy-terminal ADP-ribosytransferase activity, however ExoT only possesses 0.2-1% of 14-3-3 dependent ADP-ribsyltranseferase activity in vitro as compared to ExoS. The candidate active site residue e385 is homologous to e381 in ExoS. The amino terminal part of Exot also displays high homology to ExoS, which suggests that ExoT also harbor GAP activity [16].

P.aeruginosa also produces certain virulence factors, not translocated by type III apparatus, which contribute to the overall virulence of bacterium. These factors can cause extensive tissue damage, bloodstream invasion and dissemination. These factors are described as follows:

Exotoxin A catalyses the ADP-ribosylation and inactivation of elongation factor2, leading to inhibition of protein synthesis and cell death [17].

Phospholipase C and rhamnolipid are the two types of hemolysins produced by *P.aeruginosa.* These hemolysins act synergistically to breakdown lipids and contribute to tissue invasion. Rhamnolipid also keep fluid/nutrient channels open in the biofilms produced by the bacteria [18].

Proteases LasA and LasB has elastase activity and destroys elastin-containing human lung-tissues and cause pulmonary haemorrhage in invasive infections. LasB also degrades fibrin and collagen and inactivates immunoglobulins G and A, and complement components which not only destroy tissue components, but also interfere with host defense mechanisms [19, 20].

Type IV pili are important for colonization of the host by giving *P.aeruginosa* ability to adhere to eukaryotic cells. Tye IV pili also mediate the motility on surfaces. Pili also aid in initiation of biofilm formation [21, 22].

Adhesins are the part of the bacteria that aid them to adhere to host cells. *P.aeruginosa* adhere to the epithelial cell of its host through its fimbrae, which bind to specific receptors on host epithelial cells, such as mannose, sialic acid or galactose receptors. fimbrial adherence is required for colonization of the respiratory tract. In this case protease enzymes are used to degrade the extra cellular matrix and expose the appropriate receptors on epithelial cell surface. It has been shown that tissue injury of the respiratory tract, eyes and urinary tract is important part of colonization. Exopolysaccharide producing mucoidal strains of *P.aeruginosa* have an additional or alternative adhesin that attaches to mucin on the host cells. The adhesins present on the surface of *P.aeruginosa* are fully understood and characterized [10].

Alignate/biofilm formation allows *P.aeruginosa* to grow encapsulated in a slime layer consisting of bacteria and polysaccharide alginate. Its biofilm consist of the polysaccharides Pel, Psl and alginate, extracellular DNA and proteins like CupA, CupB and CupC, fimbrae and LecB. Biofilms help the bacterium survive on uninhabitable surfaces. After attachment to a surface, movement across that surface by twitching mobility leads to the formation of microcolnies. Biofilm matrix determines the development and evolution of biofilm architecture.

The oxygen and nutrient gradient in the biofilm affects this susceptibility to antibiotics. The matrix delays diffusion of some antibiotics into some biofilm, effectively making it more and more resistant to antibiotics [10].

Clinical manifestation of *P.aeruginosa* infections

Any part of the body can be infected by *P.aeruginosa.* This bacterium often creates biofilms in the area where they cause infection, which make it much harder to cure and more antibiotic resistant. The most vulnerable individuals to *P.aeruginosa* infections are those that have compromised immune system which include - certain populations of patients such as those with intravenous lines, burns, cancer, cystic fibrosis, diabetes, surgery trauma catheters and neonatal infants [23].

Respiratory Tract

Nosocomially acquired pneumonia is observed in patients with cystic fibrosis and is often a cause of severe decline in the health of these patients. In patients with airway affecting diseases, chronic lung colonization and chronic infection has been reported [24].

Central Nervous System

CNS infection of *P.aeruginosa* can cause meningitis and brain absecese, most often following an extension from a contiguous parameningial structure, such as an ear, a mastoid, paranasal sinus surgery or diagnostic procedures [25].

Ear

'Swimmer's ear', which is presented in patients with pain, is also caused by *P.aeruginosa* infection. This condition is worsened by friction on the ear, itching and ear discharge. Chronic otitis media (middle ear infection) is also commonly caused by *P.aeruginosa* [26] .

Eye

Infection in the eye is the most common pseudomonas infection in immune-competent patients. It can cause bacterial keratitis (infection of the cornea), endophthalmitis (infection of the intraocular cavity) and sclera abscess in adults and ophthalmia neonatrum in children. To do so it produces extracellular enzymes that creates a rapidly destructive lesion [27].

Bone and Joints

P.aeruginosa infections of the skeletal system most often involve the vertebral column, the pelvis and the sternoclavicular joint. These infection arise from intravenous drug use , pelvic infections or urinary

tract infections or contiguous from an open wound due to trauma, surgery or a soft tissue infection which most often causes blood-borne infections [28].

Gastrointestinal Tract

GI tract infection is an aspect of Pseudomonal infection that is often underestimated. GI tract infections can affect every portion of GI tract especially in very young children and adults with cancers and undergoing chemotherapy. Nursery epidemic of *P.aeruginosa* may cause contract of the in young infants. This may present symptoms of irritability, vomiting, diarrhea and dehydration. Shanghai fever is enteritis manifestation of Pseudomonal infection, which presents with headache, fever, exhaustion, enlargement of spleen, rose spots and dehydration [29].

Urinary Tract Infection

Pseudomonal UTIs, most often, are nosocomial caused due to instrumentation, surgery and catheterization. The UTIs can arise from an ascending infection or through bacteremic spread, in addition to being a source of bacteremia. Urine culture and antibiotic susceptibility test is required to distinguish a pseudomonal UTI from others [30].

Skin and Soft Tissue Infections

P.aeruginosa flourishes on moist skin, such as improperly attended wound or skin frequently submerged in water, such as with green nail syndrome. Decubiti, pressure-induced ulceration of the skin, eczema and athlete's foot are the most frequent sites of secondary wound infections by pseudomonas. The most prominent characteristic of *P.aeruginosa* that is blue-green and fruity odor is exhibited. Deep abscesses, cellulits and subcutaneous nodules may also occur. Burn victims are also highly susceptible to bacterial wound sepsis, which involves the proliferation of 100,000 organisms per gram of tissue, including the surrounding healthy tissue. These burn infections often appear incredibly discolored and symptoms may include fever, disorientation, hypotension, low urine production, bowel obstruction and decreased white blood cell count. Improperly treated hot tubs and swimming pools are frequently sources of soft tissue and skin infections, with patients presenting with varying types of skin lesions called "hot tub folliculitis" on any part of the body submerged in the water [31].

Bacteremia

P.aeruginosa bacteremia is associated with higher mortality than other Gram negative bacteremia. The mortality rate for pseudomonal bacteremia is greater than 10% and is most often acquired in a health setting. The symptoms in this case depend on the site of infection. Bacteremia can cause ecthyma gangrenosum, which painless nodular skin lesions with ulceration and hemorrhage most often in the armpit, groin or perianal area [32].

Endocarditis

P.aeruginosa can infect both native heart valve and prosthetic heart valves [33].

Antibiotic therapy for *Pseudomonas .aeruginosa*

Conventional Approaches

P.aeruginosa is attributed with intrinsic and acquired mechanism of antimicrobial resistance which makes the antimicrobial chemotherapy all the more complicated. Despite of this some antibiotics are active against this microorganism. The most frequently used antimicrobials used against *P.aeruginosa* belong to the three classes (1) Beta-Lactams, (2) Quinolones and (3) Aminoglycosides. Polymyxin therapy is also considered in some cases [34].

Beta-lactams

Beta-lactams bind to and inactivate penicillin-binding proteins (PBPs) that are transpepidases involved in bacterial cell wall synthesis. The group of beta-lactam antibiotics includes penicillins, cephalosporins, monobactams and carbapenems. The beta lactams that are most active against *P.aeruginosa* are: Pipracillin and ticarcillin (Penicillins), Ceftazidime (Third generation cephalosporin), Cefepime (Fourth generation cephalosporin), aztreonam (monobactam) and Imipenem, Meropenem and Doripenem (Carbapenems) [35].

Fluorouinolones

These are the synthetic antimicrobials that inhibit the activity of DNA gyrase and topoisomerase IV and block bacterial DNA replication. Ciprofloxacin, Levofloxacin and Ofloxacin are the fluoroquinolones with anti-pseudomonal activity [36].

Anminoglycosides

Aminoglycosides inhibit protein synthesis by binding to the 30S ribosomal subunit and degrading its structure. The drugs belonging to this class of antibiotics which show activity against *P.aeruginosa* are Tobramycin, Amikacin and Gentamicin. Aminogylcoside often show cytotoxicity and nephrotoxicity. They have narrow therapeutic range as well. Because of these reasons aminoglycosides are often used in combination with agents belonging to other classes of antibiotics. Urinary tract infections due to *P.aeruginosa* are the only case where the monotherapy aminogylcoside treatments are recommended [37].

Polymyxins

Polymyins are cyclic, positively charged peptide antibiotics derived from various species of *Paenibacillus (Bacillus) polymyxa.* Polymyxins can be classified into five major classes (polymyxinA-E). There are only two polymyxins, Polymixin B and Polymyxin E (Colistin), which have been shown to be effective against *P.aeruginosa* infections. These antimicrobials have a detergent-like activity that disrupts membrane integrity and results in leakage of intracellular components. These distinctive properties of polymyxins shelter them from cross-resistance with other anti-pseudomonal agents and are protected from rapid selection of resistance. In the wake of emergence of MDR Gram negative bacteria and the absence of new classes of antibiotics has led to the resurgence of old antibiotics like polymyxins as a last resort in the treatment of MDR *P.aeruginosa* pneumonia. Colistin has increasingly been used as salvage therapy alone or in combination with one or more anti-bacterial for the treatment of pneumonia with MDR strains. Polymyxins are associated with nephrotoxicity and neurotoxicity. The efficacy of intravenous Polymixin therapy for treating severe infections caused by MDR *P.aeruginosa* has outweighed risk associated with them in the absence of therapeutics alternatives [38].

Combinational Therapy

Combination drug therapy against MDR strains seems to be some times necessary (for example in cases PAN-resistance or resistance to all except a single agent). In such cases better results are expected by the additive or sub-additive activity of a combination or by enhancement of a single active agent by an otherwise inactive drug.

Several old and newer studies have showed the increased activity *in vitro* of various antibiotic combinations against MDR *P.aeruginosa* even though, the mechanisms of positive interaction between the various agents are rarely known [39].

Other Recent Therapeutic Approaches

As therapeutic options become restricted, the search for new agents is a priority. Several *in vitro* and *in vivo* studies evaluating the efficacy of different antimicrobials agents and development of vaccines against *P. aeruginosa* have been reported as novel approaches, such as inhibition of virulence factor expression or inhibition of their metabolic pathways [40]

Several studies have shown the efficacy of bacteriophages in the treatment of experimental infections caused by *P. aeruginosa* in animals [41].

It has been shown that when gold and silver nanoparticles are functionalized with ampicillin they became potent bactericidal agents with unique properties that subverted antibiotic resistance mechanisms of multiple-drug-resistant bacteria as *P. aeruginosa* [42]

Polymyxin B-loaded liposomes represent a successful example of liposomal antimicrobial drug delivery [43]. It has been re-ported that liposomal encapsulation of polymyxin B dramatically diminishes side effects and improves its antimicrobial activity against resistant strains of *P. aeruginosa* [44].

Mode of Resistance

Mechanism of antibiotic resistance in *P.aeruginosa* can broadly be divided into categories intrinsic resistance and acquired resistance. Intrinsic resistance refers to resistance that is attributed to a large selection of genetically-encoded mechanisms and acquired resistance is referred to resistance that is achieved through acquiring additional mechanisms or is a consequence of mutational events under selective pressure [45].

Intrinsic resistance of *P.aeruginosa*

P.aeruginosa shows inherent antimicrobial resistance through a variety of mechanisms: (1) decreased permeability of the outer membrane, (2) efflux systems which actively pump antibiotics out of the cell, and (3) production of antibiotic-inactivating enzymes.

Outer membrane permeability

Gram-negative bacteria have an outer membrane that act as a barrier and prevent large hydrophilic molecules to pass through it. Antibiotic classes such as aminoglycosides and colistin change the permeability of the outer membrane by interacting with the lipopolysaccharides of the outer membrane in order to pass into the cell cytoplasm. Porin channels are required by beta-lactams and quilonoes in order to diffuse inside the cell. Bacteria produce two major classes of porins: **general**; which allow almost any hydrophilic molecule to pass and **specific**; which have binding sites for certain molecules, allowing them to be oriented and pass in most energy-efficient way [46, 47]. Most bacteria posses' lot of general porins and relatively few specific ones, however, *P.aeruginosa* mainly the specific porins. According to Livermore *et al* the outer membrane permeability of *P.aeruginosa* is 1/100 of the permeability of *E.coli* outer membrane [48].

Efflux systems

P.aeruginosa expresses several efflux pumps that expel drugs together with other substances out of the bacterial cell. Usually these efflux pumps are made up of three different types of proteins: (1) a protein transporter of the cytoplasmic membrane that uses energy in the form of proton motive force [48], (2) a periplasmic connective protein, and [49] (3) an outer membrane porin. Except polymyxins most of the other antibiotics are pumped out by these efflux systems therefore their first two components are named multidrug efflux (Mex) along with a letter (example, Mex and MexB) [50]. Outer membrane porin is called Opr along with a letter (example, OprA) [51].

Antibiotic inactivating enzymes

P.aeruginosa belongs to the SPICE group of bacteria (*Serratia* spp. *P.aeruginosa,* Indole positive *Proteus*, *Citrobacter* spp., *Enterobacter* spp.)[52], these microbes share a common characteristic: the

ability to produce chromosomal-encoded and inducible AmpC beta-lactamases. These are cephalosprinases that hydrolyze most beta-lactams and are not inhibited by beta-lactamase inhibitors. Another endogeneous beta-lactamase produced by laboratory mutants of *P.aeruginosa* is the class D oxacillinases PoxB. This enzyme however is not clinically significant [53].

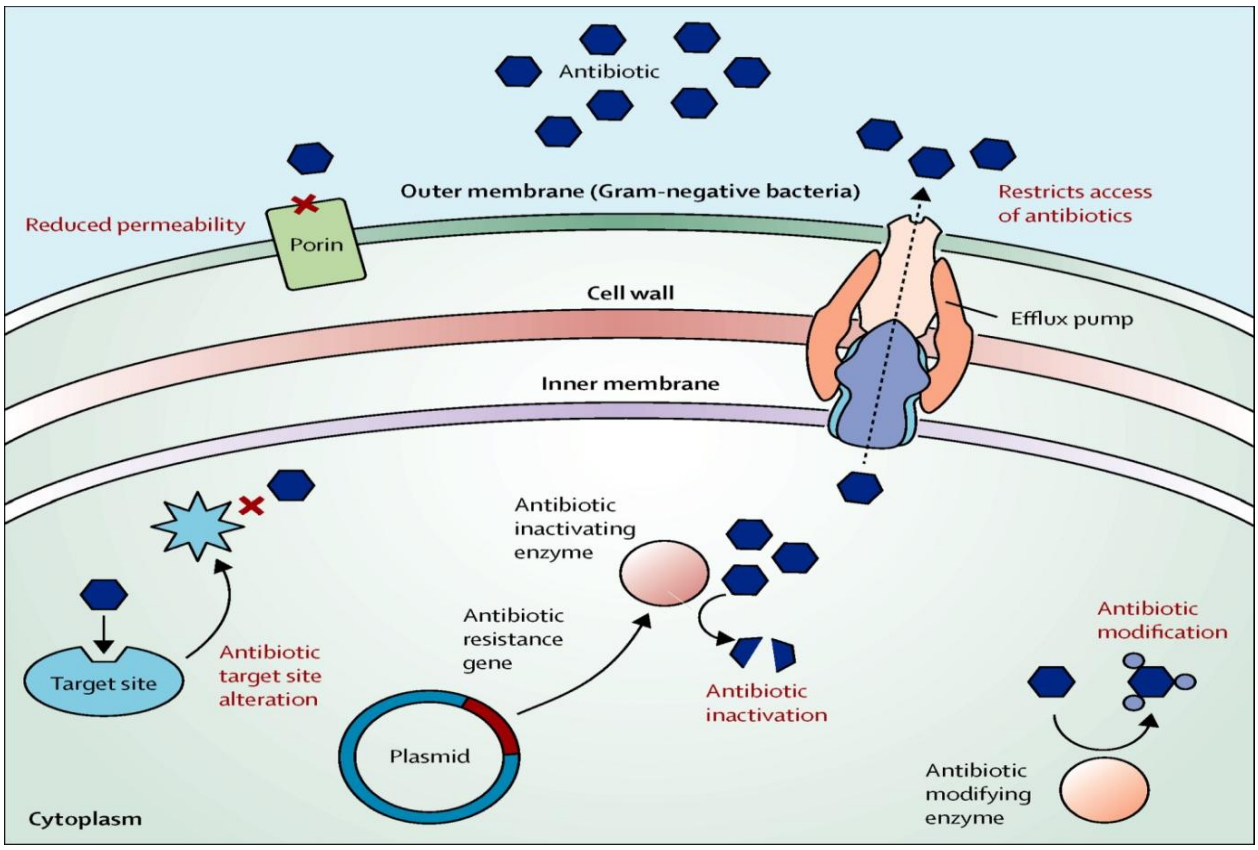


Figure 1: Diagrammatic representation of antibiotic resistance mechanism in *P.aeruginosa.* Source: The Lancet [112]

Acquired resistance in *P.aeruginosa*

Acquired resistance in *P.aeruginosa* is a consequence of mutational changes or the acquisition of resistance mechanism via horizontal gene transfer and can occur during chemotherapy [54] .Mutational events may lead to over-expression of endogenous beta-lactamases or efflux pumps, diminished expression of specific porins and target site modifications while acquisition of resistance genes mainly refers to transferable beta-lactamases and aminoglycoside-modifying enzymes.

Mode of resistance to different classes of antibiotic in *Pseudomonas aeruginosa*

Resistance to beta-lactamas

Resistance to antibiotic class beta-lactams is controlled by multiple factors. It is mainly achieved by beta-lactams inactivating enzymes known as beta-lactamases [55]. The mechanism of action of these enzymes is that they cleave the amide bond of the lactam ring, a four carbon atom ring, and cause the inactivation of the antibiotic. Beta-lactamases are classified according to their structure [56] and function [57].

Crbapenems is the subclass of beta-lactams that proven to be most effective against *P.aeruginosa* [58]. These drugs are stable to the hydrolytic effect of most of the beta-lactamases including Extended Spectrum Beta–Lactamases (ESBL) [59].

Endogenous beta-lactamases

P.aeruginosa isolated from clinical samples has been reported show resistance to beta-lactams. It is commonly due to the presence of AmpC beta-lactamases [60]. A number of beta-lactams such as benzyl penicillins, narrow spectrum cephalosporin and imipenem can induce the production of beta-lactamases in *P.aeruginosa.* In fact, mutational depression of AmpC protein is one of the most common mechanisms of resistance to beta-lactams in *P.aeruginosa* [61]. Though AmpC are not carbapenemases, however they possess a low potential to hydrolyse carbapenem. Their overproduction along with efflux-pumps over-expression and/or diminished outer membrane permeability has been proven to lead also to carbapenem resistance in *P.aeruginosa* [62].

Acquired beta-lactamases

Acquired beta-lactamases are typically encoded by genes which are located in transferable genetic elements such as plasmids or transposons [63] often on integrons [64]. Integrons are genetic elements that capture and mobiles genes [65]. Other genetic elements associated with transferable resistance in *P.aeruginosa* are the mobile insertion sequences called ISCR (Insertion Sequence Common Regions) elements. Different types of transferable beta-lactamases have been found in clinical *P.aeruginosa* isolates around the world [66].

Carbenicillin hydrolyzing Beta-lactamases

Four carbenicillin hydrolyzing Beta-lactamases of *Pseudomonas* specific enzyme type are found in *P.aeruginosa,* PSE 1 Carb2, PSE4 Carb1, Carb3 and Carb4. There substrate profile includes carboxypenicillin, uredopenicillin and cefsulodime. These enzymes belong to molecular class A and functional group to C [103]. PSE1, PSE4 and Carb3 are closely related but they are only 86.3% homologous with Carb4 (Sanschagrin et al., 1998) [108]. The bla_{CARB-4} gene is likely to have been acquired from other bacterial species, as the mol% G+C in this gene is 39.1% unlike the mol% G+C of genes that are typical for *P. aeruginosa*, which is 67%. Carbenicillinase producers show variable susceptibility to cefepime, cefpirome and aztreonam, and 100% susceptibility towards ceftazidime and carbapenems [104].

Extended Spectrum Beta-Lactamases

Unlike PSEs, ESBLs of molecular class A and functional group 2b' [103] lead to the development of resistance not only to carboxypenicillins and ureidopenicillins, but also to extended-spectrum cephalosporins (ceftazidime, cefepime, cefpirome) and aztreonam [105]. They show low affinity to carbapenems and their in vitro activity is inhibited by clavulanic acid and tazobactam [106]. Discovery of class A ESBLs in clinical isolates of *P. aeruginosa* occurred after 1990. Apart from the TEM and SHV types of enzyme that are well known in the Enterobacteriaceae family, in P. aeruginosa other enzymes that were identified are PER (mostly in clinical isolates from Turkey), VEB (from South-East Asia, France and Bulgaria), GES/IBC (France, Greece and South Africa) and BEL types. These six types have low identity at the genetic level, and yet they have similar hydrolysis profiles [105].

Carbapenemases

P.aeruginosa is the species in which all types of transferable carbapenemases, except SIM-1 have been reported. The class B carbapenemases that bear Zn^{2+} in their active sites are the most frequent around the world in *P.aeruginosa* isolates and are called metallo-beta-lactamases (MBLs) [67]. IMP and VIM type MBLs were first discovered in Japan 1991[68] and Italy [69] respectively and have spread to through all the continents since then. Other metallo-beta-lactamases are more geographical but KPC (*Klebsiella pneumonia*e carbapenemase)-producing *P.aeruginosa* isolates have not been reported from other continents except Latin America. KPC present high rates of carbapenem hydrolysis and inactivate all other beta-lactams including aztreonam [70].

Enzymes GES/IBC belongs to the same enzymatic class but their carbapenemase activity is not as high as that of KPCs. It may becaome important if combined with diminished outer membrane permeability or efflux over-expression. For *P.aeruginosa,* GES-2 has been reported in South Africa and IBC-2 in Greece. Class-D carbapenemases like Oxa-198 have been found in *P.aeruginosa* isolates [71].

Diminished permeability

OprD is a specific porin of the outer membrane of *P.aeruginosa* through which carbapenems enter into the periplasmic space [72]. Diminished expression or mutational loss of this porin is the most common mechanism of resistance to carbapenems and is frequently associated with efflux pumps and/or AmpC over-expression. Diminished expression or loss of the OprD porin is a frequent phenomenon during imipenem treatment [73].

Efflux system over expression

Though *P.aeruginosa* possess a number of efflux systems that make the bacterium resistant to different classes of anit-pseudomonal antibiotics, beta-lactam resistance is conferred by only three of these efflux systems, namely: MexAB-OprM, MexXY-OprM and MexCD-OprD [74]. Among these three, MexAB-OprM accommodates the broadest range of beta-lactams. It is by far the better exporter of meropenem and is most frequently related to beta lactam resistance in clinical isolates of *P.aeruginosa* [75]. The efflux pumps may be over expressed in some isolates contributing thus, together with other mechanisms in the development of multi-drug resistance [76].

Target modification

Modifications in the target sites of beta-lactams i.e. alterations in the structure of penicillin binding proteins is the rarest mechanism of resistance to beta-lactams. Altered PBP-4S with low affinity were reported after imipenem treatment, as well as after administration of high doses of piperacillin in patients suffering with cystic fibrosis. There are reports of reduced susceptibility to beta-lactams in *P.aeruginosa* strain with over production of PBP-3s [71].

Resistance to quinolones

Two major mechanisms that give high level of resistance to *P.aeruginosa* against quinolones are: structural changes in the target enzymes and active efflux pumps. Often these two mechanisms coexist in the bacterium.

DNA gyrase and topoisomerase IV mutations

DNA gyrase is made up of two subunits GyrA and GyrB. The genes for these proteins gyrA and gyrB, lie within the Quinolone Resistance Determining Region (QRDR) motif which is considered as enzymes active site [77]. These are the primary targets of fluoroquinolones. Modification in the primary target of fluoroquinolones is caused by mutations. This cause alteration in amino acid sequence of A and B subunits which lead to synthesis of modified DNA gyrase with low binding affinity quinolone molecule [78].

Topoisomerase IV is the secondary target of fluoroquinolones. Mutations in the genes parC and parE encoding for ParC and ParE enzyme subunit respectively causes modification in the drug target rendering the drug ineffective [79].

Efflux pumps

Four efflulx pumps contribute to fluoroquinolone resistance: MexAB-OprM, MexCD-OprJ, MexEF-OprD and MexXY-OprM as a consequence of mutational events in their repressor genes. Among these, MexAB-OprM, MexCD-OprJ and MexEF-OprN have been associated to fuoroquinolone resistance in clinical isolates whereas MexXY-OprM has only been linked rarely to such type of resistance [80].

The coexistence of efflux pump systems and mutations of gene coding for DNA gyrase and topoisomerase IV leads to high level of resistance against fluoroquinolones in *P.aeruginosa* [81].

Resistance to aminoglycosides

There are four reported aminoglycoside resistance mechanism till date. These are: enzyme modification, lower outer membrane permeability, active efflux systems and rarely target modification [82, 83].

Aminoglycoside modifying enzymes (AMEs)

These enzymes are plasmid encoded [82] and attach a phosphate, adenyl or acetyl group to the antibiotic molecule and thus decrease the binding affinity of the modified antibiotic to the target in the bacterial cell (30S ribosomal subunit) [84]. These enzymes are classified as follows: (1) aminoglycoside acetyltransferase (AACs), (2) aminoglycoside adenyltransferase (also known as nucleotidetransferase)

(AADs or ANTs) and (3) aminoglycoside phosphoryltransferase (APHs). Most commonly encountered AMEs in *P.aeruginosa* are: AAC (6')-II, AAC (3)-I, AAC (3)-II, AAC (6')-I and ANT (2')-I [85].

Outer membrane impermeability

It provides resistance to all aminoglycoside and is often associated with reduced accumulation antibiotic in the bacterial cell [86]. Numerous studies have highlighted the importance of outer membrane impermeability in aminoglycoside resistant clinical isolates, especially in isolates from cystic fibrosis patients. It is the most common aminoglycoside resistance mechanism in these types of isolates [87].

Active efflux systems

Active efflux is a relatively rare resistance mechanism that is due to MexXY proteins operating in conjunction with OprM [88], as well as with some other outer membrane proteins- OpmB, OpmG and OpmI [89] thus forming three component active efflux system.

Target modification

Methylation of the 16S rRNA of the A site of the 30S ribosomal subunit has recently emerged as a new mechanism of resistance against aminoglycoside among Gram negative pathogens belonging to the family *Enterobacteriaceae* and glucose-nonfermentative microbes, including *P.aeruginosa* and *Acinetobacter* spp. [90]. This methylation event interferes with the aminoglycoside binding and consequently promotes high-level resistance to all aminoglycosides. Different 16S rRNA methylases have been described for *P.aeruginosa:* RmtA [91], RmtD [90],ArmA and RmtB [92]. According to a study RmtD is commonly found together with the MBL SPM-1 in Brazil [92].

Recent studies and research gaps

Among 33 European countries participating in the European Antimicrobial Resistance Surveillance System in 2012 six countries reported Aminoglycoside resistance rates of >25%, five countries reported Carbapenem resistance rates of >25%, eight countries reported Fluoroquinolones resistance rates of >25% and four countries reported piperacillin resistance rates of >25%, among *P.aeruginosa.* The highest rate of emergence of resistance was reported from Romania (46%) [107].

In study conducted by Mai M. Zafer *et al* [108], 97.5 % of all the total *P.aeruginosa* isolates were sensitive to Polymixin B. This supports the evidence that Polymixin B has increasingly become the last viable therapeutic option for multi drug resistance (MDR) *P.aeruginosa* infections. This result agreed with a study done by Twafik *et al* in 2012 in which they found that all the isolates were sensitive to Polymixin [109].

High prevalence of aminoglycoside modifying enzymes among the *Pseudomonas aeruginosa* isolates in India has also been reported in burn patients. Most recent development is the appearance of metallo-beta-lactamase producers among *P.aeruginosa* [98]. In case of XDR *P. aeruginosa* higher resistance was found to ceftazimide followed by amikacin, piperacillin. In other study 6.06% isolates were PDR which were resistant to all anti-pseudomonasal drugs [100].

Mai M. Zafer *et al* [108] also reported presence of $bla_{\text{VIM-2}}$, $bla_{\text{IMP-1}}$, bla_{NDM}, and $bla_{\text{OXA-10}}$ genes in *P. aeruginosa* in Egypt. The four recent research studies done at SKIMS, Kashmir India on ESBL and MBL assays showed an alarming rise in antibiotic resistance in Kashmir, India as well results depicted 72% and 60% strains of Hospital acquired *K.pneumonae* & *E.coli* as ESBL positive respectively. Also 14% of *P. aeruginosa* proved MBL producing and thus a direct bearing on the use of 3rd generation cephalosporins and carbapenams in the area [110,111].

As new patterns of antibiotic resistance in *P.aeruginosa* are emerging across the world, constant monitoring of these changes is becoming important to prevent the outbreak of the pathogen, lower the cost of treatment for patients infected with *P.aeruginosa,* develop better antibiotic treatments, training the healthcare providers for better management of infected patients and prevention of outbreak of nosocomial infection and to reduce the overall economic and psychological cost of the infection management.

In India antibiotic susceptibility testing prior to prescription of antibiotic for microbial infections is not as prevalent as it is in developed countries. This is leading to evolution new microbial strains that are resilient to antibiotics. Reports on development of new antibiotic susceptibility patterns are not consistent enough for researchers and health-care officials to rely. This is also true for state of Himachal Pradesh where majority of population lives in distant rural areas. Our study aims to find out antibiotic susceptibility patterns and the molecular basis of the resistance in *P.aeruginosa* in the state of Himachal Pradesh where no such pervious study is reported.

Chapter 3

Aim - To perform ccomparative analysis of resistance profile of *P.aeruginosa* against clinically relevant antibiotics.

Objectives

The following are the targeted objectives of this study:

- Procurement of clinical samples from regional hospitals. Isolation of pure cultures of *P. aeruginosa* from different clinical samples.
- Antibiotic susceptibility testing of *P. aeruginoa* isolates from each sample using different classes of antibiotics.
- Construction of resistance profiles for each antibiotic and comparison these profiles among each other.

Chapter 4

Materials

Reagents

1. METHYL RED REAGENT:

Methyl red	0.1gm
Ethanol	300ml
Distilled water	200ml

2. VOGUS PROSKAUER REAGENT:

Solution A

Potasium hydroxide	40gm
Distilled water	1000ml

Solution B

α- Naphthol	5ml
Absolute alcohol	95ml

3. KOVAC'S REAGENT FOR INDOLE:

P-dimethylaminobenzaldehyde	10gm
Isoamyl alcohol	50ml
Conc. HCl	50mM

4. CATALASE REAGENT

Hydrogen peroxide	3ml
Distilled water	97ml

ROUTINE MEDIA:

Various media used in the study were prepared as referred in Mackie and Mac Carty (1966). These media were available in dehydrated form and are prepared and sterilized as per manufacturer's instructions.

PEPTONE WATER:

Peptone	10.00 gm
Sodium chloride	5.00 gm
Distilled water	1 Litre

pH = 7.4

it was autoclaved at 121^0C for 15 minutes and distributed in aliquots of 5 ml.

GLUCOSE PHOSPHATE MEDIUM:

Peptone	7.00 gm
Dipotassium Hydrogen Phosphate	5.00 gm
Glucose	5.00 gm
Distilled water	1 litre

pH = 6.9 ± 0.2

This media was available in dehydrated form and was prepared and sterilized as per manufacturer's instruction.

NUTRIENT BROTH (Dehydrated Hi-Media):

Peptone	5.00 gm
Beef extract	1.50 gm
Yeast Extract	1.50 gm
Sodium Chloride	5.00 gm
Distilled Water	1 Litre

This media was available in dehydrated form and was prepared and sterilized as per manufacturer's instructions.

NUTRIENT AGAR (Dehydrated Hi-Media):

Nutrient Broth	1 Litre
Agar (Difco)	15.00 gm

pH =7.4 ± 0.2

This media was available in dehydrated form. It was prepared and sterilized as per manufacturer's instructions. Slopes were prepared by distributing 5 ml aliquots, allowed to solidify at an angle of 10^0. If plates were to be prepared, 20 ml was poured into each plate and allowed to cool and stored at 4^0C.

MAC CONKEY' AGAR (Dehydrated Hi- Media):

Peptone	20.00 gm
Sodium taurocholate	5.00 gm
Agar	20.00 gm
Neutral red	0.04 gm
Lactose	15.00 gm
Distilled water	1 Litre

This media was available in dehydrated form and was prepared and sterilized as per manufacturer's instructions. Plates were prepared by pouring 20 ml of molten media in sterile plates, stored at 4^0C.

EOSIN METHYLENE BLUE AGAR (Dehydrated Hi- Media):

Peptic digest of animal tissue	10.000gm
Dipotassium phosphate	2.000gm
Lactose	5.000gm
Sucrose	5.000gm
Eosin - Y	0.400gm
Methylene blue	0.065gm
Agar	13.500gm
Distilled water	1 Litre

Final pH (at 25°C) 7.2±0.2

This media was available in dehydrated form and was prepared and sterilized as per manufacturer's instructions. Plates were prepared by pouring 20 ml of molten media in sterile plates, stored at 4^0C.

XYLOSE-LYSINE DEOXYCHOLATE AGAR (Dehydrated Hi- Media):

Yeast extracts	3.000gm
L-Lysine	5.000gm
Lactose	7.500
Sucrose	7.500gm
Xylose	3.500gm
Sodium chloride	5.000gm
Sodium deoxycholate	2.500gm
Sodium thiosulphate	6.800gm
Ferric ammonium citrate	0.800gm
Phenol red	0.080gm
Agar	15.000gm
Distilled water	1 Litre

Final pH (at 25°C) 7.2±0.2

This media was available in dehydrated form and was prepared and sterilized as per manufacturer's instructions. Plates were prepared by pouring 20 ml of molten media in sterile plates, stored at 4^0C.

<u>SUBSTRATE UTILIZATION MEDIA</u>:

SIMMON'S CITRATE MEDIA (Dehydrated Hi-Media)-

Sodium chloride	5.00 gm
Magnesium sulphate	0.20 gm
Ammonium dihydrogen phosphate	1.00 gm
Dipotassium hydrogen phosphate	1.00 gm
Sodium citrate	2.00 gm

Agar	15.00 gm
Bromothymol blue	0.08 gm
Distilled water	1 litre

pH = 6.8± 0.2

This media was available in dehydrated form and was prepared sterilized as per manufacturer's instructions.

TRIPPLE SUGAR IRON MEDIUM (Dehydrated Hi-Media):

Peptone	20.00 gm
Yeast Extract	3.00 gm
Beef Extract	3.00 gm
Glucose	1.00 gm
Lactose	10.00 gm
Sucrose	10.00 gm
Ferrous ammonium sulphate	0.20 gm
Sodium chloride	5.00 gm
Sodium thiosulphate	0.30 gm
Phenol red	0.025 gm
Agar	15.00 gm
Distilled water	1 Litre

pH = 7.4 ± 0.2

This media was available in dehydrated form and was prepared and sterilized as per manufacturer's instructions.

SPECIAL MEDIA

MULLER HINTON BROTH (Dehydrated Hi-Media):

Lab lemco	300 gm
Casein hydrolysate	17.50 gm
Starch	1.5 gm

This media was available in dehydrated form and was prepared and sterilized as per manufacturer's instructions.

MULLER HINTON AGAR (Dehydrated Hi-media):

Lab lemco 300 gm

Casein hydrolysate 17.50 gm

Starch 1.50 gm

Agar 15gm

This media was available in dehydrated form and was prepared and sterilized as per manufacturer's instructions.

Table1: List of conventional antibiotics tested with their code and concentration

	Antibiotic Disc Himedia	**Code**	**Concentration**
1	Norfloxacin	NX	10µg\ml
2	Cefotaxime	CTX	30µg\ml
3	Ciprofloxacin	CIP	5µg\ml
4	Ceftazidime	CAZ	30µg\ml
5	Cefepime	CPM	30µg\ml
6	Amikacin	AK	30µg\ml
7	Nalidixic acid	NA	30µg\ml
8	levofloaxcin	LE	5µg\ml
9	Ofloxacin	OF	5µg\ml
10	Tobramycin	TOB	10µg\ml
11	Streptomycin	S	10µg\ml
12	Vancomycin	VA	30µg\ml
13	Netilitin (Netimicin sulphate)	NET	30µg\ml
14	Ciftriaxone	CTR	30µg\ml

15	Tazobactan\ceftazidime	CAT	30\10 µg\ml

Table 2: List of combination drugs tested with their code and concentration

Antibiotic name	**Code**	**Stock Concentration**	**Disc content used**
Potentox (Cefepime1000mg&Amikacin 0.25mg)	I	12500µg\ml	125 µg\µl
Supime (cefepime 1000mg &Sulbactum 500mg)	II	15000µg\ml	150 µg\µl
Elores (ceftriaxone 1000mg \ disodium edeate 37mg \ sulbactum 500mg)	III	15000µg\ml	150 µg\µl
Vancoplus (ceftriaxone 1000mg &vancomycin 500mg)	IV	15000µg\ml	150 µg\µl

❖ **MISCELLANEOUS ITEMS**

1. Disposable pipettes
2. Tips
3. Rubber teats
4. Compound microscope
5. Microtitre plates round bottom
6. Forceps
7. Metal loops
8. Metal straight wires
9. Petri dishes
10. pH meter
11. Autoclave
12. Test tube strand

Method

- **Procurement of samples**

  Samples were procured from regional hospital settings in Himachal Pradesh.

- **Enrichment of samples**

  Each sample was inoculated in 5ml nutrient broth and was cultured overnight in incubator cum shaker at 37°C.

Figure 2: Enrichment of clinical samples in nutrient broth

- **Differential selection of bacterial colonies**

Isolation of different bacteria present in clinical samples was done by culturing 20 µl of cultured broth on selection and differential media MaConkey agar, Eosine methylene blue agar and Xylose-lysine-deoxycholate agar (XLD) (Hi- Media).

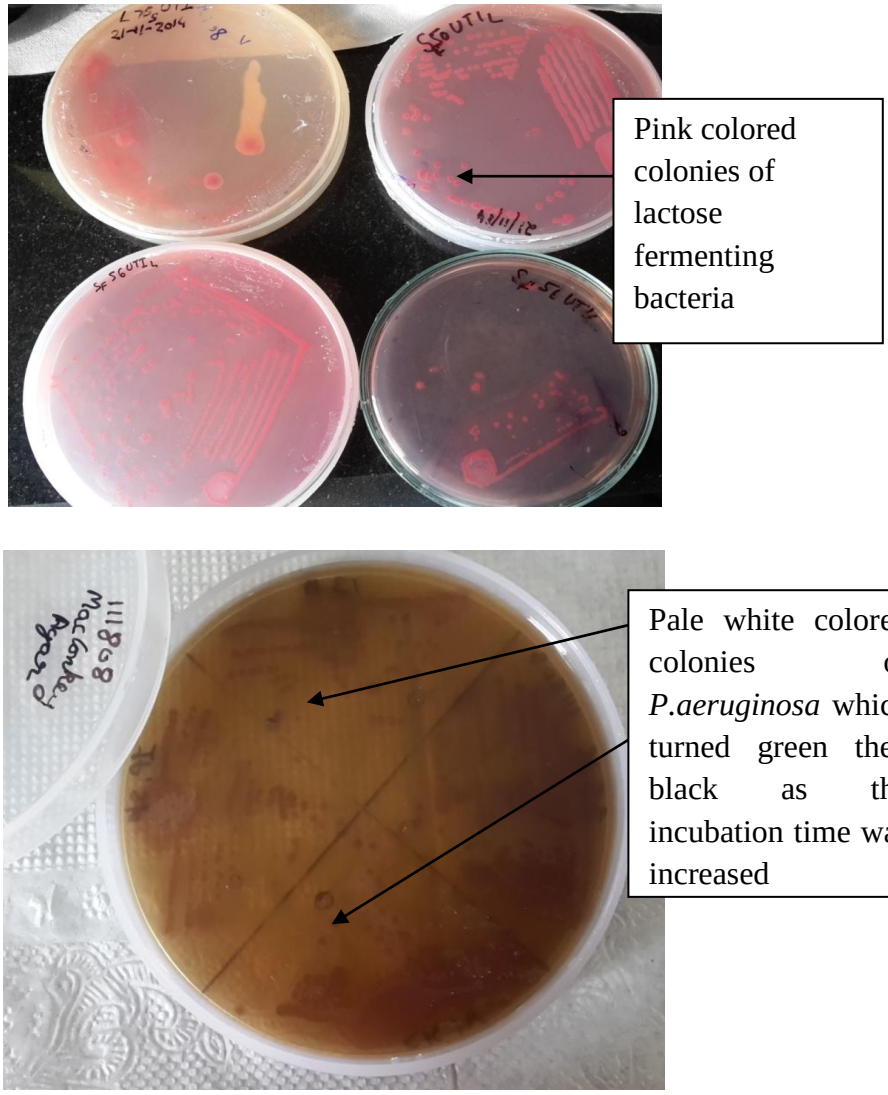


Figure 3- Lactose fermenting colonies on Mac Conkey agar

Figure 4- Bright yellow colonies on Xylose-lysin deoxycholate (XLD) agar

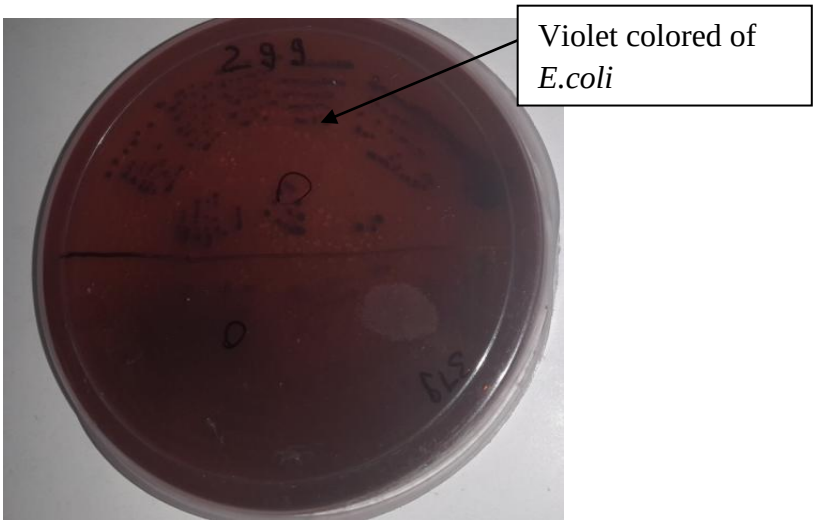


Figure 5- Sucrose and lactose fermenting colonies on EMB (Eosin-methylene blue) agar

Single isolated colony was inoculated in 5ml nutrient broth and was cultured overnight in incubator shaker at 37°C and further test were performed.

- **Biochemical characterization**

To characterize the specific bacteria following tests were done on the overnight grown culture:

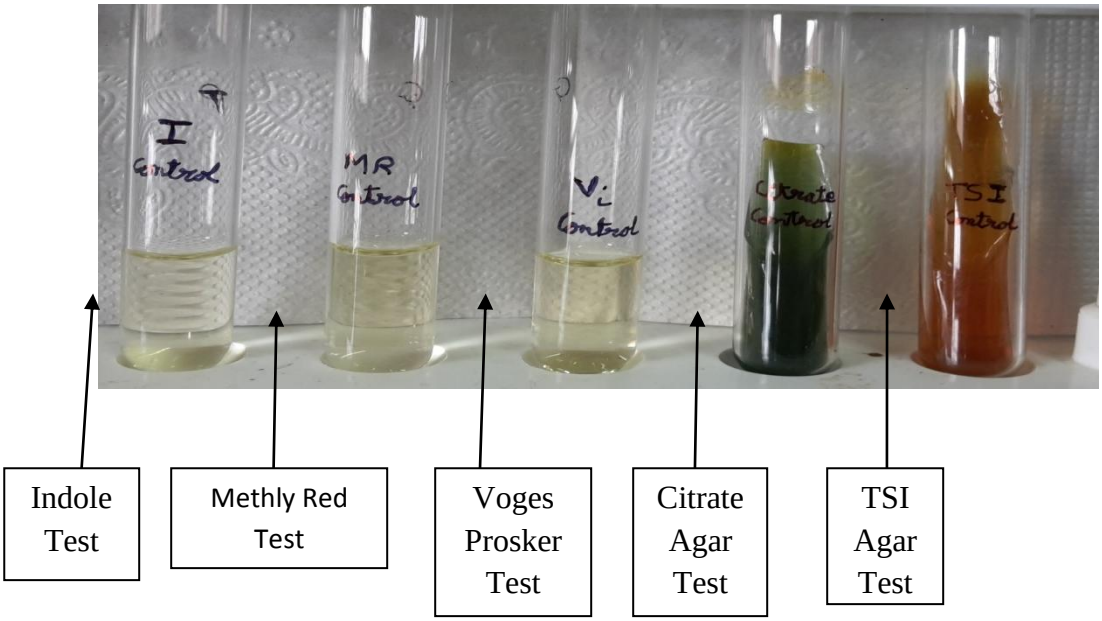
1. Indole test- 20 µl of culture was inoculated in 5 ml peptone broth. The culture was incubated overnight at 37°C. 200 µl of Kovac's reagent was added to this culture. Red ring at junction of culture and the reagent indicated positive result while a yellow ring indicated negative result.

2. Methyl Red test- 20 µl of culture was inoculated in 5 ml Glucose phosphate media broth. The culture was incubated overnight at 37°C. 200 µl of Methy Red reagent was added to this culture. Change of culture's color from yellow to red indicated positive result and no change in color indicated negative result.

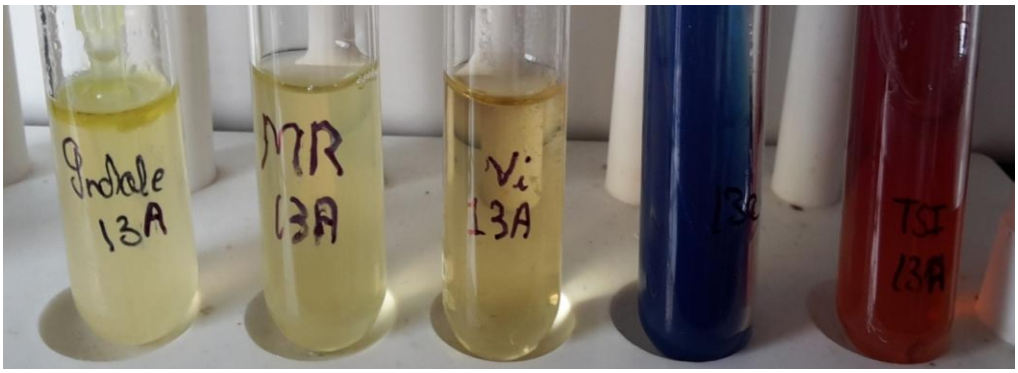
3. Vogus Proskauer test- 20 µl of culture was inoculated in 5 ml Glucose phosphate media broth. The culture was incubated overnight at 37°C. Then 5oo µl of 5% alpha naphathol and 1000 µl of 40% KOH were added to the broth. Appearance a cheery red color ring indicated the positive result and appearance yellow ring indicated negative result.

4. Citrate test- Bacteria was inoculated on Cimmon's Citrate Agar slant and incubated overnight at 37°C. Next day the change in color from green to blue indicated the positive results.

5. Triple Sugar Iron (TSI) test- Bacteria was inoculated on TSI Agar slant and butt and then incubated overnight at 37°C. the result was interpreted by observing the change in color of the slant and butt (A/A=yellow slant and yellow butt, K\A= red slant and yellow butt, A/K yellow slant and red butt and K/K red slant and red butt), production of hydrogen sulfide by change in color of culture to black and production of carbon dioxide gas by formation observing cracks in agar or levitation of the agar from the bottom of the test tube.



(a)



(b) Test Sample

Figure 6- (a) (b)- Shows biochemical characterization of isolates using Indole, Methyl Red, Voges Prausker, Citrate agar and Triple Sugar Iron agar test(test sample is ---+, k/k)

6. Catalase test- a single isolated colony of *P. aeruginosa* was immobilized on a glass slide and normal saline was added to homogenize the colony. Then absolute hydrogen peroxide was added to it. The enzyme catalase bacteria convert hydrogen peroxide into water and oxygen which results in frothing on applied area of the glass slide.

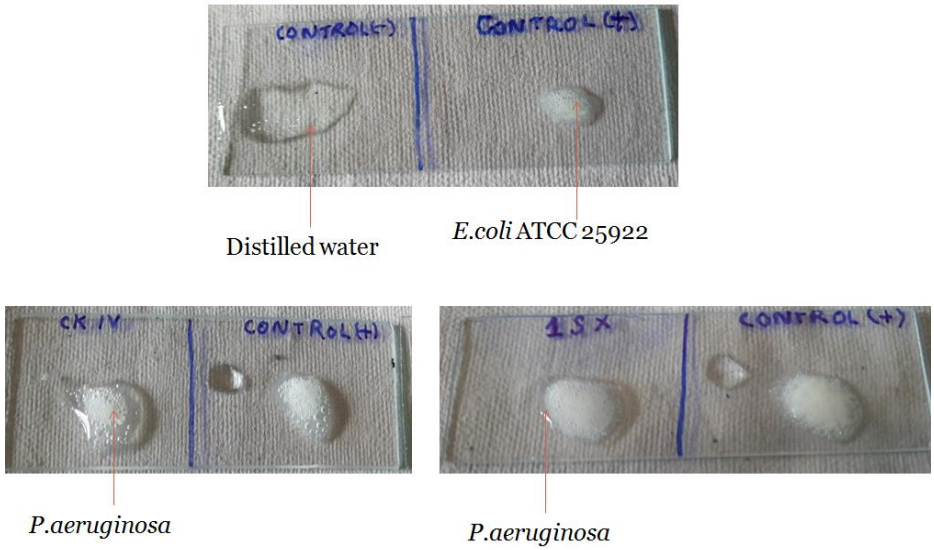


Figure 7- Shows catalase test results for *P.aeruginosa*

- **Antibiotic susceptibility testing**

After characterization of bacteria antibiotic susceptibility test was done for each isolated and characterized bacteria by following the Kerby Bauer method [93].

1. Inoculum was prepared from the cultured broth used during the biochemical test by transferring 20 µl to a tube containing 10 ml nutrient broth and allowed to grow for 3-4 hours at 37°C in incubator shaker till the cultured broth reached the desired OD_{625} of 0.5-0.6.

2. 100 µl of this culture broth was taken and spread on the Muller Hinton agar plate with the help of a glass spreader. The culture was allowed to dry for a few minutes at room temperature with the lid closed.

3. The antibiotic discs were placed on the inoculated plates using forceps.

4. These plates were then incubated at 37°C for 16-18 hours.

5. The diameter of zone of inhibition was measured using venire calipers on the under-surface of the plate containing transparent medium. The diameter of zone of inhibition was measured in mm.

6. The sizes of the zones of inhibition were interpreted by referring through 2I (Zone Diameter Interpretative Standards) of the CLSI guidelines [94].

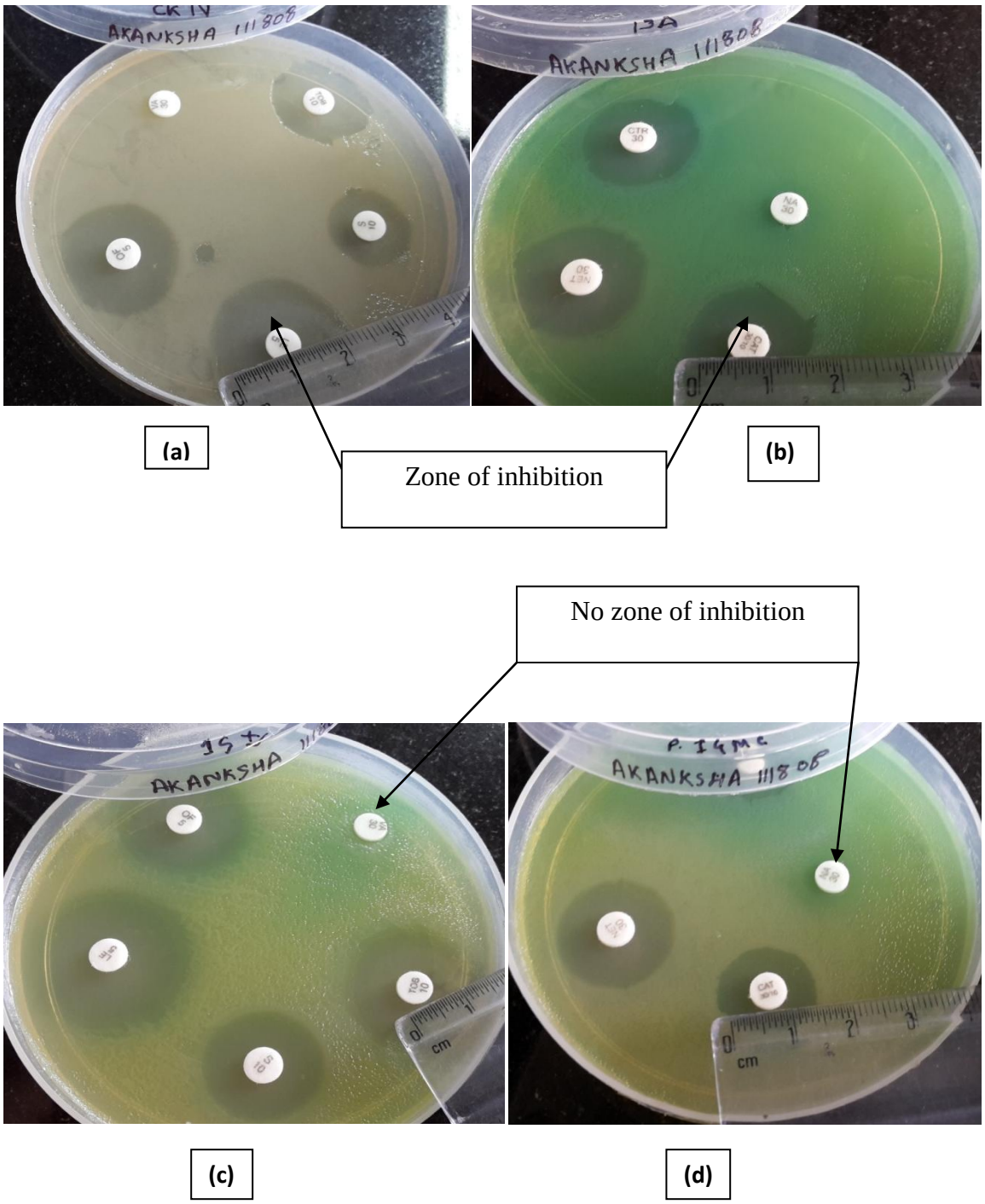


Figure 8- Depicts different diameters of zone of inhibition against different isolates of *P.aeruginosa* against tested antibiotics of class beta-lactams, aminogycoside, furoquinolone and glycopeptide.

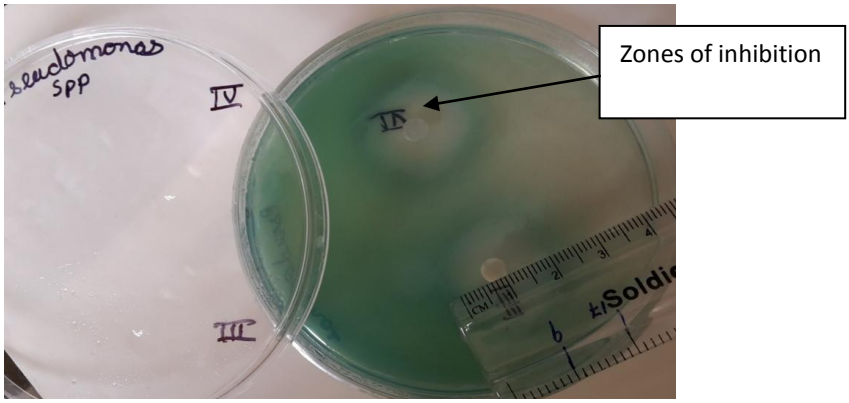


Figure 9- Shows antibiotic sensitivity test of combination drugs (ceftriaxone\disodium edeate\sulbactum III and ceftriaxone& vancomycin) against *Pseudomonas* spp.

- **MIC determination**

Minimum inhibitory concentration for each isolate was determined by the following method [95]:

1. Antibiotic stock solution was prepared by commercially available antimicrobial powders (with given potency) and the amount needed and the diluents in which it was dissolved was calculated by using the following formula to determine the amount of powder (1) or diluents (2) needed for a standard solution:

$$\text{Weight (mg)} = \frac{\text{volume (ml)} * \text{concentration (µl /ml)}}{\text{Potency (µg/mg)}}$$

$$\text{Volume (ml)} = \frac{\text{weight (mg)} * \text{potency (µg/mg)}}{\text{Concentration (µl/ml)}}$$

2. Antimicrobial agent stock solutions were prepared at concentrations of at least 1000 µg/mL or 10 times the highest concentration to be tested.

3. Small volumes of the sterile stock solutions were dispensed into eppendorf vials; carefully seal; and store (at −20 °C or below,).

4. For each antibiotic two-fold dilution range was made starting from 20mg/ml to 0.625mg/ml and preserved at 20 °C or below in sterile eppendorf vials.

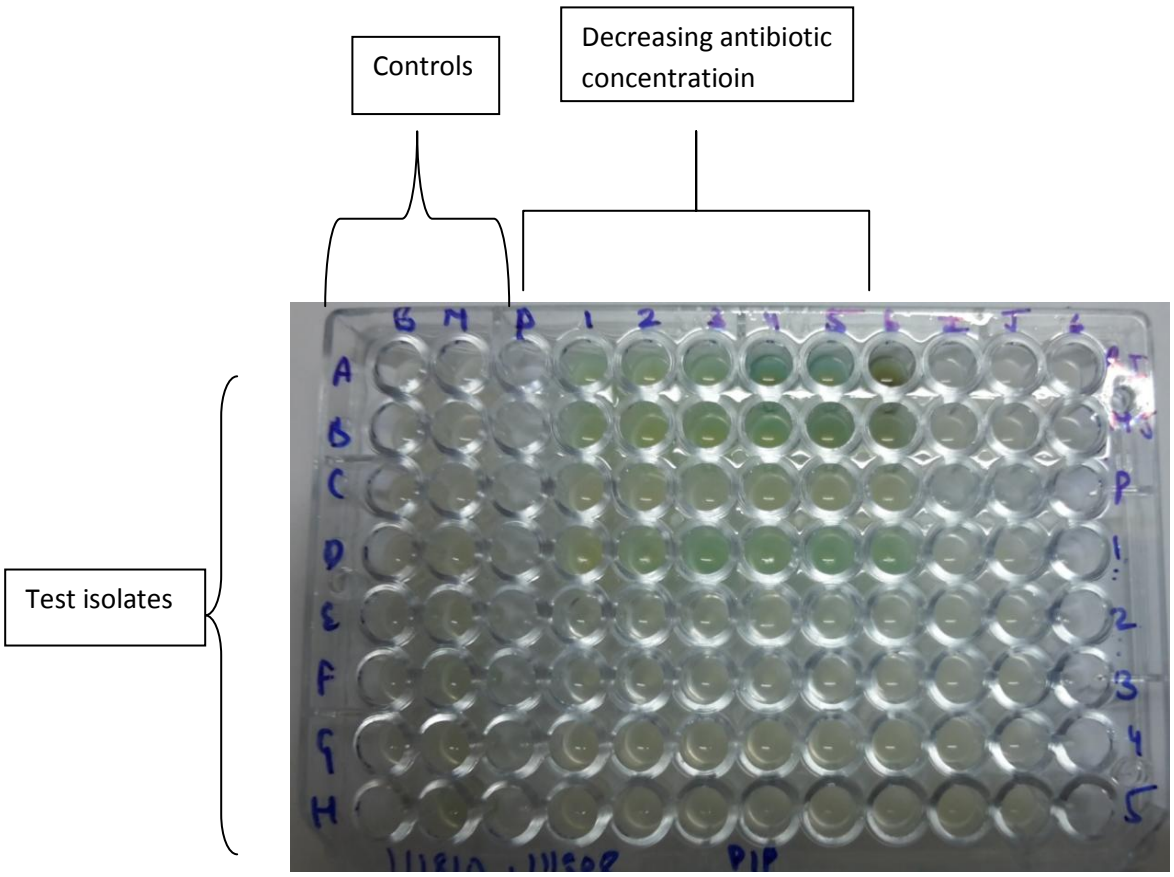
5.Single isolated colony was picked from the nutrient agar plated which was streaked with preserved stocks from previous experiment and inoculated in 10ml of Muller Hinton broth. It was then incubated at 37° C in incubator shaker till the cultured broth reached the desired OD_{600} 0.4-0.5 is reached which indicates bacterial concentration of 10^4 to 10^5 CFU/ml.

6. Then on a sterile U-bottom 96 well microtire well plate 95 µl pure bacterial culture of test organism was dispensed in column number 10 to 4. This was followed by addition of test antibiotic in the order of increasing concentration from column 10 to 4. Column 1 was taken as bacterial control in which 95µl teat bacteria broth and 5µl of sterile water was dispensed. Column two was taken as media control in which only 95µl sterile culture media (Muller Hinton broth) and 5µl of sterile water was dispensed. Column 3 was taken as the plate control which was left empty. Each row consisted of a different test bacteria and each column from 10 to 4 consisted of different concentration of the test antibiotic. A single plate was used test eight different test bacteria and one test antibiotic having six different concentrations.

7. The plates were then covered and incubated at 37^oC.

8 .When satisfactory growths were obtained (18-36 hours) the plates were scanned with an ELISA reader (Thermo Reader) at 600nm.

9. MIC was taken as the lowest concentration of drug that reduces, by more than 50% or 90% for MIC_{50} or MIC_{90} respectively.



(a)

Figure 10- Shows U-bottom microtitre 96 well plate used to determine the MIC for piperacillin (a). Column B represents bacterial control, column M represents media control and column P represents plate control. Columns 1 to 6 have decreasing antibiotic concentrations of 2000 µg/100ml , 1000 µg/100ml, 500 µg/100ml, 250 µg/100ml, 125 µg/100ml and 62.5µg/100ml. each row represents different bacterial isolate. Rows A, B, C and D have *P. aeruginosa* isolates.

- **Glycerol stocks preparation:**

For glycerol stock preparation 500µl of 30% sterile glycerol solution and 500µl of log phase culture of single isolated were mixed in a sterile eppendorf vial. The glycerol stocks were labeled; sealed; and then stored at -80°C.

Chapter 5

Results

Identification of different bacteria

Clinical samples enriched in nutrient broth were cultured on non selective and non differential nutrient agar and selective media MacConkey agar, XLD agar and EMB agar. Bacteria showing different colony morphologies were observed **(see figure 3, 4, 5)**.

Biochemical characterization of bacterial colonies

Using Indole, Methyl Red, Vogus Proskauer, Citrate agar, TSI agar and catalase test biochemical characterization was performed (**see figure 6 and 7**). Bacteria showing different biochemical profiles were identified **(see appendix 1).**

The prevalence nce rate of different bacterial species found is as follows: *E.coli* 47%, *Klebsiella* spp. 15%, *Shilgella* spp 7%, *Pesudomonas* spp 5%, *Proteus* spp. 4%, *Citrobacter* spp. 1% and organism of unknown etiology 20% (**see figure 11**). *P.aeruginosa* isolates were found in whole blood, urine and pus samples (**see table 3**).

Table 3: Prevelance of *P.aseruginosa* in different clinical samples

S.No	Sample name	Sample type
1	1P	
2	CK IV	Whole blood
3	1S X	Pus
4	13A	Urine

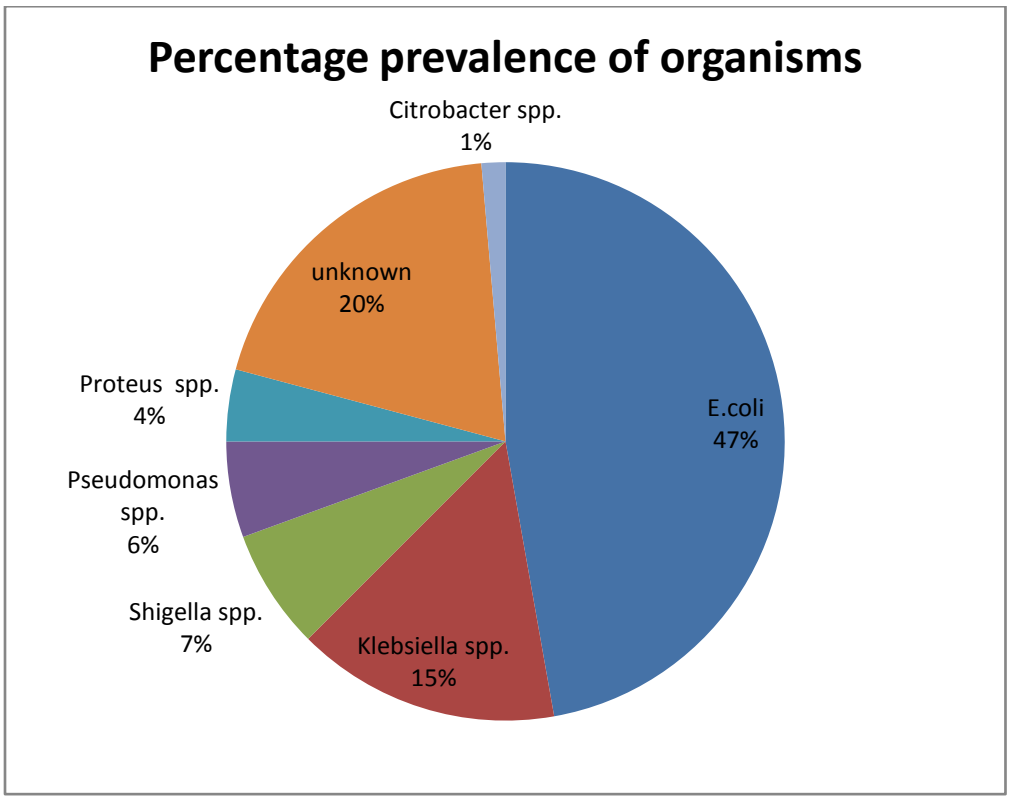


Figure 11- shows the percentage prevalence of various organisms in the tested clinical isolates

Antibiotic susceptibility testing

AST was performed on all the bacterial isolates identified using Kerby bauer method for conventional and combination drugs (**see figure 8 and 9**). The diameters of zone of inhibition for different subclasses of beta-lactams were measured and interpreted (**see table 4**). The percentage susceptibility of *Pseudomonas spp*. for cefotaxime i.e. 25% and resistance rate against the antibiotic was 50% (**see figure 12**). The percentage susceptibility of *Pseudomonas spp*. for cefepime was 25% and 75% resistance rate was observed against the antibiotic (**see figure 13**). The percentage resistance of 25% and percentage susceptibility of 50% was observed in *Pseudomonas spp*. for ceftazidime (**see figure 14**). The percentage susceptibility of *Pseudomonas spp*. for cefotaxime i.e. 25% and resistance rate against the antibiotic was 50%. The percentage susceptibility of *Pseudomonas spp*. for ceftriaxone of 75% and 0% resistance against the antibiotic were observed (**see figure 15**).

The diameters of zone of inhibition for different subclasses of amnioglycoside were measured and interpreted (**see table 5**). The percentage susceptibility of *Pseudomonas spp*. for amikacin of 75% and

25% resistance rate were observed against the antibiotic **(see figure 16).** All the isolated *Pseudomonas* spp. were 100% sensitive to tobramycin, netimicin sulphate and streptomycin (**see figure 17 and 18**).

The diameters of zone of inhibition for different subclasses of fluoroquinolones and glycopeptides were measured and interpreted (**see table 6**). The percentage susceptibility of *Pseudomonas spp*. for ciprofloaxcin and norfloaxcin 50% and 75% respectively and the resistance rate of 25% were observed for both the antibiotics **(see figure 19 and 20**). 100% susceptibility was seen in case of ofloaxcin and levofloaxcin (**see figure 21**). The isolated *Pseudomonas* spp. were 100% resistant to nalidixic acid and vancomycin (**see figure 22**).

The diameters of zone of inhibition for different combinational drugs were also measured. There interpretation was done on the basis of comparison with the diameter of zone of inhibition observed for their individual drug constituents (**see tables 7, 8, 9 and 10**). The combinational drugs were found to be slightly more sensitive than their conventional drugs.

Table 4- Diameter of zone of inhibition (in mm) for each isolate against all the beta-lactams tested along with their interpretation

	Beta-Lactamas				
	CPM	**CTX**	**CTR**	**CAT**	**CAZ**
1P	30mm\S	26mm\S	18mm\S	16mm	25mm\S
CKIV	18mm\R	19mm\I	22mm\S	21mm	18mm\S
1SX	15mm\R	12mm\R	15mm\I	19mm	15mm\I
13a	7mm\R	11mm\R	20mm\S	15mm	6mm\R

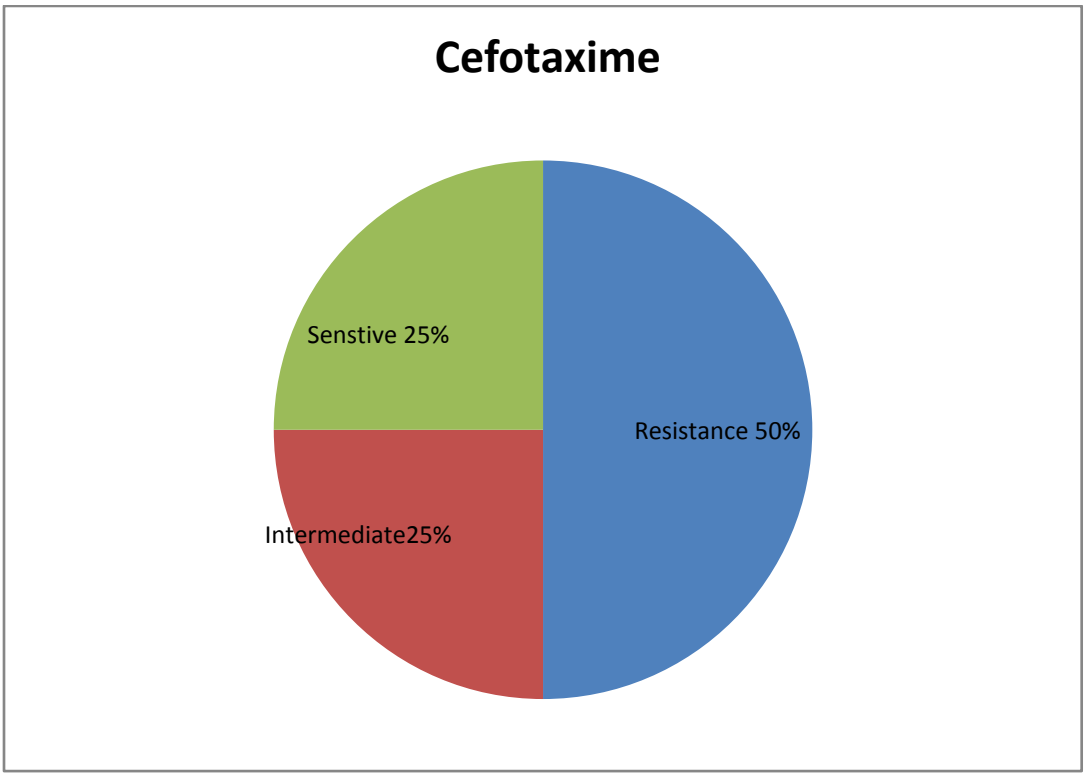


Figure 12- Sensitivity distribution patterns of *Pseudomonas spp*. for cefotaxime

The percentage susceptibility of *Pseudomonas spp*. for cefepime was 25% and 75% resistance rate was observed against the antibiotic.

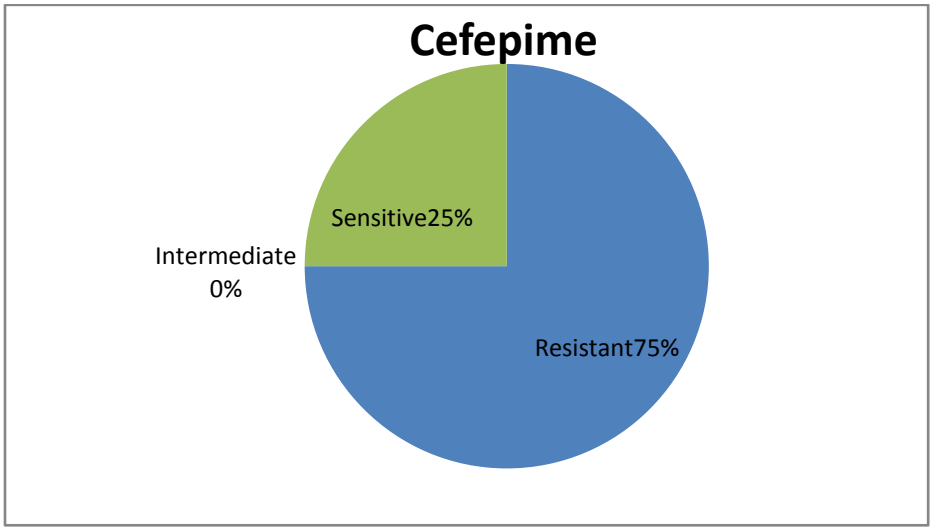


Figure 13- Shows sensitivity distribution patterns of *Pseudomonas spp*. for cefepime

The percentage resistance of 25% and percentage susceptibility of 50% was observed in *Pseudomonas spp*. for ceftazidime.

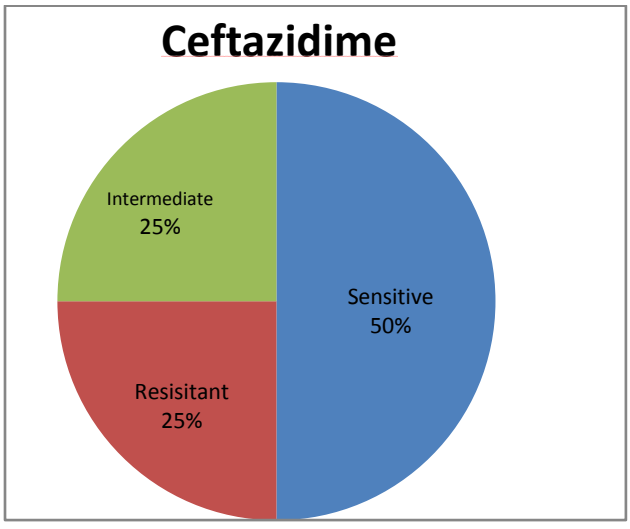


Figure 14-Shows sensitivity distribution patterns of *Pseudomonas spp*. for ceftazidime

The percentage susceptibility of *Pseudomonas spp*. for ceftriaxone of 75% and 0% resistance against the antibiotic were observed

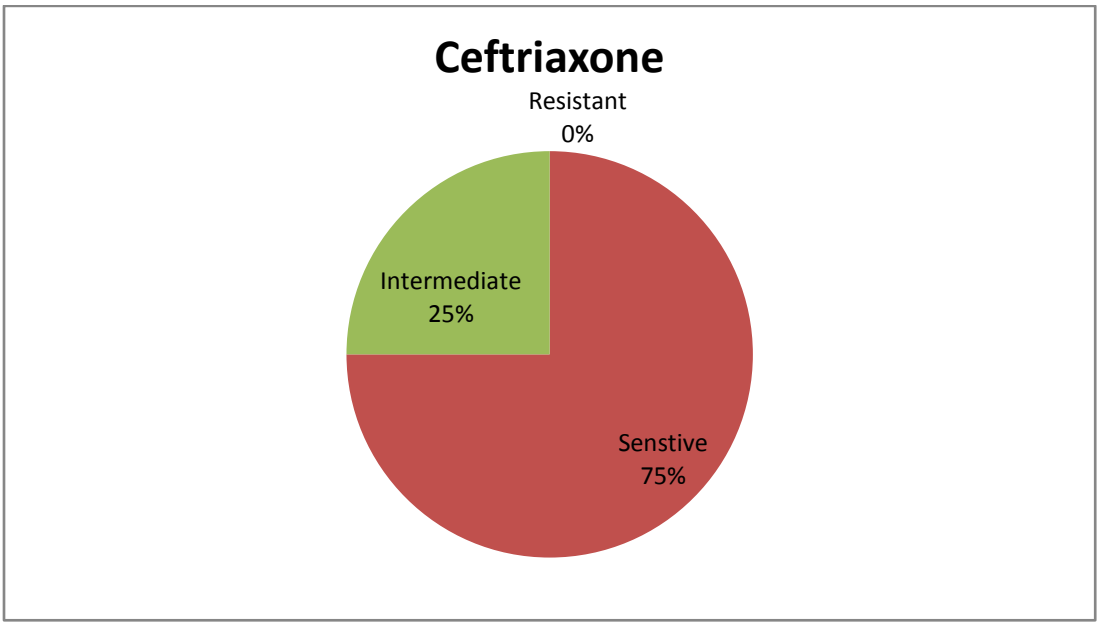


Figure 15- Shows sensitivity distribution patterns of *Pseudomonas spp*. for ceftriaxone

Table 5: Diameter of zone of inhibition (in mm) for each isolate against all the aminoglycosides tested along with their interpretation

			Aminoglycosides	
	TOB	**NET**	**AK**	**S**
1P	22mm\S	20mm\S	24mm\S	20mm\S
CKIV	21mm\S	16mm\S	20mm\S	16mm\S
1SX	20mm\S	21mm\S	20mm\S	18mm\S
13a	26mm\S	20mm\S	5mm\R	20mm\S

The percentage susceptibility of *Pseudomonas spp*. for amikacin of 75% and 25% resistance rate were observed against the antibiotic.

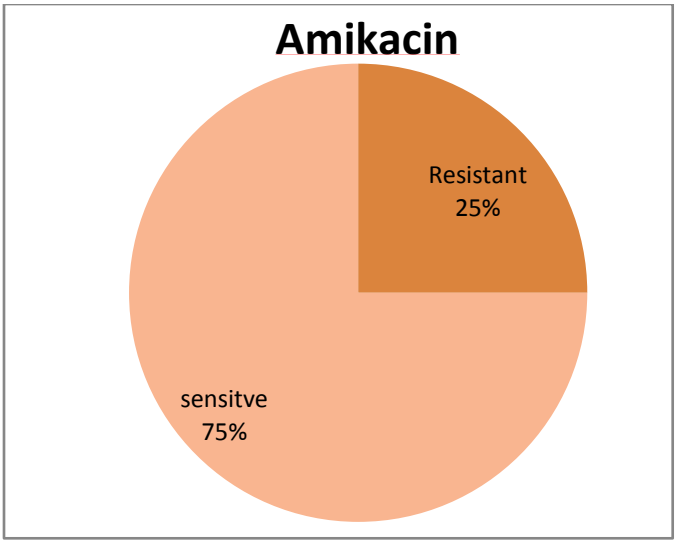


Figure 16- Shows sensitivity distribution patterns of *Pseudomonas spp*. for amikacin

All the isolated *Pseudomonas* spp. were 100% sensitive to tobramycin, netimicin sulphate and streptomycin.

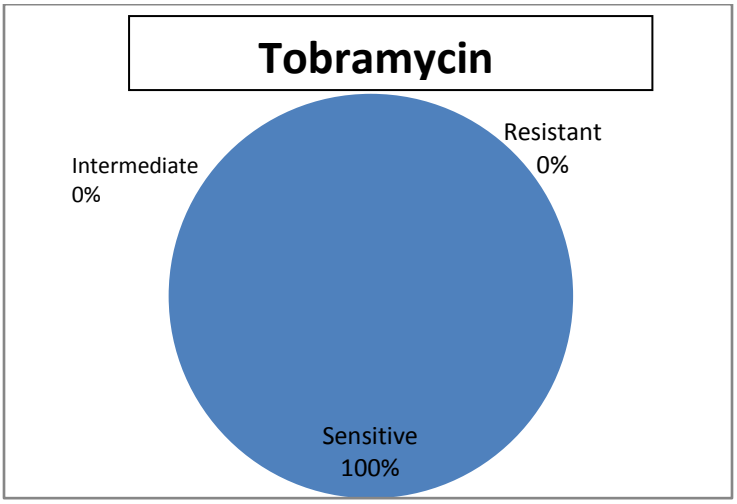
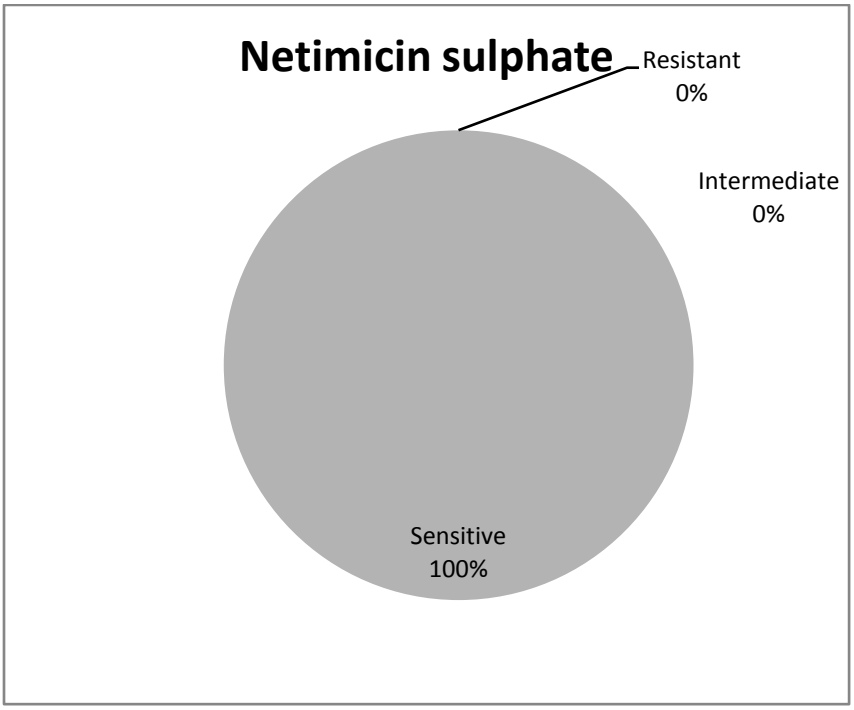


Figure 17- Shows sensitivity distribution patterns of *Pseudomonas spp*. for tobramycin



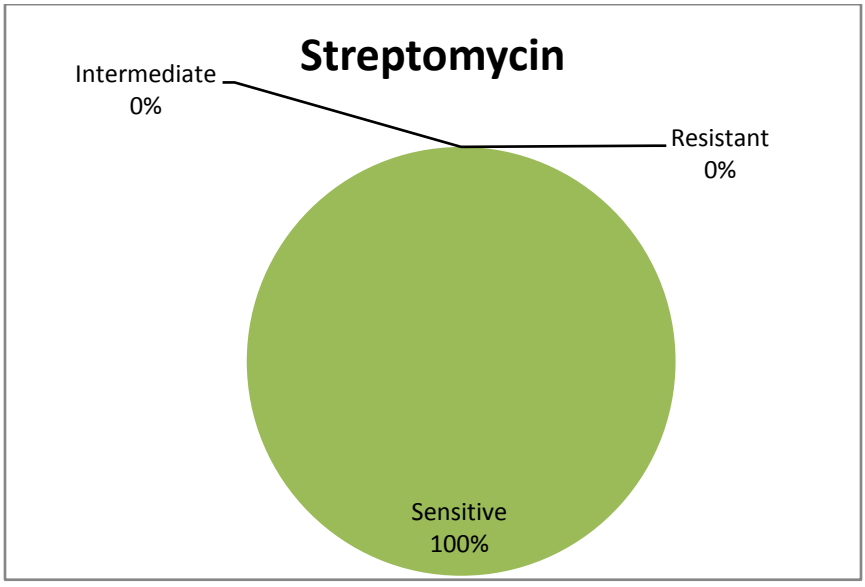


Figure 18- Shows sensitivity distribution patterns of *Pseudomonas spp*. for netimicin sulphate and streptomycin

Table 6- Diameter of zone of inhibition (in mm) for each isolate against all the fluoroquinolone and glycopeptides tested along with their interpretation

	Quinolnes					Glycopeptides
	NX	**NA**	**OF**	**LE**	**CIP**	**VA**
1P	29mm\S	0mm\R	20mm\S	25mm\S	32mm\S	00mm\R
CKIV	22mm\S	11mm\R	21mm\S	24mm\S	18mm\I	00mm\R
1SX	19mm\S	0mm\R	19mm\S	26mm\S	28mm\S	00mm\R
13a	5mm\R	0mm\R	21mm\S	22mm\S	6mm\R	00mm\R

The percentage susceptibility of *Pseudomonas spp*. for ciprofloaxcin and norfloaxcin 50% and 75% respectively and the resistance rate of 25% were observed for both the antibiotics

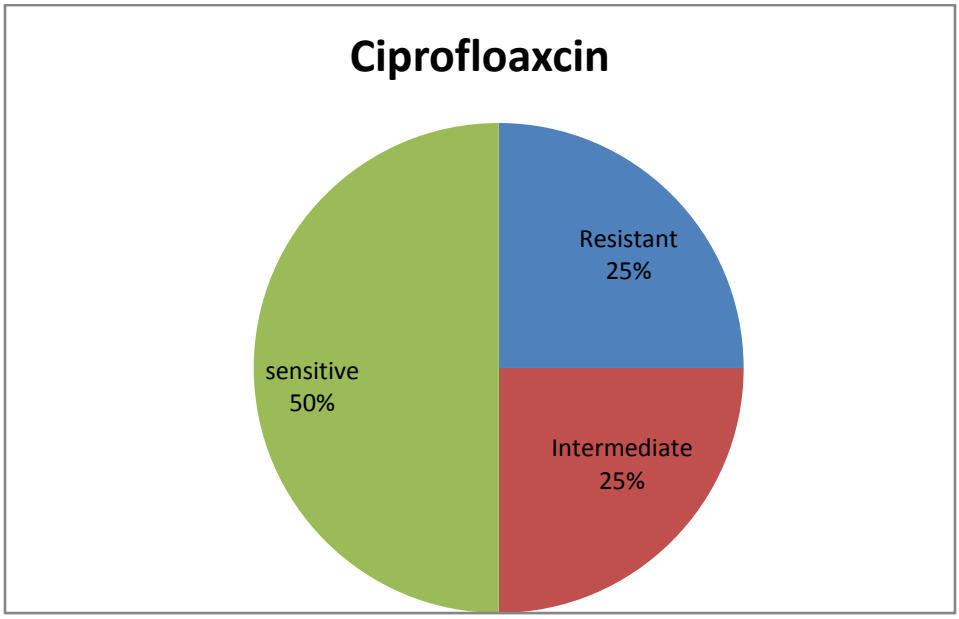


Figure 19- Shows sensitivity distribution patterns of *Pseudomonas spp*. for ciprofloxacin

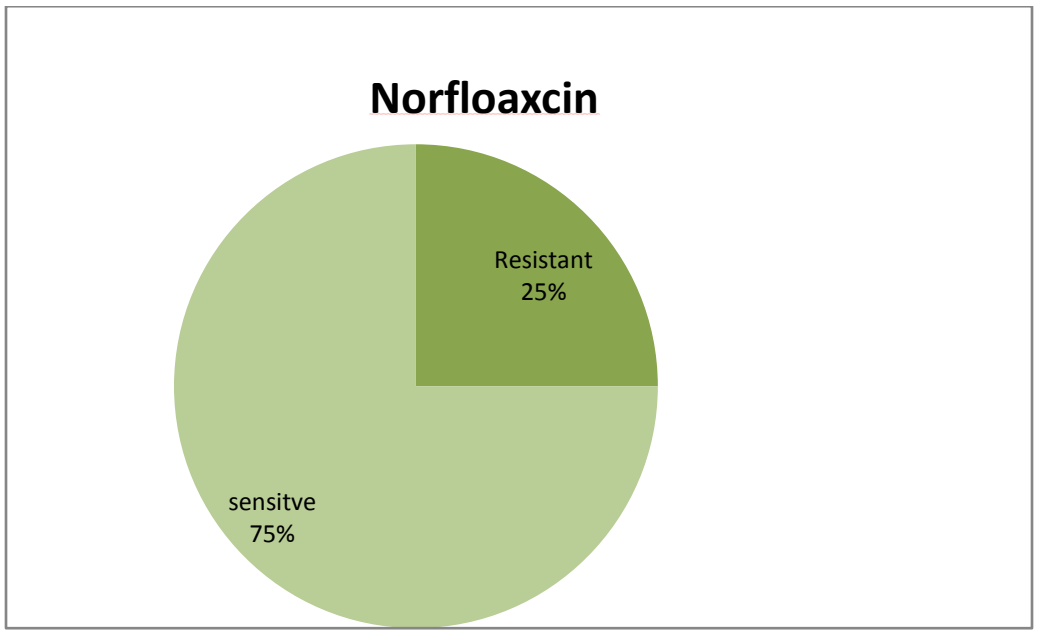
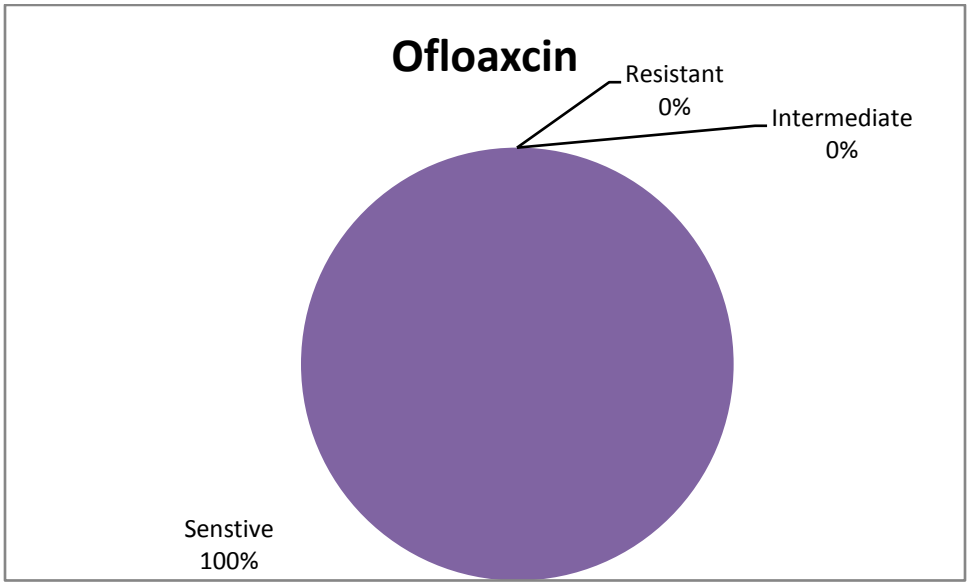


Figure 20- Shows sensitivity distribution patterns of *Pseudomonas spp*. for Norfloaxcin

100% susceptibility was seen in case of ofloaxcin and levofloaxcin.



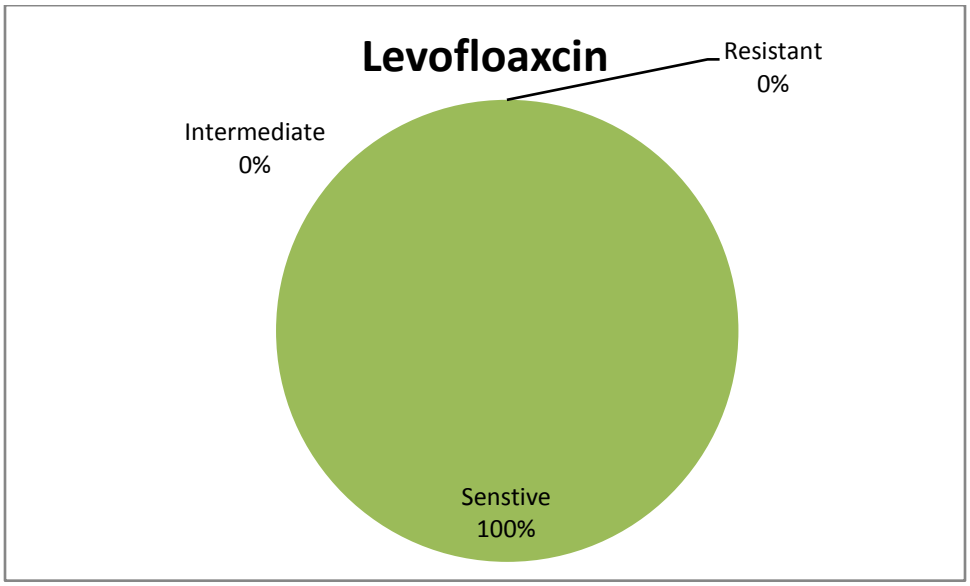
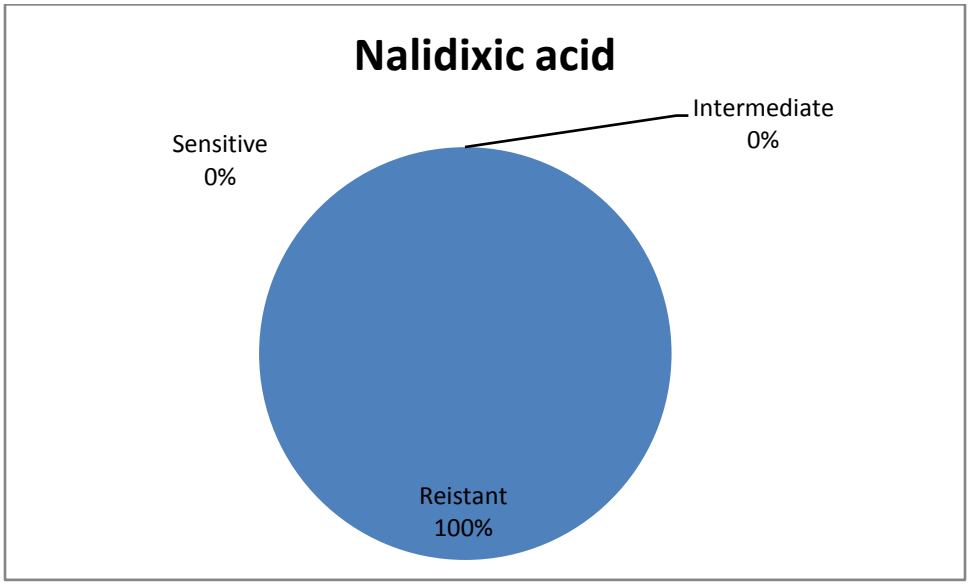


Figure 21- Shows sensitivity distribution patterns of *Pseudomonas spp*. for ofloaxcin and levofloaxcin

The isolated *Pseudomonas* spp. were 100% resistant to nalidixic acid and vancomycin.



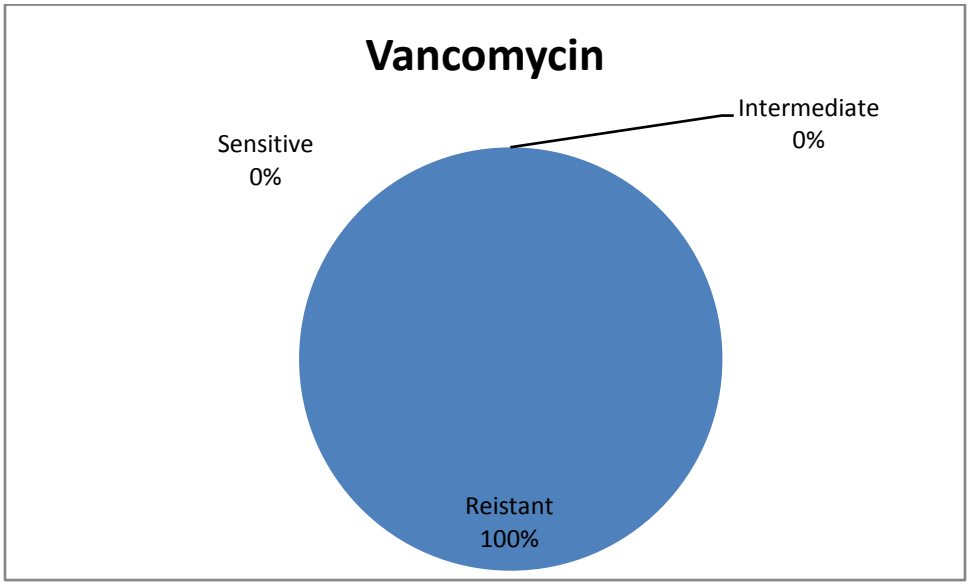


Figure 22- Shows sensitivity distribution patterns of *Pseudomonas spp*. for nalidixic acid and vancomycin

Table 7 - Interpretation of combination drug Potentox (cefepime and amikacin)

Sample name	CPM	AK	I	Inference
1P	30mm\S	24mm\S	26mm	Comparable
CK IV	18mm\R	20mm\S	19mm	Comparable
1S X	15mm\R	20mm\S	25mm	Synergism
13A	7mm\R	5mm\R	17mm	Synergism

Table 8- Interpretation of combination drug Supime (cefepime and sulbactam)

Sample name	CPM	II	Inference
1P	30mm\S	26mm	Comparable
CK IV	18mm\R	21mm	Comparable
1SX	15mm\R	28mm	Synergism
13A	7mm\R	18mm	Synergism

Table 9- Interpretation of combination drug Elores (cefrtiaxone, disodium EDTA and sulbactam)

Sample name	CTR	III	Inference
1P	18mm\S	22mm	synergism
CK IV	22mm\S	15mm	synergism
1SX	15mm\S	29mm	Synergism
13A	20mm\S	20mm	comparable

Table 10- Interpretation of combination drug Vancoplus (ceftriaxone and vancomycin)

Sample name	CTR	VA	IV	Inference
1P	18mm\S	0mm\R	20mm	comparable
CK IV	22mm\S	0mm\R	16mm	synergism
1S X	15mm\S	0mm\R	29mm	synergism
14A	20mm\S	0mm\R	22mm	comparable

Minimum inhibitory concentration breakpoints for aminoglycosides amikacin were observed at the concentration of 250µg/100ml and for gentamycin it was observed at 125µg/100ml. In case of beta-lactam piperacillin MIC breakpoint was observed at 1000µg/100ml and for fluoroquinolone ciprofloxacin it was found at 1000µg/100ml. this shows that lower dosage of amikacin and gentamycin are effective against inhibiting the growth of *P.aeruginosa* whereas high dosage maybe required in case of treatment with beta-lactams and fluoroquinolone piperacillin and ciprofloxacin respectively.

Discussion

Biochemical tests for characterization of clinical isolates were performed on 73 samples among them 4 were tested positive for *P. aeruginosa*, thus having a prevalence rate of 6%. *E.coli* was observed as the dominant bacteria having occurrence rate of 47% where as *Klebsiella* spp. was the subdominant bacteria with prevalence of 15% followed by *Shigella* spp. 7%, *Proteus* spp 4% and *Citrobacter* spp. 1%. 20% bacteria were of unknown etiology and require further testing. The prevalence rate of *P. aeruginos* observed in our study is significantly lower than those found by Gunjan Shrivastava *et al.*, 2014 [101], 21.85%, Indu Biswal *et al.*, 2014 [98], 66.07% and Ved Prakash *et al*., 2014 [102], 21.85%.

In our study we found the isolates were sensitive to aminoglycosides (amikacin 75%, tobramycin 100%, streptomycin 100%, and netlitin 100%). The percentage of sensitive isolates were markedly higher than the findings of other groups like Shrivastav et al., 2014 [101] reported percentage sensitivity of 71.7% and 43% for tobramycin and amikacin respesctively. In onaother study conducted by Indu Biswal *et al.*, 2014 [98], *P. aeruginosa* showed 18.96% sensitivity towards amikacin and 31.3% sensitivity towards netilitin. In a study conducted by Ved Prakash *et al*., 2014 [102], susceptibility of 62.7% for amikacin was reported. Low susceptibility rate of 23.67% was reported for streptomycin by Indu Biswal *et al.*, 2014 [98] against *P. aeruginosa.*

In case of fluoroqinolones percentage resistance rate of 25% was found for ciprofloxacin which is comparable with the findings of Shrivastav et al., who reported percentage resistance of 22.3%. This is also in agreement with the findings of Akhiles *et al* 2014, and Indu Bisawal et al 2014, [98,99] who reported lower rates of resistance for ciprofloxacin in *P. aeruginosa*, 13% and 12.06% respectively, in their recent studies. For ofloaxcin and levofloaxcin 100% sensitivity was found in our study. This finding is in agreement with the findings of Nakade et al., who reported high sensitivity of *P. aeruginosa* towards levofoaxcin and moderately high sensitivity towards ofloaxcin. Jombo *et al*., [96] also reported high rate of sensitivity 92% for ofloaxcin in his study in 2008. *P. aeruginosa* was found to be totally resistance to nalidixic acid this proves the failure of antibiotic activity of the drug against it. Resistance rate of 25% was found for norfloaxcin.

Different generations of beta-lactam class cephalosporin were tested in our study. Fourth generation cephalosporin cefepime showed sensitivity rate of 25% and third generation cephalosporin ceftazidime which showed sensitivity rate of 50% against *P. aeruginosa.* This is comparable with the findings of and Indu Biswal *et al*., 2014 and Ved Prakash *et al*., 2014, [98,102] who reported low rate of sensitivity for these aforementioned antibiotics in their studies. Cefotazime showed percentage resistance of 50% this agrees with the reported percentage resistance for cefotazime (60.47%) by Ved Prakash *et al*., 2014 [102]. High Sensitivity rate of 75% was observed in case of ceftriaxone. Similar sensitivity rate was reported by Olayinkal *et al*. in his study [97].

P.aeruginosa isolates in our findings showed highest sensitivity towards all sub- classes of aminoglycosides followed by fluoroquinolones levofloaxcin, ofloaxcin, ciprofloxacin and norfloaxcin. Isolates were totally resistant to naldixic acid and glycopeptides vancomycin. Increase in resistance rates were observed in case of beta-lactams cefepime and ceftazidime among the isolates.

Among the combination drugs tested Potentox (cfepime and amikacin), Supime(cefeipme and sulbactum), Elores (ceftriaxone, EDTA and sulbactum) and Vancoplus(ceftriaxone and vancoplus) none of the combination showed slight synergistic effect as the diameter of zone of inhibition for individual drug is comparable with the combination drug and no marked increase in the zone of inhibiton was observed. This finding in our study contradicts with the findings of Srinivas *et al*., 2014 and Akhilesh *et al*., 2014 [99,100], who showed synergism in their antibiotic combinations.

Minimum inhibitory concentration for aminoglycoside amikacin was observed at the concentration of 250µg/100ml and for gentamycin it was observed at 125µg/100ml, this shows that these isolates were

sensitive towards these antibiotics as their MIC are well below the breakpoint recommended by CLSI. Elevated MIC were observed in case of beta-lactam piperacillin: 1000µg/100ml and for fluoroquinolone ciprofloxacin it was found at 1000µg/ 100ml, which were above the CLSI recommended breakpoints indicating that the isolates were resistant against to these antibiotics. These results co-relate with the observations made after the analysis AST results i.e. the isolates showed sensitivity towards aminoglycosides and increased resistance towards flouroquniolones and beta-lactams.

Conclusion

Though the prevalence rate of *P.aeruginosa* in our finding is relative lower compared to findings reported in other parts of the country. However these isolates showed higher rate of resistance against different classes of antibiotics than other species isolated during the course of the study. So, it becomes important to maintain a constant surveillance on *P.aeruginosa* to deduce the recent trends of prevalence and antibiotic resistance to help prevent the outbreak of this pathogen.

Determination of antibiotic susceptibility pattern can help to choose the best choice of antimicrobial therapy. *P.aeruginosa* isolates resistant to various classes of antibiotics are emerging worldwide and the recent resistance or reduced susceptibility to carbapenems is considered a serious clinical threat due to their role as first choice of therapy. The antimicrobials are losing their efficacy because of the spread of resistant organisms due to indiscriminate use of antibiotics, lack of awareness and lack of antibiotic testing facilities. So continuous monitoring of emergence of resistance trends of *P.aeruginosa* is essential in health care centers.

As *P.aeruginosa* is becoming resistant to more and more antibiotics, mono-therapy of *P.aeruginosa* infections is becoming redundant. Rational combination drug therapy, combining different classes of antipseudomonal drugs and beta-lactamse inhibitors should be used cure the infections and prevent the development of resistant strains of the bacteria.

The experience with the isolates suggested that the surveillance for multi-drug-resistant *P.aeruginosa* should be maintained and careful infection control measures and cautious use of antibiotics must be promoted. The solution can be planned by continuous efforts of clinicians, microbiologist, pharmacists and community to promote great understanding of this problem.

Research in the area of finding novel approaches in overcoming the development and spread of multi-drug-resistant pathogens, such as development anti-pseudomonal vaccines and nanodrugs should be promoted by the government and academic and research institutes.

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Appendix 1

Biochemical test results for different bacteria isolated from clinical samples

							BIOCHEMICAL TEST RESULTS
S.No	**Sample name**	**Indole**	**MR**	**VP**	**Citrate**	**TSI**	**Organism**
1	1SI	NEV	NEV	NEV	POS	A\A	*Proteus.mirabilus*
2	1SII	NEV	POS	NEV	POS	A\A	*unk*
3	1SIII	NEV	POS	NEV	NEV	A\A	*Shigella.spp*
4	1SIVR	POS	NEV	NEV	NEG	A\A	*Proteus.mirabilus*
5	1SIVW	NEV	NEV	POS	POS	A\A	*Klebsiella.pneumonia*
6	1SVIII	NEV	POS	POS	POS	A\A	*Klebsiella.pneumonia*
7	1SX	NEV	NEV	NEV	POS	K\K	*Pseudomonas.aeruginosa*
8	NAVDEEP	NEV	POS	NEV	NEV	A\A	*Shigella.app*
9	SFUTIL	NEV	POS	NEV	NEV	A\A	*E.coli*
10	PUS3S	NEV	NEV	NEV	NEV	A\A	*UNK*
11	2LF	NEV	POS	POS	POS	A\A	Klebsiella.pneumonia
12	SF50LUTI	NEV	POS	POS	POS	A\A	*Klebsiella.pneumonia*
13	L75LUTI	POS	POS	NEV	NEV	A\A	*E.coli*
14	SF56SUTI	POS	POS	NEV	NEV	A\A	*E.coli*
15	SF56LUTI	POS	POS	NEV	NEV	A\A	*E.coli*

16	SF50SUTI	NEV	POS	POS	POS	A\A	*Klebsiella.pneumonia*
17	L75SUTI	POS	POS	NEV	NEV	A\A	*E.coli*
18	1NLFL	NEV	NEV	NEV	NEV	A\A	*UNK*
19	NARANLS	NEV	NEV	POS	NEV	A\A	UNK
20	1NLFS	NEV	POS	POS	POS	A\A	*Klebsiella.pneumonia*
21	PUSNAV	NEV	POS	NEV	NEV	A\A	*Shigella.spp*
22	PUSND1L	NEV	POS	NEV	NEV	A\A	*Shigella. spp*
23	PUSND1S	NEV	POS	NEV	NEV	A\A	*E.coli*
24	PUSND2L	NEV	POS	POS	POS	A\A	*Klebsiella.pneumonia*
25	PUSND2S	NEV	POS	POS	NEV	A\A	UNK
26	NARANS	NEV	POS	POS	POS	A\A	*Klebsiella.pneumonia*
27	NARANLL	NEV	POS	NEV	POS	A\A	*Proteus.mirabilus*
28	CKIII	POS	POS	NEV	NEV	A\A	*E.coli*
29	CKII	NEV	POS	POS	POS	A\A	*Klebsiella.pneumonia*
30	CKIV	NEV	NEV	NEV	POS	K\K	*Pseudomonas.aeruginosa*
31	CKI	POS	POS	POS	POS	A\A	*Klebsiella.pneumonia*
32	CRIKIII'	POS	POS	POS	POS	A\A	*Klebsiella.pneumonia*
33	CRIKI''	NEV	POS	POS	NEV	K\A	*UNK*
34	CRIKIII''W	NEV	NEV	NEV	NEV	A\A	*UNK*
35	CRIKII''	NEV	NEV	POS	POS	K\A	*UNK*
36	CRIKI'	NEV	NEV	POS	POS	K\A	*UNK*
37	CRIKII'	NEV	POS	NEV	POS	K\A	*UNK*
38	CRIKIII''R	POS	POS	NEV	NEV	A\A	*E.coli*
39	11A	POS	POS	NEV	NEV	A\A	*E.coli*
40	11B	POS	POS	NEV	NEV	A\A	*E.coli*
41	13A	NEV	NEV	NEV	POS	K\K	*Pseudomonas.aeruginosa*
42	13B	POS	POS	NEV	NEV	A\A	*E.coli*
43	1P	NEV	NEV	NEV	POS	K\K	*Pseudomonas.aeruginosa*
44	321iII	POS	POS	NEV	NEV	A\A	E.coli
45	321iI	POS	POS	NEV	NEV	k\k	Unk
46	321iiI	POS	POS	NEV	NEV	A\A	E.coli

47	321iiII	POS	POS	NEV	NEV	A\A	E.coli
48	344iiIII	NEV	POS	NEV	NEV	A\A	E.coli
49	276iiII	POS	POS	NEV	NEV	A\A	E.coli
50	275iiII	NEG	POS	NEV	NEV	A\A	E.coli
51	278ii	POS	POS	NEV	NEV	A\A	E.coli
52	299i	POS	POS	NEV	NEV	A\A	E.coli
53	344iiI	POS	POS	NEV	NEV	A\A	E.coli
54	379iiII	POS	POS	NEV	POS	A\A	Citrobacter spp.
55	276iiI	POS	POS	NEV	NEV	A\A	E.coli
56	299ii	POS	POS	NEV	NEV	A\A	E.coli
57	275i	POS	POS	NEV	NEV	A\A	E.coli
58	344iII	NEV	POS	NEV	NEV	A\A	E.coli
59	379i	POS	POS	NEV	NEV	A\A	E.coli
60	275iiI	POS	POS	NEV	NEV	A\A	E.coli
61	344iI	POS	POS	NEV	NEV	A\A	E.coli
62	276i	POS	POS	NEV	NEV	A\A	E.coli
63	379iiII	POS	POS	NEV	NEV	A\A	E.coli
64	321iiIII	POS	POS	NEV	NEV	k\k	Unk
65	369X	POS	POS	NEV	NEV	A\A	E.coli
66	369E	POS	POS	NEV	NEV	K/A	E.coli
67	278X	POS	POS	NEV	NEV	A\A,GAS	E.coli
68	278E	POS	POS	NEV	NEV	A\A,GAS	E.coli
69	329X	POS	POS	NEV	POS	A\A,GAS	Citrobacter spp.
70	329E	POS	POS	NEV	NEV	A\A	E.coli
71	322XI	NEV	POS	NEV	NEV	K\A	Shigella spp
72	322XII	NEV	POS	POS	NEV	K\A	unk
73	322XIII	NEV	NEV	NEV	POS	K\K	unk
74	322EI	POS	POS	NEV	NEV	A\A	E.coli
75	322EII	POS	POS	NEV	NEV	A\A,GAS	E.coli
76	322EIII	POS	POS	NEV	NEV	A\A,GAS	E.coli

Appendix 2

Diameter of zone of inhibition (in mm) for all isolated bacteria against all the beta-lactams tested along with their interpretation

			Beta Lactams				
	Sample	**Organism**	**CAZ**	**CAT**	**CPM**	**CTR**	**CTX**
1	321iII	E.coli	8mm/R	20mm	0mm/R	0mm/R	0mm/R
2	321iI	Unk	0mm/R	16mm	0mm/R	0mm/R	0mm/R
3	321iiI	E.coli	0mm/R	13mm	0mm/R	0mm/R	0mm/R
4	321iiII	E.coli	0mm/R	16mm	0mm/R	0mm/R	0mm/R
5	344iiIII	E.coli	0mm/R	12mm	0mm/R	0mm/R	0mm/R
6	276iiII	E.coli	0mm/R	14mm	0mm/R	0mm/R	0mm/R
7	275iiII	E.coli	0mm/R	11mm	0mm/R	0mm/R	17mm/R
8	278ii	E.coli	0mm/R	16mm	10mm\R	0mm/R	0mm/R
9	299i	E.coli	0mm/R	10mm	0mm/R	0mm/R	0mm/R
10	344iiI	E.coli	0mm/R	12mm	0mm/R	0mm/R	0mm/R
11	379iiII	Citrobacter spp.	0mm/R	17mm	25mm\S	25mm\S	20mm/R
12	276iiI	E.coli	17mm/R	15mm	26mm\S	20mm\S	25mm/S
13	299ii	E.coli	0mm/R	13mm	13mm\R	0mm/R	0mm/R
14	275i	E.coli	0mm/R	14mm	0mm/R	0mm/R	0mm/R
15	344iII	E.coli	11mm/R	15mm	17mm\S	10mm\R	12mm/R
16	379i	E.coli	0mm/R	18mm	9mm/R	0mm/R	0mm/R
17	275iiI	E.coli	10mm/R	10mm	0mm/R	0mm/R	0mm/R

18	344iI	E.coli	0mm/R	13mm	13mm\R	0mm/R	0mm/R
19	276i	E.coli	0mm/R	0mm/R	0mm/R	0mm/R	0mm/R
20	379iiII	E.coli	16mm/R	16mm	12mm\R	25mm\S	16mm/R
21	321iiIII	Unk	0mm	12mm	0mm	0mm	0mm
22	369X	E.coli	0mm/R	14mm	0mm/R	0mm/R	0mm/R
23	369E	E.coli	0mm/R	16mm	0mm/R	0mm/R	0mm/R
24	278X	E.coli	0mm/R	12mm	0mm/R	0mm/R	0mm/R
25	329X	klebsiella	12mm\R	13mm	0mm/R	0mm/R	0mm/R
26	329E	E.coli	0mm/R	16mm	0mm/R	0mm/R	0mm/R
27	322X I	Shigella	20mm\I	18mm	20mm\R	24mm\S	20mm/R
28	322X II	unk	11mm	15mm	13mm	20mm	15mm
29	322X III	Unk	0mm	20mm	0mm	0mm	0mm
30	322 E II	E.coli	0mm/R	14mm	0mm/R	0mm/R	0mm/R

				Beta-Lactams		
S.No	**Sample name**	**location**	**Organism**	**CPM**	**CTX**	**CAZ**
1	SF56LUTI	IGMC	*E.coli*	17mm\R		
2	SF56SUTI	IGMC	*E.coli*	18mm\R	15mm\R	13mm\R
3	L75SUTI	IGMC	*E.coli*	16mm\R		
4	PUSND2L	IGMC	*E.coli*	13mm\R	14mm\R	12mm\R
5	CKIII	IGMC	*E.coli*	15mm\R	10mm\R	15mm\R
6	CRIKIII''R	IGMC	*E.coli*			
7	SFUTIL	IGMC	*E.coli*	20mm\R	21mm\R	21mm\S
8	11a	IGMC	*E.coli*	21mm\R	22mm\R	20mm\I
9	11b	IGMC	*E.coli*	4mm\R	4mm\R	4mm\R
10	13b	IGMC	*E.coli*	20mm\R	22mm\R	20mm\I
11	E.coli IGMC	IGMC	*E.coli*	18mm\R	20mm\R	18mm\I
12	SF50LUTI	IGMC	*Klebsiella.spp*	15mm\R	19mm\R	15mm\R
13	SF50SUTI	IGMC	*Klebsiella.pneumoniae*	18mm\R	19mm\R	17mm\R
14	PUSNAV	IGMC	*Klebsiella.pneumoniae*	15mm\R	18mm\R	11mm\R
15	NARANS	IGMC	*Klebsiella.pneumoniae*	10mm\R	5mm\R	4mm\R

16	CKII		*Klebsiella.pneumoniae*	20mm\R	20mm\R	19mm\I
17	CKI		*Klebsiella.pneumonia*	23mm\I	22mm\R	18mm\I
18	CRIKIII'		*Klebsiella.pneumonia*	20mm\R	19mm\R	19mm\I
19	1SVIII		*Klebsiella.pneumonia*	20mm\R	19mm\R	19mm\I
20	2LF		*Klebsiella.pneumonia*	18mm\R	17mm\R	10mm\R
21	1SVIw		*Klebsiella.pneumonia*	18mm\R	20mm\R	18mm\R
22	1P		*P. aeruginosa*	30mm\S	26mm\S	25mm\S
23	CKIV	Kasauli	*Pseudomonas.aeruginosa*	18mm\R	19mm\I	18mm\S
24	1SX	Shimla	*Pseudomonas.aeruginosa*	15mm\R	12mm\R	15mm\I
25	13a	Shimla	*Pseudomonas.aeruginosa*	7mm\R	11mm\R	6mm\R
26	PUSND1L	Shimla	*Shigella.boydii*	19mm\R	16mm\R	14mm\R
27	PUSND1S	Shimla	*Shigella.boydii*	29mm\ S	30mm\S	4mm\R
28	Navdeep	Shimla	*Shigella.boydii*	22mm\ I	24mm\I	21mm\S
29	1SIII	Shimla	*Shigella.boydii*	18mm\R	20mm\R	18mm\S
30	NARANLL	Shimla	*Proteus.mirabilus*	21mm\R	20mm\R	10mm\R
31	1SI	Shimla	*Proteus.mirabilus*	21mm\R	24mm\I	20mm\S
32	1SVIR	Shimla	*Proteus.mirabilus*	20mm\R	19mm\R	19mm\S
33	1NLFL	Shimla	*unk*	20mm/ R	12mm/R	14mm/ R
34	1NLFS	Shimla	*unk*	15mm/R	15mm/ R	11mm/R
35	PUSND2S	Shimla	*unk*	10mm/ R	6mm/ R	8mm/R
36	CRIKI''	Kasauli	*unk*	8mm/R	11mm/ R	4mm\R
37	CRIKIII''W	Kasauli	*unk*			
38	CRIKII''	Kasauli	*unk*			
39	CRIKI'	Kasauli	*unk*			
40	CRIKII'	Kasauli	*unk*	15mm/ R	20mm/ R	20mm/ I
41	Pus 3s	Shimla	*unk*	20mm/ R	28mm/ S	10mm/ R
42	1SII	Shimla	*unk*			

43	NARANLS	Shimla	*unk*	29mm/S	26mm/s	25mm/S

Appendix 3

Diameter of zone of inhibition (in mm) for all isolated bacteria against all the fluoroquinolones tested along with their interpretation

				fluorouinolnes			
	Sample	**Organism**	**NX**	**CIP**	**OF**	**NA**	**LE**
1	321iII	E.coli	0mm/R	10mm/R	10mm/R	0mm/R	14mm/I
2	321iI	Unk	0mm/R	0mm/R	0mm/R	0mm/R	7mm/R
3	321iiI	E.coli	0mm/R	0mm/R	0mm/R	10mm/R	10mm/R
4	321iiII	E.coli	0mm/R	0mm/R	0mm/R	0mm/R	8mm/R
5	344iiIII	E.coli	0mm/R	0mm/R	9mm/R	0mm/R	10mm/R
6	276iiII	E.coli	0mm/R	0mm/R	0mm/R	0mm/R	10mm/R
7	275iiII	E.coli	17mm/S	15mm/R	17mm/S	0mm/R	17mm/S
8	278ii	E.coli	0mm/R	12mm/R	12mm/R	0mm/R	12mm/R
9	299i	E.coli	0mm/R	0mm/R	0mm/R	0mm/R	0mm/R
10	344iiI	E.coli	0mm/R	8mm/R	8mm/R	0mm/R	10mm/R
11	379iiII	Citrobacter spp.	0mm/R	10mm/R	10mm/R	0mm/R	14mm/I
12	276iiI	E.coli	25mm/S	25mm/S	23mm/S	26mm/S	25mm/S
13	299ii	E.coli	0mm/R	9mm/R	0mm/R	0mm/R	11mm/R
14	275i	E.coli	0mm/R	0mm/R	9mm/R	0mm/R	10mm/R
15	344iII	E.coli	0mm/R	0mm/R	10mm/R	0mm/R	15mm/I
16	379i	E.coli	0mm/R	0mm/R	9mm/R	0mm/R	12mm/R

17	275iiI	E.coli	0mm/R	0mm/R	9mm/R	0mm/R	10mm/R
18	344iI	E.coli	0mm/R	0mm/R	0mm/R	0mm/R	10mm/R
19	276i	E.coli	0mm/R	0mm/R	9mm/R	0mm/R	11mm/R
20	379iiII	E.coli	0mm/R	10mm/R	12mm/R	0mm/R	13mm/R
21	321iiIII	Unk	0mm	0mm	0mm	0mm	9mm
22	369X	E.coli	0mm/R	0mm/R	6mm\R	0mm/R	8mm/R
23	369E	E.coli	0mm/R	0mm/R	6mm\R	0mm/R	11mm/R
24	278X	E.coli	0mm/R	0mm/R	0mm/R	0mm/R	8mm/R
25	329X	klebsiella	0mm/R	0mm/R	6mm\R	0mm/R	9mm/R
26	329E	E.coli	0mm/R	13mm\R	0mm/R	0mm/R	6mm\R
27	322X I	Shigella	18mm\S	24mm\S	18mm	18mm\S	24mm\S
28	322X II	unk	19mm	19mm	20mm	16mm	20mm
29	322X III	Unk	0mm	0mm	0mm	0mm	0mm
30	322 E II	E.coli	0mm/R	0mm/R	0mm/R	0mm/R	10mm/R

			fluorouinolnes	
			NX	CIP
1	SF56LUTI	IGMC	19mm\S	15mm\R
2	SF56SUTI	IGMC	13mm\I	19mm\I
3	L75SUTI	IGMC	17mm\S	11mm\R
4	PUSND2L	IGMC	9mm\R	9mm\R
5	CKIII	IGMC	10mm\R	13mm\R
6	CRIKIII''R	IGMC		
7	SFUTIL	IGMC	11mm\R	10mm\R
8	11a	IGMC	4mm\R	4mm\R
9	11b	IGMC	4mm\R	4mm\R
10	13b	IGMC	4mm\R	4mm\R
11	E.coli IGMC	IGMC	4mm\R	4mm\R
12	SF50LUTI	IGMC	13mm\I	17mm\I
13	SF50SUTI	IGMC	14mm\I	15mm\R
14	PUSNAV	IGMC	8mm\R	8mm\R
15	NARANS	IGMC	7mm\R	8mm\R
16	CKII		28mm\S	26mm\S

17	CKI		16mm\I	20mm\S
18	CRIKIII'		28mm\S	27mm\S
19	1SVIII		25mm\S	26mm\S
20	2LF		20mm\S	20mm\I
21	1SVIw		21mmS	29mm\S
22	1P		29mm\S	32mm\S
23	CKIV	Kasauli	22mm\S	18mm\I
24	1SX	Shimla	19mm\S	28mm\S
25	13a	Shimla	5mm\R	6mm\R
26	PUSND1L	Shimla	10mm\R	17mm\I
27	PUSND1S	Shimla	4mm\R	29mm\S
28	Navdeep	Shimla	10mm\R	6mm\R
29	1SIII	Shimla	4mm\R	4mm\R
30	NARANLL	Shimla	33mm\S	32mm\S
31	1SI	Shimla	4mm\R	4mm\R
32	1SVIR	Shimla	4mm\R	4mm\R
33	1NLFL	Shimla	20mm/ S	20mm/ I
34	1NLFS	Shimla	10mm/ R	8mm/ R
35	PUSND2S	Shimla	7mm/R	9mm/ R
36	CRIKI''	Kasauli	10mm/ R	16mm/ R
37	CRIKIII''W	Kasauli		
38	CRIKII''	Kasauli		
39	CRIKI'	Kasauli		
40	CRIKII'	Kasauli	18mm/ S	17mm/ I
41	Pus 3s	Shimla	10mm/I	13mm/ R
42	1SII	Shimla		
43	NARANLS	Shimla	13mm/ I	33mm/ S

Appendix 4

Diameter of zone of inhibition (in mm) for all isolated bacteria against all the aminoglycoside tested along with their interpretation

					Aminoglycosides	
	Sample	Organism	S	NET	AK	TOB
1	321iII	E.coli	14mm/I	17mm\s	18mm/S	15mm/S
2	321iI	Unk	18mm/S	18mm\s	19mm/S	15mm/S
3	321iiI	E.coli	14mm/I	14mm\I	20mm/S	16mm/S
4	321iiII	E.coli	12mm/I	17mm\S	14mm/R	12mm/R
5	344iiIII	E.coli	15mm/S	17mm\S	16mm/I	16mm/S
6	276iiII	E.coli	9mm/R	15mm\S	15mm/I	14mm/I
7	275iiII	E.coli	15mm/S	15mm\S	17mm/S	10mm/R
8	278ii	E.coli	15mm/S	15mm\S	15mm/S	10mm/R
9	299i	E.coli	9mm/R	15mm\S	13mm/R	10mm/R
10	344iiI	E.coli	15mm/S	22mm\S	20mm/S	19mm/S
11	379iiII	Citrobacter spp.	15mm/S	17mm\S	15mm/I	16mm/S
12	276iiI	E.coli	20mm/S	20mm\S	20mm/S	16mm/S
13	299ii	E.coli	18mm/S	18mm\S	18mm/S	16mm/S
14	275i	E.coli	15mm/S	16mm\S	20mm/S	16mm/S
15	344iII	E.coli	12mm/I	19mm\S	15mm/I	11mm/R
16	379i	E.coli	13mm/I	16mm\S	19mm/S	17mm/S

17	275iiI	E.coli	14mm/I	13mm\I	18mm/S	15mm/S
18	344iI	E.coli	15mm/S	15mm\S	15mm/I	15mm/S
19	276i	E.coli	13mm/I	18mm\S	18mm/S	17mm/S
20	379iiII	E.coli	17mm/S	16mm\S	15mm/I	11mm/R
21	321iiIII	Unk	14mm	15mm	13mm	12mm
22	369X	E.coli	15mm/S	11mm\R	15mm\I	16mm/S
23	369E	E.coli	14mm/I	6mm\R	15mm\I	13mm
24	278X	E.coli	14mm/I	8mm\R	15mm\I	10mm/R
25	329X	klebsiella	15mm/S	0mm\R	16mm/I	14mm/I
26	329E	E.coli	16mm	18mm\s	16mm/I	16mm/S
27	322X I	Shigella	18mm/S	0mm	20mm/S	19mm/S
28	322X II	unk	17mm	0mm	18mm	20mm
29	322X III	Unk	26mm	0mm	22mm	23mm
30	322 E II	E.coli	12mm/I	9mm	16mm/I	16mm/S

		aminoglycosides
		AK
SF56LUTI	IGMC	
SF56SUTI	IGMC	13mm\R
L75SUTI	IGMC	
PUSND2L	IGMC	13mm\R
CKIII	IGMC	20mm\S
CRIKIII''R	IGMC	
SFUTIL	IGMC	20mm\S
11a	IGMC	18mm\S
11b	IGMC	4mm\R
13b	IGMC	19mm\S
E.coli IGMC	IGMC	16mm\I
SF50LUTI	IGMC	15mm\I
SF50SUTI	IGMC	11mm\R
PUSNAV	IGMC	13mm\R

NARANS	IGMC	4mm\R
CKII		24mm\S
CKI		22mm\S
CRIKIII'		20mm\S
1SVIII		22mm\S
2LF		22mm\S
1SVIw		20mm\S
1P		24mm\S
CKIV	Kasauli	20mm\S
1SX	Shimla	20mm\S
13a	Shimla	5mm\R
PUSND1L	Shimla	13mm\R
PUSND1S	Shimla	4mm\R
Navdeep	Shimla	18mm\S
1SIII	Shimla	18mm\S
NARANLL	Shimla	22mm\S
1SI	Shimla	17mm\S
1SVIR	Shimla	18mm\S
1NLFL	Shimla	18mm
1NLFS	Shimla	15mm
PUSND2S	Shimla	4mm
CRIKI''	Kasauli	21mm
CRIKIII''W	Kasauli	
CRIKII''	Kasauli	
CRIKI'	Kasauli	
CRIKII'	Kasauli	18mm/ S
Pus 3s	Shimla	18mm/ S
1SII	Shimla	
NARANLS	Shimla	22mm/ S

Appendix 5

Diameter of zone of inhibition (in mm) for all isolated bacteria against the tested glycopeptides vancomycin along with their interpretation

			Glycopeptides
	Sample	**Organism**	**VA**
1	321iII	E.coli	11mm/I
2	321iI	Unk	16mm/S
3	321iiI	E.coli	10mm/R
4	321iiII	E.coli	0mm/R
5	344iiIII	E.coli	0mm/R
6	276iiII	E.coli	0mm/R
7	275iiII	E.coli	0mm/R
8	278ii	E.coli	0mm/R
9	299i	E.coli	0mm/R
10	344iiI	E.coli	0mm/R
11	379iiII	Citrobacter spp.	0mm/R
12	276iiI	E.coli	0mm/R
13	299ii	E.coli	9mm/R
14	275i	E.coli	11mm/I
15	344iII	E.coli	0mm/R
16	379i	E.coli	0mm/R
17	275iiI	E.coli	0mm/R
18	344iI	E.coli	0mm/R
19	276i	E.coli	0mm/R
20	379iiII	E.coli	0mm/R
21	321iiIII	Unk	0mm
22	369X	E.coli	16mm/S
23	369E	E.coli	11mm/I
24	278X	E.coli	16mm/S
25	329X	klebsiella	15mm\s
26	329E	E.coli	6mm\R
27	322X I	Shigella	20mm\S

28	322X II	unk	24mm
29	322X III	Unk	23mm
30	322 E II	E.coli	10mm/R

Appendix 6

Diameter of zone of inhibition (in mm) for all isolated bacteria against all the combination drugs tested

			VMRC antibiotics			
			I	II	III	IV
1	SF56LUTI	*E.coli*				
2	SF56SUTI	*E.coli*	30mm	29mm	32mm	30mm
3	L75SUTI	*E.coli*	30mm	35mm	34mm	30mm
4	PUSND2L	*E.coli*				
5	CKIII	*E.coli*	20mm	16mm	20mm	18mm
6	CRIKIII''R	*E.coli*	19mm	24mm	22mm	21mm
7	SFUTIL	*E.coli*	29mm	26mm	24mm	27mm
8	11a	*E.coli*	28mm	34mm	30mm	26mm
9	11b	*E.coli*	8mm	6mm	9mm	4mm
10	13b	*E.coli*	31mm	32mm	29mm	30mm
11	E.coli IGMC	*E.coli*	25mm	24mm	28mm	24mm
12	SF50LUTI	*Klebsiella.spp*	30mm	25mm	30mm	30mm
13	SF50SUTI	*Klebsiella.pneumoniae*	32mm	38mm	30mm	29mm
14	PUSNAV	*Klebsiella.pneumoniae*				
15	NARANS	*Klebsiella.pneumoniae*	12mm	20mm	4mm	4mm
16	CKII	*Klebsiella.pneumoniae*	12mm	10mm	14mm	10mm
17	CKI	*Klebsiella.pneumonia*	20mm	25mm	21mm	14mm
18	CRIKIII'	*Klebsiella.pneumonia*	26mm	26mm	24mm	27mm

19	1SVIII	*Klebsiella.pneumonia*	25mm	25mm	30mm	26mm
20	2LF	*Klebsiella.pneumonia*	30mm	28mm	24mm	27mm
21	1SVIw	*Klebsiella.pneumonia*	25mm	27mm	21mm	23mm
22	1P	*P. aeruginosa*	26mm	26mm	22mm	20mm
23	CKIV	*Pseudomonas.aeruginosa*	19mm	21mm	15mm	16mm
24	1SX	*Pseudomonas.aeruginosa*	25mm	28mm	29mm	29mm
25	13a	*Pseudomonas.aeruginosa*	17mm	18mm	20mm	22mm
26	PUSND1L	*Shigella.boydii*				
27	PUSND1S	*Shigella.boydii*	36mm	35mm	30mm	29mm
28	Navdeep	*Shigella.boydii*	25mm	20mm	28mm	22mm
29	1SIII	*Shigella.boydii*	24mm	20mm	19mm	18mm
30	NARANLL	*Proteus.mirabilus*	22mm	23mm	22mm	12mm
31	1SI	*Proteus.mirabilus*	25mm	26mm	29mm	25mm
32	1SVIR	*Proteus.mirabilus*	24mm	26mm	25mm	21mm
33	1NLFL	*unk*				
34	1NLFS	*unk*				
35	PUSND2S	*unk*				
36	CRIKI''	*unk*	15mm	15mm	14mm	17mm
37	CRIKIII''W	*unk*	5mm	5mm	5mm	5mm
38	CRIKII''	*unk*	20mm	21mm	16mm	15mm
39	CRIKI'	*unk*	17mm	15mm	11mm	14mm
40	CRIKII'	*unk*	21mm	17mm	18mm	16mm
41	Pus 3s	*unk*	22mm	23mm	24mm	22mm
42	1SII	*unk*	24mm	24mm	26mm	24mm
43	NARANLS	*unk*	5mm	5mm	5mm	17mm
44	321iII	E.coli	16mm	19mm	21mm	0mm
45	321iI	Unk	12mm	16mm	15mm	0mm
46	321iiI	E.coli	17mm	18mm	15mm	0mm
47	321iiII	E.coli	15mm	12mm	18mm	0mm
48	344iiIII	E.coli	19mm	24mm	20mm	0mm
49	276iiII	E.coli	14mm	20mm	15mm	0mm
50	275iiII	E.coli	20mm	22mm	20mm	0mm

51	278ii	E.coli	15mm	20mm	21mm	0mm
52	299i	E.coli	14mm	16mm	14mm	0mm
53	344iiI	E.coli	20mm	21mm	13mm	0mm
54	379iiII	Citrobacter spp.	25mm	24mm	15mm	0mm
55	276iiI	E.coli	26mm	27mm	26mm	26mm
56	299ii	E.coli	20mm	22mm	9mm	10mm
57	275i	E.coli	16mm	17mm	14mm	0mm
58	344iII	E.coli	17mm	22mm	25mm	0mm
59	379i	E.coli	15mm	21mm	19mm	0mm
60	275iiI	E.coli	0mm	20mm	20mm	20mm
61	344iI	E.coli	19mm	20mm	16mm	9mm
62	276i	E.coli	18mm	15mm	15mm	0mm
63	379iiII	E.coli	26mm	27mm	0mm	25mm
64	321iiIII	Unk	12mm	18mm	18mm	0mm
65	369X	E.coli	11mm	14mm	12mm	0mm
66	369E	E.coli	15mm	17mm	16mm	0mm
67	278X	E.coli	12mm	15mm	13mm	0mm
68	329X	klebsiella	12mm	16mm	12mm	0mm
69	329E	E.coli	13mm	20mm	16mm	0mm
70	322X I	Shigella	25mm	21mm	20mm	19mm
71	322X II	unk	24mm	20mm	18mm	16mm
72	322X III	Unk	26mm	24mm	21mm	16mm
73	322 E II	E.coli	14mm	18mm	15mm	0mm