Evaluation of antimicrobial resistance pattern against beta-lactams and its current derivatives

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Certificate

This is to certify that project report entitled " Evaluation of antimicrobial resistance patterns and its current derivatives.", submitted by Ms. Shilpa 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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Signature of student Date

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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

Summary

The widespread use of β-lactam antibiotics has led to the worldwide appearance of drug-resistant strains of pathogens. Large number of bacteria had developed resistance against β-lactams by following main mechanisms; the beta-lactamases production accompanied by decrease of outer membrane permeability, and production of low affinity drug-resistant penicillin-binding proteins (PBPs). PBPs always remain the attractive target for developing new antibiotic targets because they are the core enzyme catalyzing biosynthesis of peptidoglycan, which is unique to bacteria and lies outside the cytoplasmic membrane.

A large number of studies in recent years have documented significant rates of resistance among wide range of antibiotics especially beta-lactams. One of the prominent reason for this abrupt resistance increase is due to often irrational prescription or inappropriate usage; in terms of dosage, duration and redundant or have potential for adverse interactions with other drugs. This fact is documented in several hospital based and city-based studies of antibiotic use in India.

Resistance against certain antibiotics is already at high levels in certain places in India, but the problem has remained largely unknown because relatively few studies were published and nationwide surveillance is not being carried out. The use of antibiotics can be rationalized by proper surveillance systems in India; first the surveillance for antibiotic resistance and second surveillance for antibiotic use. The ministry of health care and welfare task force report also recommended the development of a laboratory network, beginning from New Delhi and expanding thereafter as well as prescription and sales monitoring in New Delhi public sector hospitals. Extended spectrum beta-lactamases producing bacteria have become threat in India and other parts of globe.

Study mainly focuses on evaluating the antimicrobial resistance patterns against the beta-lactam antibiotics including all classes of beta-lactams, its current derivatives and combination drugs. Total 73 clinical samples were tested for the resistance against beta lactams by Kirby Bauer's disc diffusion methods and MIC was determined according to Clinical and Laboratory Standards Institute guidelines (CLSI 2014). Percentage prevalence of *E.coli* (47%) in clinical samples was predominant one and rest *Klebsiella sp., Shigella spp, Pseudomonas spp., Citrobacter spp, Proteus spp.* and 20% bacteria of unknown etiology were found.

Signature of student Signature of Supervisor

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Chapter 1

Introduction

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INVESTORS' SOAPBOX PM

EU Needs New Drugs to Fight Antibiotic Resistance

A recent conference focused on old drugs at higher doses or in combination where traditional therapies have failed.

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April 29, 2015

Wedbush

In our view, the greatest focus of this year's European Congress of Clinical Microbiology and Infectious Diseases meeting was growth in the rate of resistance to antibiotics; as a reminder, Europe currently experiences higher rates of resistance than the U.S.

An overall survey of carbapenem resistance (CRE) showed a stabilization of meropenem susceptibility over the past few years in Europe, likely due to increased stewardship, with an increase in resistance in the U.S. Although overall CRE rates are still low in the U.S. (less than 10%), Klebsiella pneumoniae carbapenemase (KPC)-producing strains appear to be the most prevalent. In our view, the unmet need is clear -- due to the dearth of current options, many presentations and posters focused on how to use old drugs at higher doses or in combination to treat patients where traditional therapies have failed.

Diagnostics are improving, but not there yet. Another theme of the meeting, in our view, was the push towards rapid diagnostics. Some methods, such as mass spectrometry, appear to be rapid, but are likely cost prohibitive. Others still require enrichment of pathogens, taking 24-48 hours; in our view, though progress is being made, rapid pathogen screening is not yet a reality.

Gram-negative antibiotics were in focus at clinical sessions. The newly U.S.-approved

Beta-lactams antibiotics were introduced into the health care system in later stages of World War II which is the most important contribution to the medical science history. Today beta- lactams remain the most widely used antibiotics against bacterial infections owing to their higher efficiency, low cost, ease of delivery, and minimal side effects. Low cost of production of beta lactams antibiotic contributes to its easy availability, thus it is imperative to preserve the power of this valuable clinical resource. [1]

These antibiotics are the broad class of antibiotics consisting of the agents that contain β-lactams ring in their molecular structure. These include penicillins, cephalosporins, monobactams and carbepenems. [2] Penicillin was the first antibiotic which was clinically used in 1941 by Alexander Flemings, Scottish bacteriologist.

These agents are active against many gram negative, gram positive and anaerobic organisms. Beta-lactams antibiotics generally target the transpeptidase enzyme that is responsible for the synthesis of bacterial cell wall. These enzymes are localized to the outer leaflet of bacterial cytoplasmic membrane and they are specific to bacteria. [3]

They do not have any structural and functional counterpart in human host. For most of the antibiotics, concerns about resistance were tempered due to the newer, more potent agents being developed by the dozens of companies in the business of making antibiotics. We no longer have the luxury of anticipating the imminent introduction of the solution to our resistance problems. The number of large pharmaceutical corporations actively engaged in antibiotic discovery has dwindled to the single digits, and the number of new antimicrobial agents introduced has been reduced to a trickle over the past decade. [4, 41]

Chapter 2

Review of literature

Bacteria of all the species rely on a heavily cross-linked peptidoglycan layer for the preservation of cell shape and rigidity. Bacterial cell wall integrity is important for maintaining the cell shape in hypertonic and hostile environments. Cell wall comprised of basic repeating units of alternating disaccharides-N-acetyl glucosamine and N-acetyl mumramic acid. Latter sugar in disaccharide is modified by pentapeptide. Termination always occurs in the form of D-alaline residues. Individual peptidoglycan units are produced inside the cell, but their final cross-linking is catalyzed outside the cytoplasmic membrane by a group of membrane anchored bacterial enzymes known as cell wall traspeptidases. During the cross linking reaction peptide bond is formed between the penultimate D-alanine on one chain and free amino end of diamino pimelic acid or l-lysine residue on the other chain. Linkage formed during the reaction causes the cleavage of terminal D-alaline. Traspeptidases enzymes utilize an active site serine and perform their catalytic cycle by the way of acylation/deacylation pathway. Beta-lactams inhibit the activity of these enzymes therefore often termed as penicillin binding proteins or PBPs. They are able to do this because of their stereo chemical similarity with the D-alanine-D-alanine substrate. In the presence of beta-lactams the traspeptidases for the lethal covalent penicilloyl-enzyme complex that serves to block the normal traspeptidases reaction, which results in weakly cross-linked peptidoglycan which makes the growing bacteria highly susceptible to cell lysis and cell death.[11,12]

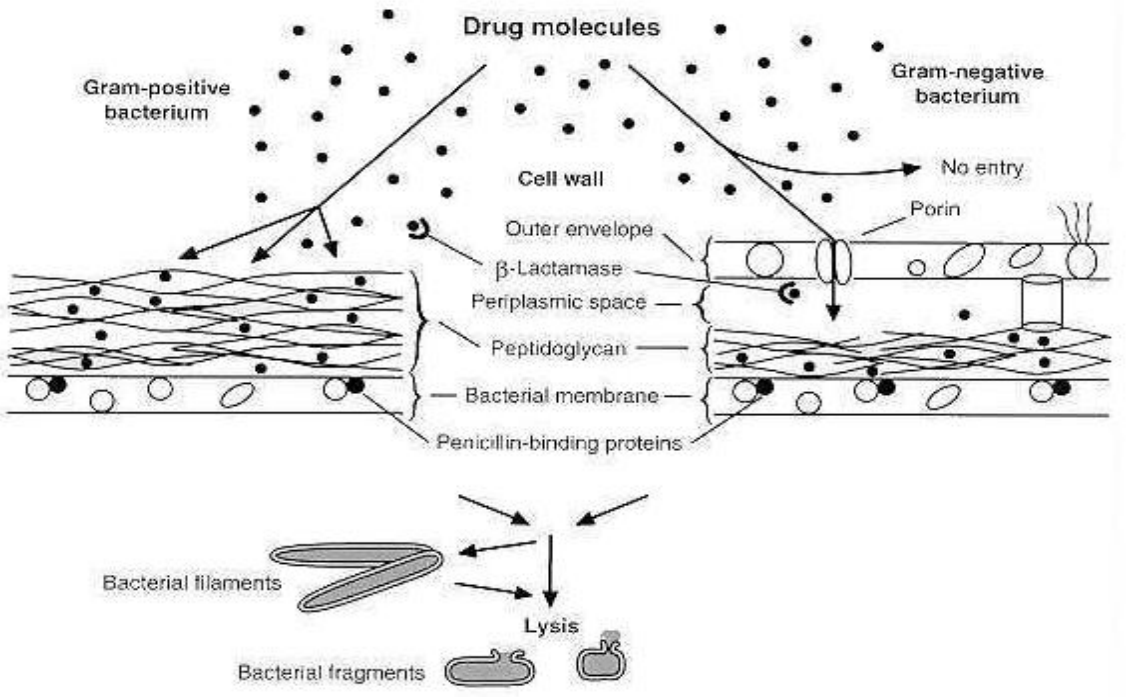


Figure 1: Mechanism of action of beta-lactams

Source- R.Lakshmi et al.Int.Res.J.Pharma.2014

Different classes of beta-lactam

These include penicillin derivatives, cephalosporin, monobactams, carbepenems, beta lactamase inhibitors and combination drugs.

Penicillin:-Penicillin is the earliest class of antimicrobial drugs among beta-lactams. They are future sub classified on the basis of their chemical structure, spectrum i.e. narrow spectrum and broad spectrum, source i.e. natural, synthetic, semi synthetic and on the basis of susceptibility to beta-lactamase destruction. Narrow spectrum beta-lactamase sensitive penicillins include the naturally occurring penicillin-G and few of the biosynthetic acid stable penicillins intended for the oral use. [5]This class is active against various gram-positive bacteria and limited number of gram-negative bacteria. This class is mainly susceptible to beta-lactamase. Broad spectrum beta-lactamase sensitive penicillins are derived semi-synthetically and are active against many gram-positive and gram-negative bacteria. They can readily be destroyed by beta-lactamases produced by many bacteria. Several ampicillin precursors that are completely absorbed from gastrointestinal tract also belong to this class example hetacillin, pivampicillin. Organisms which are usually sensitive to in-vitro penicillin-G include *Streptococci*, penicillin- sensitive *Staphylococci*, *Arcanobacterium pyogens*, *Clostridium* spp etc. The combination of beta lactamase inhibitors and penicillins enhances the spectrum and efficacy against both gram positive and gram negative pathogens. Clavulanate-potentiated amoxicillin is an excellent example of synergistic association the drugs. [6, 14]

Cephalosporins:-Cephlaosporins includes cephamycins, they are generally classified by their generation i.e. first generation to fourth generation, later generations are more resistance to beta – lactamases destruction and are characterized by their extended spectra. They can also be classified on the basis of spectra and susceptibility to beta-lactamases. First generation Cephlaosporins-includes Cephaloridine, Cepharine, Cefazolin, Cefadroxil. They are quite active against many gram-positive bacteria and moderately active against gram-negative bacteria. They are less susceptible to beta-lactamases but are susceptible to cephalosporinases. Second generation Cephlalosporins include Cefamandole, Cefoxitin, Cefuroxime, Ceforanide. This class is generally active against most of gram-positive and gram-negative bacteria. They are ineffective against *Enterococci*, pseudomonas with exception of Cefoxitin, *Actinobacter spp* and many obligate anaerobes. [10] Third generation Cephlaosporins includes ceftiofur, ceftriaxone, cefsulodin, cefotaxime. Fourth generation antibiotics include cefepime. Both third and fourth generation antibiotics are active against most of gram-positive bacteria and active against variety of gram-negative bacteria. Cefpodoxime and cefovecin are effective against *Staphylococcus intermedius*. Cephlalosporins class of antibiotic is highly resistant to beta-lactamases enzymes. Microbes are increasingly developing resistance against these antibiotics which

led to the emergence of new class of antibiotics or new generation of antibiotics. Third and fourth generation antibiotics Cephlalosporins are often able to penetrate the blood-brain barriers and are frequently indicated in bacterial meningitis infections [7].

Carbepenems: - Imipenem i.e. carbepenems class of beta lactam antibiotics; it has shown potent activity against most clinically important species of bacteria, including isolates resistant to other antibiotics. This drug is well distributed to most tissues and fluids after intravenous administration however its level in cerebral fluids is modest. Most of the drugs are eliminated in the urine, where it is metabolized by an enzyme on the brush border of renal tubular cells; cilastin is generally given simultaneously to inhibit this inactivation. Clavulanate, sulbactam, tazobactam are beta-lactamase inhibitors which show little intrinsic antibacterial activity but inhibit the activity of number of plasmid-mediated beta-lactamases [8]. They do not inhibit chromosomally mediated beta lactamases. Combination of beta-lactamases inhibitors and ampicillin, amoxicillin, or piperacillin results in antibiotics with an enhanced spectrum of activity against organisms containing plasmid encoded beta-lactamases. In addition these compounds also inhibit chromosomal beta-lactamase of various bacteroides species, extending the spectrum of coverage of these organisms.

Combination drugs- Various combination drugs like Amoxicillin-Clavulanate inhibit most of the strains of oxacillin-sensitive *Staphylococcus aureus* and beta-lactamase producing *Haemophilus influenzae* in addition to usual organisms inhibited by amoxicillin alone. The various combination drugs are also effective against beta-lactamase producing Enterobacteriaceae. Combination drug Amoxicillin-Clavulanate also known as augmentin is used as a oral therapy for patients with otitis media, sinusitis, lower respiratory infections and for bite wounds. Ampicillin-sulbactam is used to treat patients with diabetic foot ulcers. This combination has also been used for the treatment of intra-abdominal and pelvic infections. Ticarcillin- Clavulanate and piperacillin-tazobactam are used for extended spectrum of organisms producing beta-lactamases and this combination is preferred over ampicillin-sulbactam for intra-abdominal infection when an agent from this class is chosen. [7, 8]

Vancomycin also comes under the beta lactams class of antibiotics. They are complex glycopeptides that binds to precursor of peptidoglycan layer in bacterial cell-wall and generally given in combination with other beta-lactams antibiotics. This effect prevents the cell wall synthesis and produces bactericidal effects in dividing bacteria. It is active against most of gram-positive bacteria but it is not effective against gram-negative bacteria cells because of their large size and poor penetrability this drug is widely distributed in body. Excretion occurs by kidneys; in renal insufficiency, striking accumulations may develop. [13]

Mechanism of resistance:

Resistance can be achieved either through gene mutation or through the acquisition of exogenous resistance determinants. Mechanisms by which resistance genes are acquired vary. Transferable plasmids may be very large (150 kb) and contain a variety of resistance gene.7 Plasmids may form co-integrates with transposons that incorporate one or more resistance genes. Some plasmids encode their own transfer machinery, whereas others can be mobilized by a co resident transferable plasmid. Chromosomal elements may also transfer on their own or be mobilized by transferable plasmids. The large chromosomal transfers among *Enterococcus faecalis* result from mobilization of segments of the chromosome by conjugative plasmids through co-integration across identical insertion sequences located on both replicons. These findings suggest that virtually any part of the genome can be mobilized, emphasizing the fluidity of many bacterial genomes.
[15]

There are four different mechanisms of resistance mechanisms of resistance in beta-lactams

1) Production of beta-lactamases enzyme – the first beta-lactamase was identified in *E. coli* before the clinical use of penicillin. In the paper published nearly 70 years ago, E.P.Abraham and E.Chain described the penicillinase. At that time the enzyme was not thought clinically relevant, since penicillin was targeted to treat staphylococcal and streptococcal infections, and scientist were not able to isolate that enzyme from these gram-positive organisms. After that 4 years later Kirby successfully extracted these cell-free penicillin inactivators from *Staphylococcus aureus* which foreshadowed the emergence of significant clinical problems. [14] The growing number of beta-lactam antibiotics has since increased the selective pressure on bacteria, promoting the survival of organisms with multiple beta-lactamases. It is the most common and major mechanism of resistance in gram-negative bacteria- beta-lactamases gene has been greatly exacerbated by their integration within mobile genetic elements such as plasmid or transposons, which facilitate the rapid transfer of genetic material between the microbes.

   Beta-lactamases are secreted into the periplasmic space (in gram-negative bacteria), bound to cytoplasmic membrane, or excreted (in gram-positive bacteria).[40] Beta-lactamases are organized into four classes (A to D) on the basis sequence similarity. Classes A, C, and D share a similar fold and all have a mechanism that involves creation of serine nucleophile by deprotonation of an active site of beta-lactam ring to form an acyl-enzyme intermediate, and hydrolysis of the intermediate using a general base activated water molecule. The difference between the catalytic mechanism of serine beta-lactamase classes centre around the type of

general base residues used in acylation and deacylation. The class B of beta-lactamases is zinc metalloenzymes and is completely distinct from the serine beta-lactamases in term of sequence, fold and mechanisms. There are three subclasses of class B metallo-β-lactamases (B1-B3). Classes B1 and B3 are able to bind to two or more zinc ions whereas class B2 appear to be mononuclear. In the binuclear metallo-β-lactamases, the zinc ions are proximal to each other and are separated by bridging hydroxide that has been proposed to be the attacking nucleophile in β-lactam hydrolysis. [13]

New generation of beta-lactamases enzymes: b-lactamases are ancient enzymes that were relatively rare until beta -lactam antibiotics were introduced into medicine and agriculture half a century ago. The widespread use of carbapenems, the monobactam aztreonam, cephamycins and oxyimino-cephalosporins in the past few decades has led to the evolution of a new generation of b-lactamases, which have an extended substrate spectrum (i.e. extended-spectrum b-lactamases or ESBLs), as well as the development of novel carbapenemases and plasmid-mediated AmpC b-lactamases .

The most common types of β-lactamases include; ESBLs, Ampcs, TEM, OXA, SHV etc.

Extended spectrum β-lactamases (ESBLs)-they are mutant enzymes with wide range of activity than their parent molecules. They can hydrolyze third and fourth generation cephalosporins and aztreonam but do not affect second generation cephalosporins and remain susceptible to beta-lactamases inhibitors. Most common plasmid mediated β-lactamases in Enterobacteriaceae are TEM-1, TEM-2 and SHV-1 enzymes. Classical ESBLs are found in *E. coli* and *Klebsiella* species. Non-classical ESBLs are less common than classical ESBLs. It includes CTX-M AND OXA. [12]

AmpCs: They are not inhibited by beta-lactamase inhibitors that are normally repressed and are produced at low levels. Plasmid-mediated AmpCs are also inducible. Two mechanisms responsible for Ampc activity in *E. coli* are mutations in AmpC over expression and acquisition of plasmid-carried Ampc genes. [18]

Sulfhydryl variants: these are the most prominent β-lactamases produced by Enterobacteriaceae are Sulfhydryl family. The first reported SHV had a narrow spectrum of activity. Derivatives of SHV have been evolved due to accumulations of point mutations at the active site of the

enzyme. These derivatives have extended spectrum of activity which are capable of inactivating third-generation Cephalosporins.

Plasmid-encoded transposable element beta-lactamases (TEM1)- it is one of the most common and well known in producing antibiotic resistance. It confers resistance to penicillins and early cephalosporins. It is commonly found in gram-negative bacteria. Almost 90% of ampicillin resistance in *E. coli* is due to production of TEM-1. It is mostly found in *E. coli* and *K.pneumoniae.* By opening the active site to beta-lactam substrates enhances the susceptibility of the enzyme to beta-lactamases inhibitors like clavulanic acid. Currently 140 TEM type enzymes are identified [16]

Oxacillinases (OXA) - The OXA type (oxacillin hydrolyzing) enzymes are produced by Enterobacteriaceae and *P.aerogenosa.* They pose resistance against amino and ureidopenicillin and high levels hydrolytic activity against cloxacillin, oxacillin, and methilicillin. Clavulanic acid strongly inhibits the activity of this enzyme. They belong to ambler class-D and thus posses an active site as classes A and C beta lactamases. [17]

Recently, several CTX-M structures have been made available including inhibitor-bound structures, which provide snapshots of two reaction cycle transition states, and the acyl enzyme intermediate, which can aid in the design of inhibitors. In addition, several atomic resolution CTX-M structures demonstrate that the enhanced ceftazidimase activity of these enzymes is a result of the increased active site flexibility; however, this increase in flexibility is at the cost of protein stability. Carbapenemases are derived from classes A, B and D and They provide resistance to carbapenems as well as to oxyimino-cephalosporins and cephamycins. The class B metallo-b-lactamase CphA is a carbapenamase. Its crystal structure in complex with the carbapenem substrate biapenem has been determined; this might prove useful in the design of inhibitors or of non-hydrolyzable antibiotics. [33]

2) Changes in the active site of penicillin binding proteins can lower the affinity for β-lactam antibiotics and subsequently increases resistance to these agents, such as those seen in PBP2x of *Streptococcus pneumoniae.* Through natural transformation and recombination with DNA from other organisms, *Neisseria spp* and *streptococcus spp* have acquired highly resistance, low affinity PBPs. Similar in case of penicillin resistance in *Streptococcus sanguis, Streptococcus mitis* developed from horizontal gene transfer of PBP2b gene from *streptococcus pneumoniae*.

PBPs are divides into two sub groups: low molecular mass (LMM) and high molecular mass (HMM) enzymes.

The HMM enzymes are further subdivided into bifunctional class A enzymes. The soluble LMM PBPs have no identified role in beta-lactam resistance and are primarily involved in carboxypeptidase reaction and peptidoglycan trimming. The mutation in PBP2X in PRSP has been studied extensively resistance occurs in mosaic pattern with many mutations occurring in different clinical isolates. This causes problems in isolation of main determinants of resistance and also presents the requirements for screening crystallization conditions anew. Strain sp328 harbors most clinically important mutation, T338A, which was found to result in the loss of an important active site water molecule, thus weakening the hydrogen binding network that stabilizes the acyl-enzyme complex.The S389L and N514H mutations that are also present in this strain were found to sterically hinder favourable interactions with the b-lactam, reducing the acylation rate. An additional mutation, M339F, confers higher-level resistance to strains that possess the T338A mutation. The structure of this variant was found to re-orientate the S337 nucleophile and lower the reaction rate by 4–10-fold. [27]

PBP2x from PRSP strain sp5259 has also been structurally characterized and appears to offer an alternating mechanism for resistance that shares features with other class b enzymes. The mutations of Q552 to glutamate introduces a negative charge near the edge of active site; this might act like a similarly positioned residue in PBP5fm, disfavoring interaction with negatively charged beta-lactam. It is also prudent to note that more PBP1A and PBP2A are involved in PRSP resistance. Sequences of 40 clinical isolates containing *S.pneumoniae* PBPS confirmed the correlation mutations in these proteins to beta-lactam minimum-inhibitory concentration. Mutations in PBP2B resulted in higher MICS for penicillins and carbepenems, whereas MIC increases for cephalosporins were associated with PBP2x. PBP2a of MRSA is encoded by mecA gene, which is believed to have arisen as a result of horizontal transfer from an undisclosed species.

When challenged with beta-lactams, MRSA will utilize the transglycosylase activity of PBP2 (the only class A enzyme of S. aureus) and the transpeptidase functionality of PBP2a to synthesize the cell wall. It has recently been shown that PBP2 is able to mutate to a resistant form in the laboratory, but thankfully it is not responsible for the emergence of a second alternate form of MRSA in the environment. The structure of PBP2a suggested that the poor acylation by b-lactams was caused by b-strand 3 alterations, and a route to more effective antibiotics could be to increase the length of the beta-lactam compound to improve non-

covalent interactions. In contrast to S. aureus and S. pneumoniae, the bacterium *E. faecium* is naturally resistant to b-lactam antibiotics.

The PBP responsible for this resistance, PBP5fm, has been structurally characterized, although no co-ordinates for this enzyme are available in the PDB at present. The reason for the endogenous low-level affinity for b-lactams in *E. faecium* isn't immediately apparent, but could be owing to the reduced active site accessibility and also to charge repulsion with the b-lactam carboxylate group. *E. faecium* can demonstrate higher-level resistance by mutation of PBP5fm, including an insertion after S466 that is present in a loop that shifts upon acylation. The role of PBP1b in β-lactam resistance is minor at best, but its structure can be used as a model for mutational effects in the resistant PBP1a enzyme (45% sequence identity between the transpeptidase region of PBP1a and PBP1b). It is also noted that crystallization conditions for PBP1a have been reported .Like PBPs2x, 2a and 5fm, the enzyme possesses a classical transpeptidase domain, which is flanked by regions of unknown function that might play a role in association with other cell wall modifying proteins. [1]

The postulation of multi-enzyme complexes for HMM PBPs is particularly interesting when applied to this structure as the active site appears closed and activity might be regulated by interaction with other proteins. One consequence of open and closed active sites in these enzymes is that resistant PBPs are expected to favour open conformations, allowing transpeptidation of the bulkier substrates that result from inhibition of the LMM PBP carboxypeptidases by beta-lactams. *Streptococcus pneumoniae* takes advantage of its capacity to take up DNA to become resistant to -lactams. 28 Resistant strains exhibit a variety of "mosaic" genes derived from recombination between native pneumococcal PBP genes and those from less susceptible viridians streptococci.

Resistance achievable by this mechanism is limited by the levels of resistance expressed by the native PBPs that contribute to the mosaic and generally remains at low levels that affect the efficacy of intravenous antibiotics only in the cerebrospinal fluid. Other naturally transformable species, such as *Neisseria gonorrhoeae*, also exhibit mosaic PBP genes. Recently, the first gonococcal strain exhibiting high-level resistance to cephalosporins was shown to mediate resistance through a mosaic PBP gene. It stands to reason that a mosaic gene would be less efficient at performing its function than the native gene and that therefore reductions in the selective pressure favouring persistence of these genes (i.e., reduction in use of beta-lactam antibiotics) would result in reduced prevalence of resistance. Systematic attempts to reduce use of antibiotics in the community have been associated with reductions in *S pneumoniae* resistance; however, specific correlations between reductions in use of beta-lactams and penicillin resistance have been difficult to demonstrate. Moreover, antimicrobial usage analyses

have been complicated by the widespread use of the 7-valent pneumococcal conjugate vaccine, which has played a major role in reducing rates of penicillin resistance in *S pneumoniae*32 by targeting serotypes with a high prevalence of resistance. [1, 9]

3) Decreased expression of outer membrane proteins (OMPs) is another mechanism of resistance, in order to access PBPs on the inner plasma membrane, beta-lactams have to diffuse through or directly transverse porin channels in the outer membrane of gram-negative bacteria cell walls. Some Enterobacteriaceae exhibits resistance to carbepenems based on loss of these OMPs; the loss OprD is associated with imipenem resistance and reduced susceptibility to meropenem in nonfermenter *P.aerogenosa*. Point mutations or insertions in sequences in porin-encoding genes can produce proteins with decreased function and thus lower the permeability to beta-lactams. Alone this mechanism is not sufficient for producing resistance typically this mechanism is in combination with the expression of beta-lactamases.[14]

4) Efflux pumps are the part of an acquired or intrinsic resistance phenotype and are capable of exporting a wide range of substrates from periplasm to surrounding environments. With the exception of some strains of the *Streptococci*, *Enterococci* and *Staphylococci* 'superbugs', Gram-negative bacteria are generally more resistant to a large variety of antibiotics and chemotherapeutic agents than are Gram-positive bacteria. It is now recognized that a major contribution to antibiotic resistance in Gram-negative specie is the presence of broad-specificity drug-efflux pumps

One of the best-characterized of these is the drug efflux system MexAB–OprM of the opportunistic pathogen, Pseudomonas aeruginosa. This tripartite pump (composed of the inner membrane RND transporter 'pump' MexB, the outer membrane porin OprM, and the soluble periplasmic MexA) acts on a wide range of antibiotics, including tetracycline, chloramphenicol, quinilones, novobiocin, macrolides and trimethoprim, as well as b-lactams and b-lactamase inhibitors such as clavulanic acid. The orthologous outer membrane porin and inner membrane pump components TolC and AcrB from *E. coli* have been determined to 2.1 and 3.5 A ° resolution, respectively, and the periplasmic component MexA from Pseudomonas aeruginosa to 3.5A ° resolution. A structure of the inner membrane pump AcrB in the presence of several hydrophobic small molecule compounds has also been determined, which implies a diverse binding mode for individual ligands, at least in this component of the efflux pump. Although these structures have provided a tremendous new level of understanding of the distinct architecture of the three proteins that make up these pumps, there are still many unanswered questions with regard to the way in which these components interact to form a single path for extruded antibiotic ligands. These systems represent logical targets for novel antibiotic design, and development of lead compounds in this area is evolving rapidly. [20, 21]

Research groups worldwide:

In May 2010, a case of infection with *E. coli* expressing NDM-1 was reported in the United Kingdom. The patient was a man of Indian origin who had visited India 18 months previously, where he had undergone dialysis. In initial assays the bacterium was fully resistant to all antibiotics tested. The majority of the beta-lactamase enzymes are effective on some, or most, of the older antibiotics like cephalosporins and penicillins. NDM1 however, is effective on both newer and older antibiotics that contain a beta-lactam ring. Klebsiella were the first bacteria identified in 2009 to produce NDM-1 in a person who traveled from India to England with an infection that failed to respond to several antibiotics. [17]

Prevalence of the organisms expressing inhibitor-resistant enzyme varies throughout the world. French study conducted in 1993 on 2,972 *E. coli* isolates from urinary tract infections showed 25% and 10% of hospital and community isolates, showed amoxicillin-clavulanate MICs of >16/2 µg/ml. Characterization of these isolates, including MICs profiles and DNA-DNA hybridization suggested that 27.5% and 45% of hospital and community isolates were TEM-1 derived and had a substitution at amino acid positions that confers the IRT phenotype. In 1998, geriatric department of French hospital reported an outbreak of amoxicillin-clavulanate resistant isolates which all produced the same IR TEM β-lactamase enzyme. In the study published in 2000 determined the molecular mechanism of amoxicillin-clavulanate resistance in *E. coli* isolates from three French hospitals from 1996 to 1998. The overall resistance rate was 5% and majority of these resistant organisms were found infected with respiratory tract infections. Isolates producing IRT are more frequently reported in Europe than in United States. This may be due to the discrepancy between combinations of factors like environmental, methodological and clinical factors. The phenomenon does not appear to be related to large difference in the use of β-lactam and β-lactamases inhibitors combinations, as the agents are widely used in Europe and United States and likely to produce similar pressures on bacteria. The detection of IRTs presents a significant number of operational challenges. Disagreement between the results of MIC testing and disk diffusion assays is often seen in many studies, especially among the isolates with intermediate resistance phenotypes. Currently, an international standard for the amount of β-lactam for detection of IR enzymes is not in use, and different combinations can significantly alter the assigned results. [40]

A serine of monobactam derivatives was recently developed to target the pathogens harboring multiple β-lactamases. Some of the monobactam derivatives act as very effective bactericidal agents for difficult to treat gram-negative pathogens, while others are bridged monobactams such as BAL29880 that are inhibitors of AmpC enzymes. Other monobactam are the one which

bears siderophore side chain which can enhance cell entry through bacterial iron uptake systems which include BAL19764 and BAL30072. They show antibacterial activity against carbapenem-resistant *P.aerogenosa, Acinetobacter spp, S.maltophilia* and *S.marcescens* as well as gram-negative bacilli expressing VIM and IMP β-lactamases. However the activity of carbapenems is still superior to that of these new monobactams for many ESBL-producing isolates. [42, 44]

Publication in 2013 by infection innovative medicines units on Avibactam which is a derivative of beta-lactamase inhibitors possess the broad spectrum of activity against currently employed beta-lactamases. It is in phase III clinical development in combination with third generation cephalosporins i.e. ceftazidime. The addition of Avibactam to ceftazidime has shown to restore the antibacterial activity against the strains which express wide range of beta-lactamases enzyme of classes A, C and some class D. [19]

Recent study done in USA in department of chemistry in Wesleyan University showed that all three classes of serine beta-lactamases are inhibited at micromolar levels by 1:1 complex of catechols with vanadate. Typical examples of all three classes are TEM-2 and OXA-1 enzymes. The inhibition was moderately enhanced by hydrophobic substituents on catechol. The inhibition was modestly enhanced by hydrophobic substituents on the catechol. 1:1 vanadate is better inhibitors of P99 beta-lactamases than 1:1 complex of catechol with boric acid. [36]

Research groups focused from India:

In India research work is being going on beta-lactams resistance patterns in clinical isolates. Each year, approximately 600 deaths result from infections caused by the two most common type carbapenem-resistant *Klebsiella spp*. and carbapenem-resistant E. coli. NDM-1 was first detected in a Klebsiella pneumoniae isolate from a Swedish patient of Indian origin in 2008. It is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics. These include the antibiotics of the carbapenem family, which are a main stay for the treatment of antibiotic-resistant bacterial infections. [30, 38]

Resistance to β-lactam and β-lactamase inhibitors challenges the successful treatment for serious urinary tract, respiratory, and bloodstream infections caused by pathogenic microbes. In the University of Allahabad in 2011 One hundred ten samples were tested for Beta-lactamases production, collected from different tertiary care hospitals at Allahabad. Out of the 110 patient's samples, 89 (80.91%) isolates were found to be gram-negative bacteria. Of 89 clinical isolates from different samples, *Escherichia coli* was found to be most common organism (58.42%) followed by *Klebsiella pneumoniae* (20.22%), *Pseudomonas aeruginosa* (12.35%), *Proteus vulgaris* (3.37%), *Proteus mirabilis* (2.24%) and *Enterobacter aerogenes* (2.24%). A single

strain of *Candida albicans* was also isolated. Of these 89 isolates studied, 48(53.93%) were found to be beta-lactamase producers. High incidences of beta-lactamase producing strain have been found in *Klebsiella pneumoniae* (61.11%) and *Escherichia coli* (57.69%). [37]

AmpC b-lactamase producing multidrug resistant strains in *Klebsiella* spp. & *Escherichia* coli was isolated from children under five in Chennai by scientists in India. AmpC b-lactamases are Group I cephalosporinases that confer resistance to a wide variety of b-lactam drugs. Plasmid mediated AmpC b- lactamases has been discovered most frequently in isolates of *Klebsiella pneumoniae, K. oxytoca, Salmonella, Proteus mirabilis* and *Escherichia coli*. A study in Chennai found 31 percent MRSA among 805 S. aureus strains from the Sri Ramachandra Medical College and Research Institute. Among these, high resistance was found to gentamicin, co-trimoxazole, and erythromycin. [28, 35]

In 2008, a study from the microbiology department at the Post Graduate Institute of Medical Sciences in Rohtak found that 54 percent of 628 *S. Aureus* strains from inpatients and outpatients were MRS.

Most of the studies that included tests for Vancomycin resistance found that it was either very low or non-existent; however, one study from the Sher-i-Kashmir Institute of Medical Sciences found that 22 of 120 MRSA strains from clinical samples had intermediate sensitivity to vancomycin, although none was fully resistant. [39]A few studies have looked at resistance rates in multiple hospitals. One early study found that 32 percent of 739 Staphylococci strains were MRSA (Mehta et al. 1996). This study included hospitals in New Delhi, Mumbai, and Bangalore. Sixty-six percent of MRSA strains were resistant to ciprofloxacin, 22 % were resistant to rifampicin, and all were sensitive to vancomycin. [27, 43]

Research gaps:

Since the beginning of antibiotic era there have been reports on novel beta-lactams which are sensitive to all kinds of enzymatic destructions, but with the time there is need to study the resistance patterns among the clinical isolates at the microorganism are developing resistance against the drug at fast pace. This study mainly focuses on the evaluation of resistance patterns against the beta-lactams in the Himachal Pradesh (India) region where epidemic outbreaks of infection occur seasonally. In India there is a need of regular census of local sensitivity patterns to formulate and upgrade the antibiotic policies and need of upgrading the faster laboratory facilities for better and faster detection of isolates, proper collection, analyses and sharing of data.

Chapter 3

Aim: To evaluate antimicrobial resistance pattern against beta-lactams and its current derivatives

Objectives:

Following are the objectives of the project:

1) Procurement of samples from the regional hospital Isolation of different pathogenic bacteria from the clinical samples obtained from regional hospitals.

2) Beta lactams susceptibility test for different clinical isolates.

3) Construction of resistance profile against the beta lactams.

4) Determining the MIC breakpoints for beta-lactam.

Chapter 4

Material

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	50ml

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 extract	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.

SUBSTRATE UTILIZATION MEDIA:

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	1.5 gm
Agar	15gm

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

❖ **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 stand

Table 1: List of beta-lactams tested

S.NO	Antibiotic discs		Concentration
1	Ceftazidime	CAZ	30µg\ml
2	Cefepime	CPM	30µg\ml
3	Ceftriaxone	CTR	30µg\ml
4	Cefotaxime	CTX	30µg\ml
5	Tazobactam\ceftazidime	CAT	30\10 µg\ml

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

Antibiotic name	**Code**	**Concentration**
Potentx R(Cefepime&Amikacin)	I	12500µg\ml
Supime(cefepime&Sulbactum)	II	15000µg\ml
Elores(ceftriaxone\disodium edeate\sulbactum)	III	15000µg\ml
Vancoplus(ceftriaxone&vancomycin)	IV	15000µg\ml

Methodology:

Procurement of samples: Samples were procured from regional hospital (Indira Ghandhi Medical College, Shimla) and CRI Kasauli.

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

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

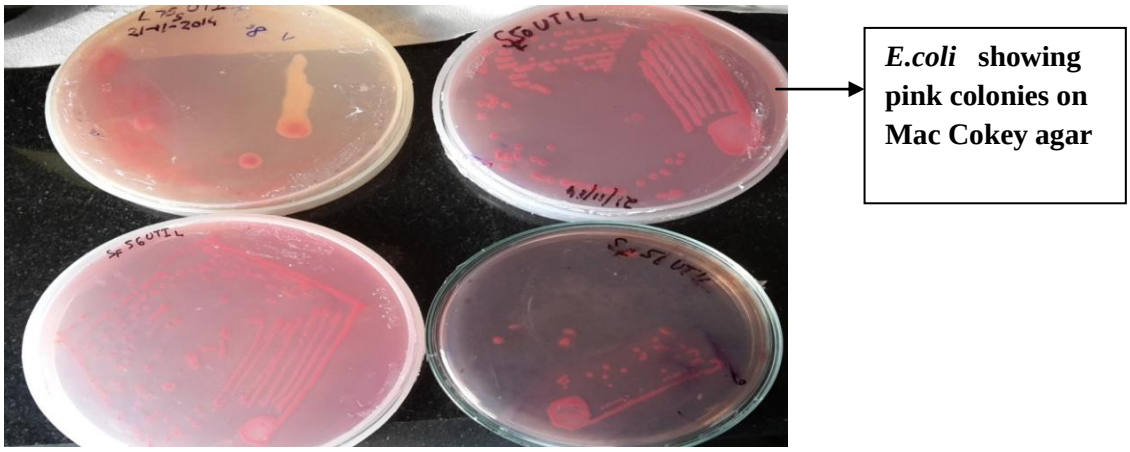


Figure2- Lactose fermenting colonies of clinical isolates on Mac Conkey agar

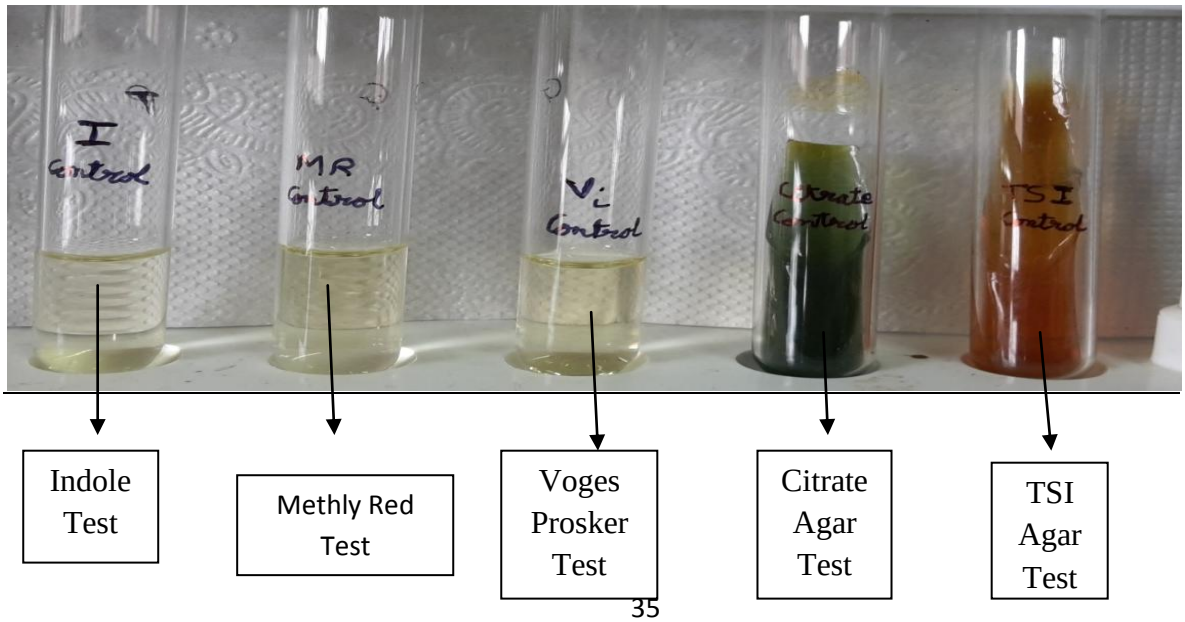
Figure 3- Bright yellow colonies of *E.coli* on Xylose-lysin deoxycholate (XLD) agar

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

- **Biochemical characterization:**

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.

Figure 4- Depicts the control tubes of biochemical test



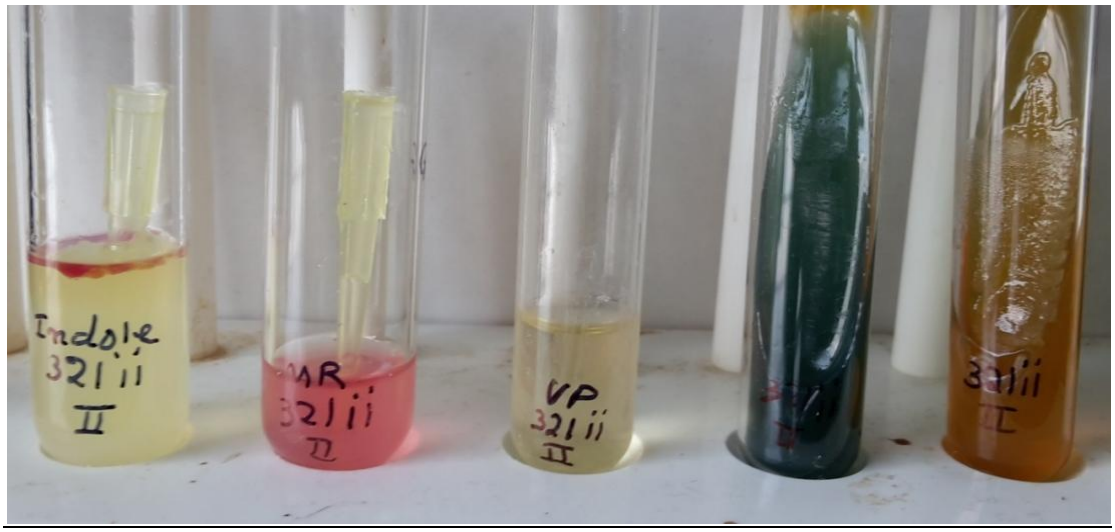


Figure 5 - Shows biochemical characterization of *E.coli* using Indole, Methyl red, Voges Prausker and Triple sugar iron agar test(++--, A/A)

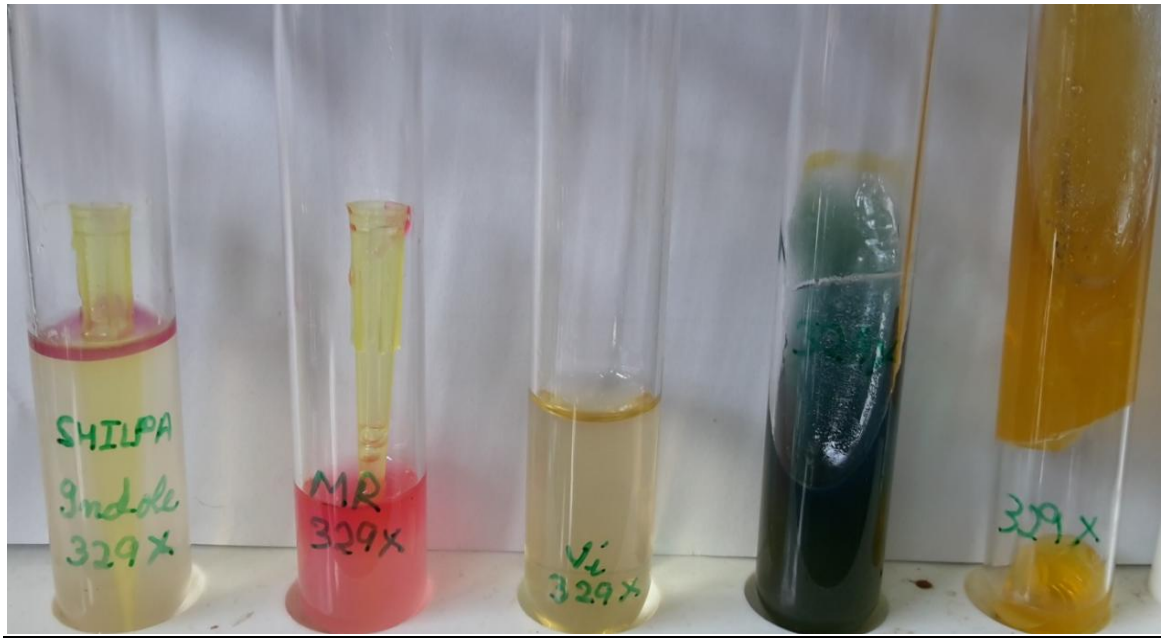


Figure 6: Shows biochemical characterization of *Klebsiella spp.* using Indole, Methyl red, Voges Prausker and Triple sugar iron agar test(++--, A/A, gas)

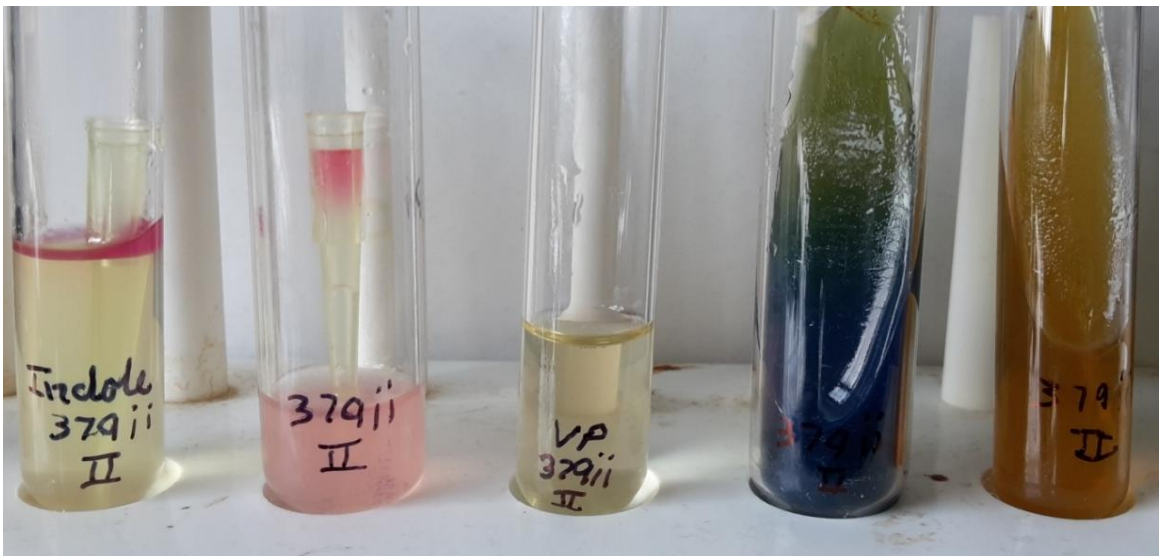
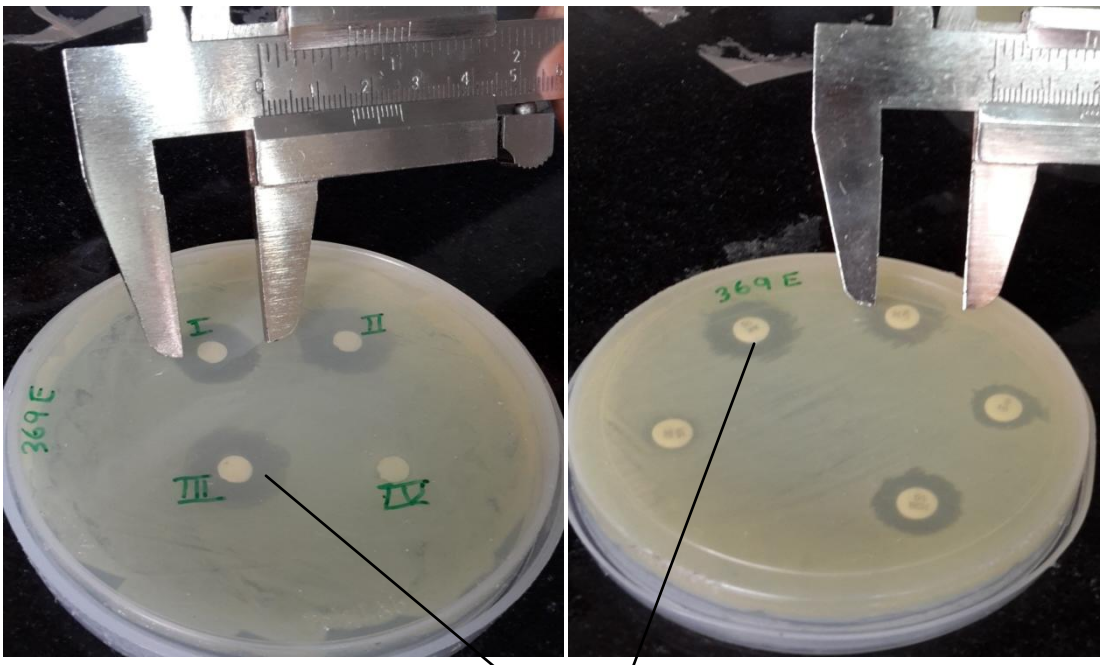


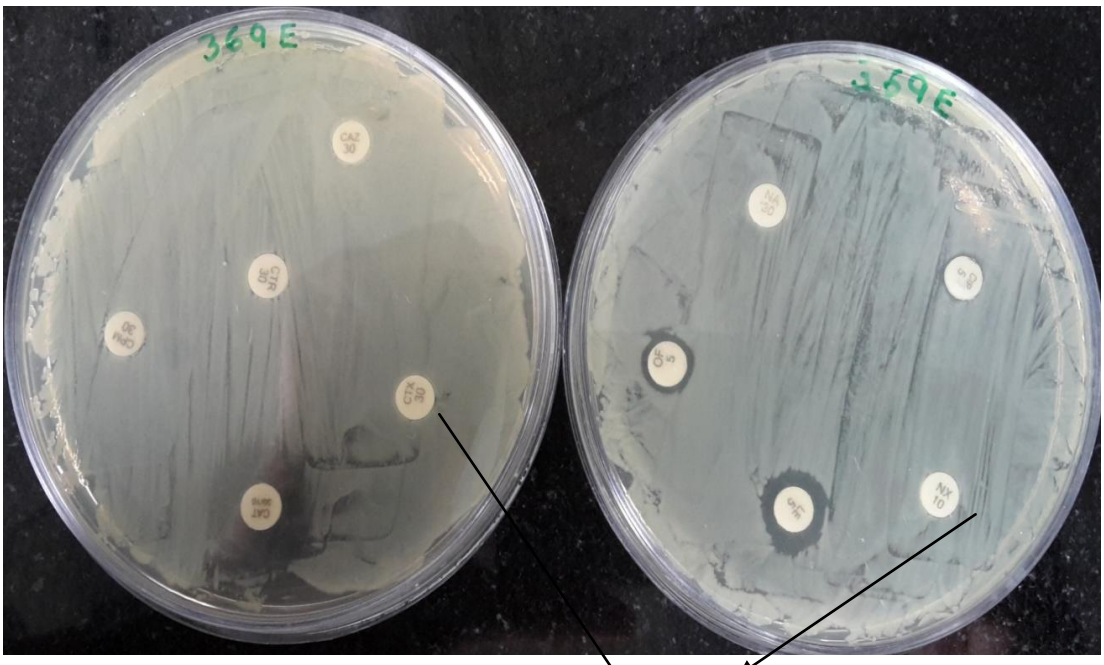
Figure 7- Shows biochemical characterization of *Citrobacter* spp. using Indole, Methyl red, Voges Prausker and Triple sugar iron agar test(++-+, A/A)

Antimicrobial susceptibility testing:

- After characterization of bacteria antibiotic susceptibility test was done each isolated and characterized bacteria by following the Kerby Bauer method.
- Inoculums was prepared from the cultured broth used during the biochemical test by transferring 20 µl to a tube containing 10 ml NB broth and allowed to grow for 24 hours till the cultured broth reaches the desired OD625 of 0.5-.6.
- 100 µl of this culture broth was taken and spread on the Muller Hinton agar plate with the help of a glass spreader. The inoculums were allowed to dry for a few minutes at room temperature with the lid closed.
- The antibiotic discs were placed on the inoculated plates using forceps.
- These plates were then incubated at 35°C for 16-18 hours.
- The diameter of zone of inhibition was measured using a ruler on the under-surface of the plate containing transparent medium. The diameter of zone of inhibition will be measured in mm.
- The sizes of the zones of inhibition are interpreted by referring through 2I (Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints) of the CLSI. [32]

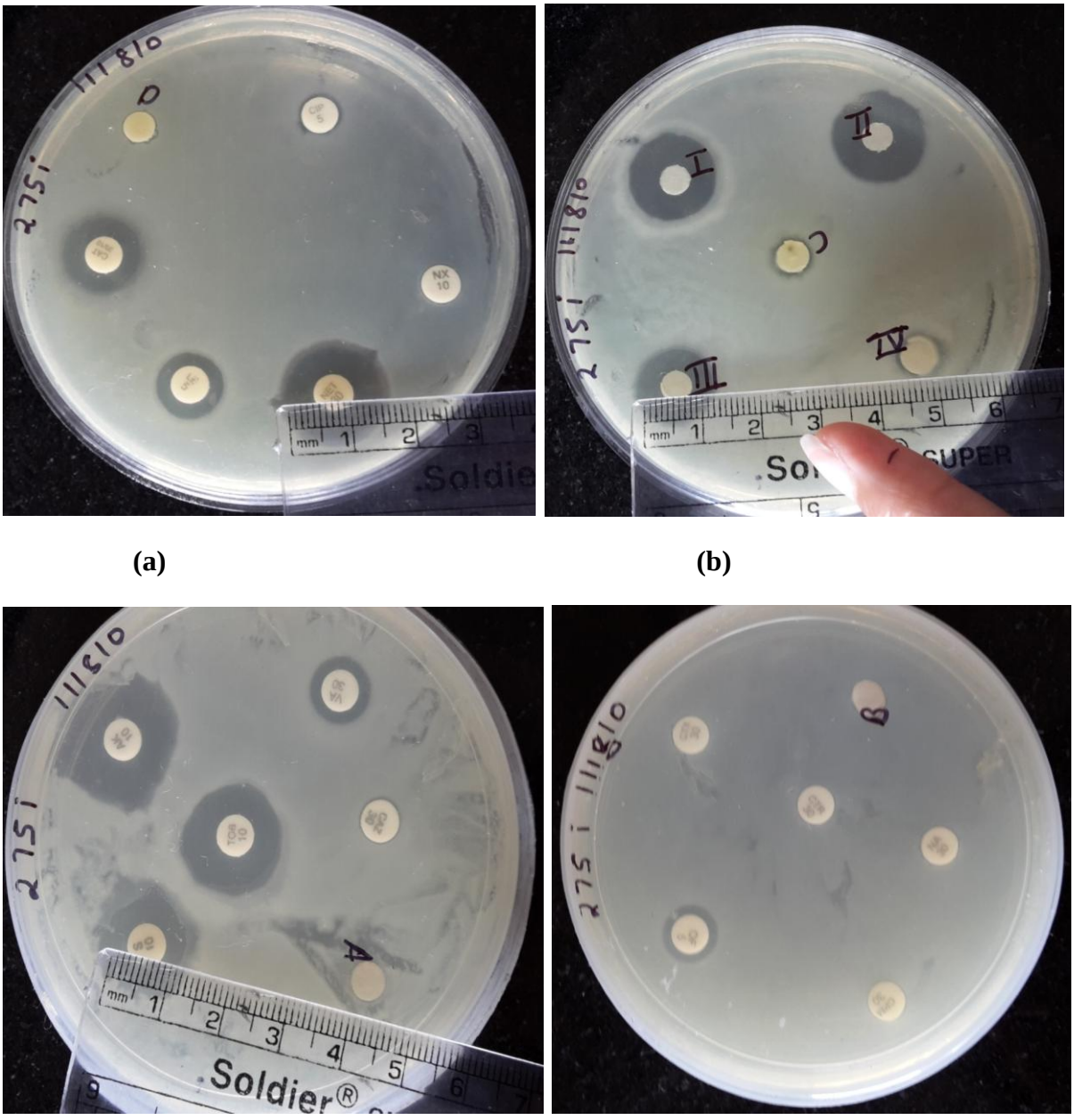


(a) Zone of inhibition **(b)**



(c) No zone of inhibition (resistance)

Figure 8: (a): shows antibiotic sensitivity test of combination drugs (Cefepime&Amikacin (I) cefepime&Sulbactum (II) ceftriaxone\disodium edeate\ sulbactum(III) and ceftriaxone& vancomycin(IV)) against *E.coli* and **(b) and (c)** depicts different diameters of zone of inhibition against *E.coli* tested for beta-lactams and others antibiotics



(a) **(b)**

(c) **(d)**

Figure 9 : (a): shows antibiotic sensitivity test of combination drugs (Cefepime&Amikacin (I) cefepime&Sulbactum (II) ceftriaxone\disodium edeate\ sulbactum(III) and ceftriaxone& vancomycin(IV)) against *E.coli* and (b), (c) and (d**)** depicts different diameters of zone of inhibition against *E.coli* tested for beta-lactams and others antibiotics

- **MIC (Minimum inhibitory concentration) determination was done –**

1) Preparation of antibiotic stock solution- antibiotic stock solution was prepared by commercially available antibiotic powder (with potency) and the amount needed and diluents in which it can dissolved can be calculated by using either of the following formulas to determine the amount of powders (1) or diluent (2) needed for standard solution.

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

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

2) Antibiotic stock solutions were prepared for antibiotics;

- Amikacin, Gentamycin, Ciprofloaxin, Piperacillin.
- Combination drugs provided by Venus medical research centre i.e.
- 1)Potentox (Cefepime&Amikacin)
- 2)Supime(cefepime&Sulbactum)
- 3)Elores(ceftriaxone\disodiumedeate\sulbactum)
- 4) Vancoplus (ceftriaxone&vancomycin)

1) Small volumes of the sterile stock solutions were dispensed into eppendorfs vials; carefully seal; and store (at −20 °C or below,).
2) 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 eppendorfs vials.
3) 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.
4) 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.

5) The plates were then covered and incubated at 37^{o}C.
6) When satisfactory growth was obtained (18-36 hours) the plates were scanned with an ELISA reader (Thermo Reader) at 600nm.
7) MIC was taken as the lowest concentration of drug that reduces, by more than 50% or 90% for MIC_{50} or MIC_{90} respectively.[34]

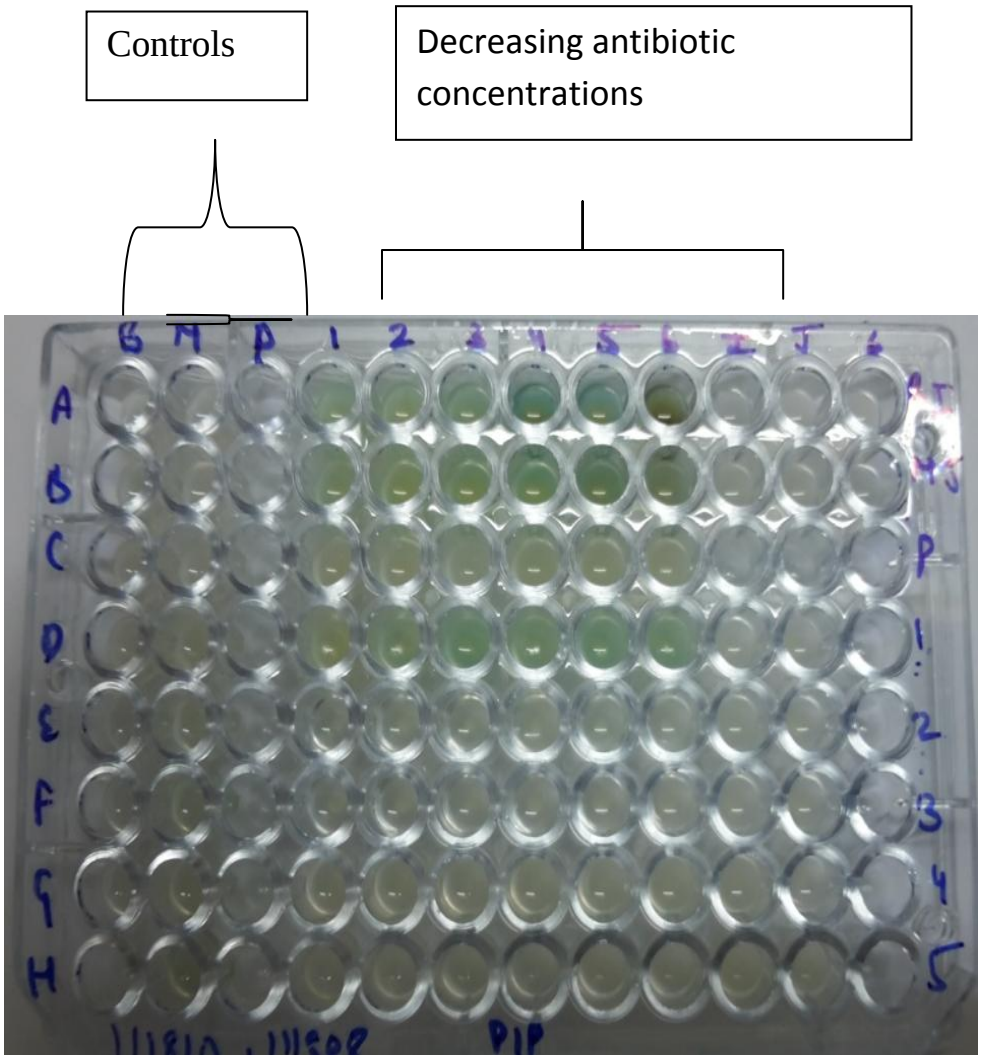


Figure 10- Shows U-bottom microtitre 96 well plate used to determine the MIC for piperacillin Column B represents bacterial control, column M represents media control and column P represents plate control. Columns 1 to 6 have decreasing antibiotic concentrations of 2000 µg/100ml , 1000 µg/100ml, 500 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 i*solates, row D, E, F, G have *E.coli* and I, J has *Shigella spp.*

Glycerol stock preparation: Glycerol stock preparation: For glycerol stock preparation; 30% glycerol solution was prepared and autoclaved it. Then the processed culture was inoculated in nutrient broth and then next day prepared glycerol stocks in well labeled eppendorfs by adding 500µl of glycerol solution and 500µl of culture and prepared it in duplicates. The glycerol stocks were then stored at -80℃. [33, 32]

Chapter 5

Results

(5.1) Isolation of different bacteria on nutrient agar and identification on selective media:

Clinical samples were inoculated in nutrient broth and sample were the characterized by streaking on different selective media as shown in figure 2 and figure 3 of methodology section.

(5.2) Characterization of isolates based on biochemical testing:

Isolates were characterized on the bases of the IMViC and TSI patterns shown by them. See figure 4, 5, 6, 7 and table 3,4,5,6. *E.coli* was found as the predominant one among other isolates.

(5.3) Antimicrobial susceptibility testing of characterized bacteria by Kirby Bauer method:

Antimicrobial susceptibility testing was performed by the Kirby Bauer method and zone of inhibitions were determined according to which the resistance and susceptibility of isolates was determined (see figure 8,9 and table 6,7). Different classes of beta-lactams tested showed different susceptibility patterns against the clinical isolates. Ceftazidime has shown high rate of sensitivity against the isolates i.e. 25%, ceftriaxone shown 14.20% sensitivity rate against the isolates. Cefepime had shown high rate of resistance against clinical isolates (see figure 12).

E.coli has shown 87% sensitive for ceftazidime and 82% sensitivity for cefotaxime while 12% resistance against ceftazidime and 18% resistance against cefotaxime. *E.coli* is highly resistance against cefepime showing 99% of resistance and 91% resistance against ceftriaxone. (see figure 13 and 14).

Klebsiella spp. isolated from the clinical samples has shown high resistance against all the beta-lactams tested. It has shown 100% resistance against cefotaxime and ceftriaxone. It has shown 36% and 9% intermediacy for ceftazidime and cefepime respectively while 64% and 91% of resistance against ceftazidime and cefepime respectively.(see figure 15 and 16).

Proteus spp. has shown 100% resistance against cefepime and 67% resistance against cefotaxime. It has shown 33% intermediacy for cefotaxime. It is sensitive for ceftazidime and has shown 67% sensitivity and 33 % resistance against ceftazidime. On the other hand proteus spp has shown 30% sensitivity and 70% resistance against ceftriaxone. (see figure 21 and 22)

Shigella spp. has shown 60% resistance against cefotaxime and cefepime while it has shown 40% and 0% resistance against ceftazidime and ceftriaxone respectively. It has shown 20% intermediacy cefepime, cefotaxime and ceftazidime. It is 20% sensitive for cefepime and cefotaxime while it has

shown 40% and 100% sensitivity for ceftazidime and ceftriaxone respectively (see figure 17 and 18)

Pseudomonas spp.. has shown 25%, 75%, 50%, and 25% sensitivity for cefotaxime, ceftriaxone, ceftazidime and cefepime respectively. It has shown 50%, 0%, 25%, and 75% resistance against cefotaxime, ceftriaxone, ceftazidime and cefepime respectively. It has shown 25% intermediacy for cefotaxime, ceftriaxone and ceftazidime.(see figure 19 and 20)

(5.4) Minimum inhibitory concentration determination:

MIC breakpoint determined for the Piperacillin antibiotic was observed at 10µg/ml in case of Pseudomonas isolates while in case of *E.coli* it was observed at 2.5µg/ml and in case of Shigella spp. it was observed at 20µg/ml. these concentrations determined were well above the ClSI recommended breakpoints concentrations. (see figure 10).

Table 3: Shows the biochemical results and the identified organisms in clinical isolates.

S.NO		I	MR	VP	Citrate	TSI	Organism
1	321iill	POS	POS	NEV	NEV	A\A	E.coli
2	321il	POS	POS	NEV	NEV	k\k	Unk
3	321iil	POS	POS	NEV	NEV	A\A	E.coli
4	321iiill	POS	POS	NEV	NEV	A\A	E.coli
5	344iillll	NEV	POS	NEV	NEV	A\A	E.coli
6	276iill	POS	POS	NEV	NEV	A\A	E.coli
7	275iill	NEG	POS	NEV	NEV	A\A	E.coli
8	278ii	POS	POS	NEV	NEV	A\A	E.coli
9	299i	POS	POS	NEV	NEV	A\A	E.coli
10	344iil	POS	POS	NEV	NEV	A\A	E.coli
11	379iill	POS	POS	NEV	POS	A\A	Citrobacter spp.
12	276iil	POS	POS	NEV	NEV	A\A	E.coli
13	299ii	POS	POS	NEV	NEV	A\A	E.coli
14	275i	POS	POS	NEV	NEV	A\A	E.coli
15	344ill	NEV	POS	NEV	NEV	A\A	E.coli
16	379i	POS	POS	NEV	NEV	A\A	E.coli
17	275iil	POS	POS	NEV	NEV	A\A	E.coli
18	344il	POS	POS	NEV	NEV	A\A	E.coli
19	276i	POS	POS	NEV	NEV	A\A	E.coli
20	379iill	POS	POS	NEV	NEV	A\A	E.coli
21	321iiill	POS	POS	NEV	NEV	k\k	Unk
22	369X	POS	POS	NEV	NEV	A\A	E.coli
23	369E	POS	POS	NEV	NEV	K/A	E.coli
24	278X	POS	POS	NEV	NEV	A\A,GA:	E.coli
25	278E	**POS**	**POS**	**NEV**	**NEV**	**A\A,GA**	**E.coli**
26	329X	POS	POS	NEV	POS	A\A,GA:	Citrobacter spp.
27	329E	POS	POS	NEV	NEV	A\A	E.coli
28	322XI	NEV	POS	NEV	NEV	K\A	Shigella spp
29	322XII	NEV	POS	POS	NEV	K\A	unk
30	322XIII	NEV	NEV	NEV	POS	K\K	unk
31	322EI	POS	POS	NEV	NEV	A\A	E.coli
32	322EII	POS	POS	NEV	NEV	A\A,GA:	E.coli
33	322EIII	POS	POS	NEV	NEV	A\A,GA:	E.coli

Table 4: Shows biochemical results and bacteria found in clinical isolates

S.No	Sample	Indole	MR	VP	Citrate	TSI	Organism
1	1SI	NEV	NEV	NEV	POS	A\A	*Proteus.mirabilus*
2	1SII	NEV	POS	NEV	POS	A\A	*Salmonella.paratyhpi*
3	1SIII	NEV	POS	NEV	NEV	A\A	*Shigella.boydii*
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.boydii*
9	SFUTIL	NEV	POS	NEV	NEV	A\A	*E.coli*
10	PUS3S	NEV	NEV	NEV	NEV	A\A	*Pasteurella.haemolytica*
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	*Pasteurella.haemolytica*
19	NARANLS	NEV	NEV	POS	NEV	A\A	
20	1NLFS	NEV	POS	POS	POS	A\A	*Klebsiella.pneumonia*
21	PUSNAV	NEV	POS	NEV	NEV	A\A	*Shigella.boydii*
22	PUSND1L	NEV	POS	NEV	NEV	A\A	*Shigella.boydii*

Table 5: Shows the biochemical results and identified organism according to IMViC pattern

		BIOCHEMICAL TEST RESULTS					
S.No	Sample	Indole	MR	VP	Citrate	TSI	Organism
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	
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	
34	CRIKIII''W	NEV	NEV	NEV	NEV	A\A	*Pasteurella.haemolytica*
35	CRIKII''	NEV	NEV	POS	POS	K\A	*Serratia.marcescens\Corneybacteria.pyogenes*
36	CRIKI'	NEV	NEV	POS	POS	K\A	*Serratia.marcescens\Corneybacteria.pyogenes*
37	CRIKII'	NEV	POS	NEV	POS	K\A	*salmonella.tyhpi\Pseudomonas. aeruginosa*
38	CRIKIII''R	POS	POS	NEV	NEV	A\A	*E.coli*

Out of 73 samples tested the percentage prevalence of *E.coli* was predominant one among all the clinical isolates. Percentage distribution of *E.coli* was 47 % other isolates found were *Klebsiella* spp. (15%), *Proteus spp*. (4%), *Citrobacter spp*. (1%), *Pseudomonas spp.*. (6%), *Shigella spp.* (7%) and bacteria from unknown etiology (20%).

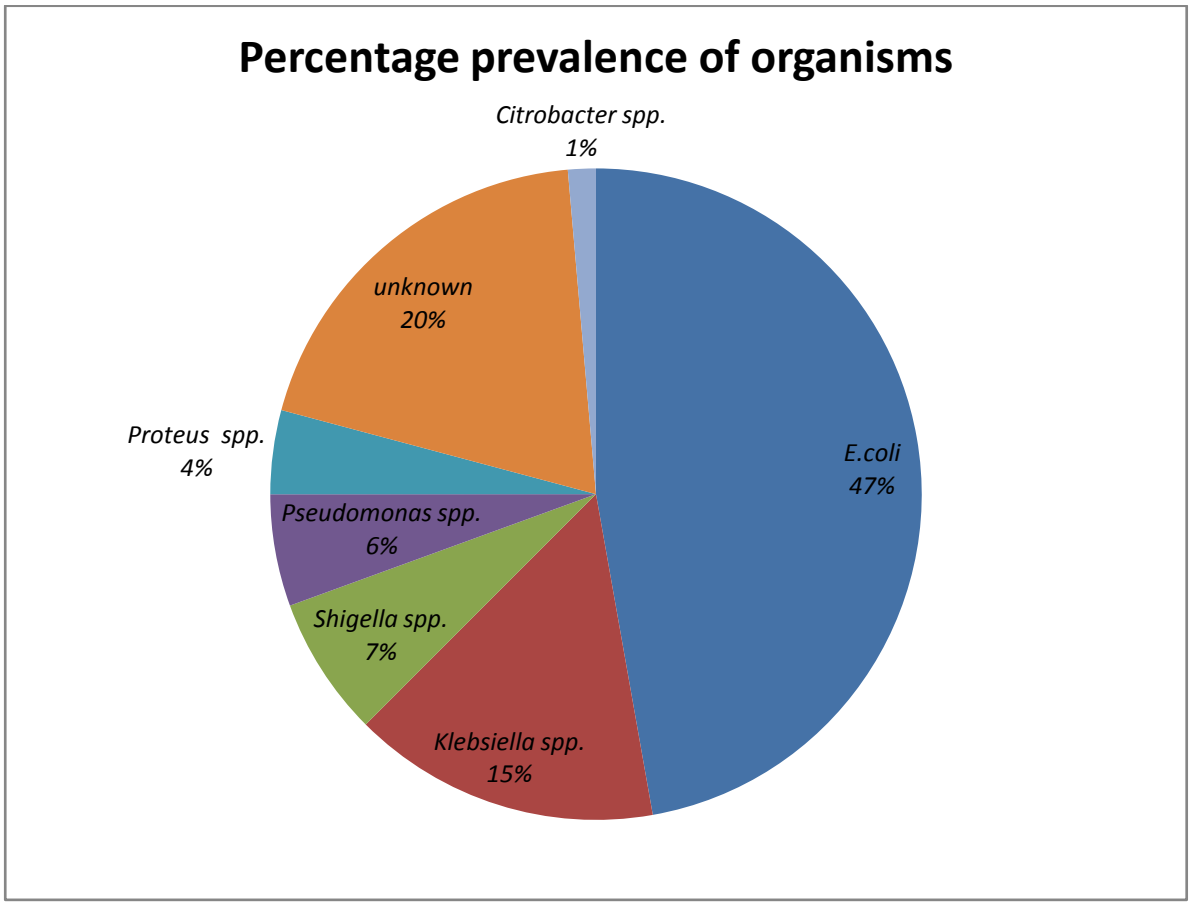


Figure 11- Shows the percentage prevalence of various organisms in the clinical samples antibiotic susceptibility test results

Table 6: Shows the antimicrobial susceptibility results along with the interpretations for beta –lactam tested on the samples

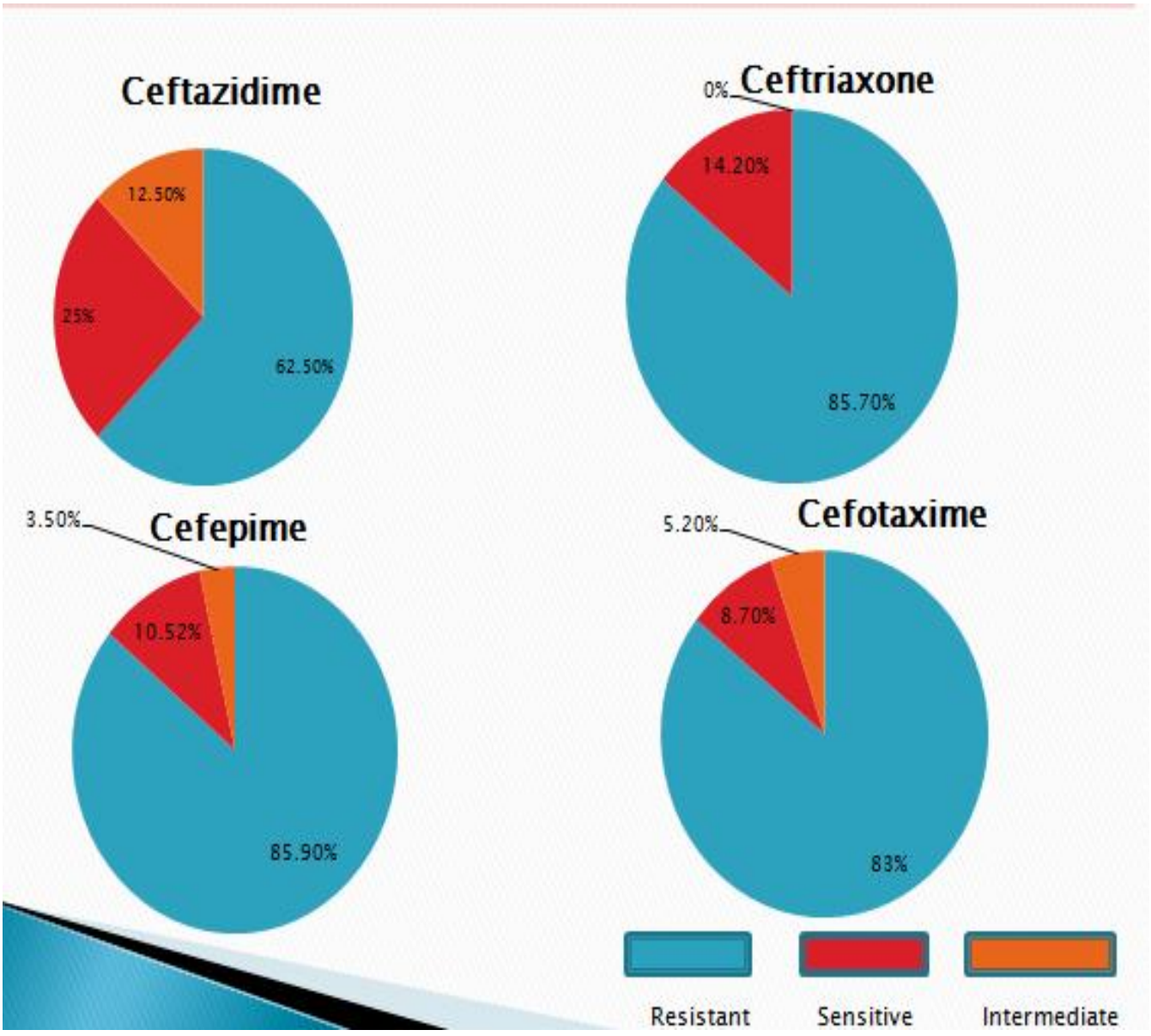
			Beta Lactams				
	Sample	Organis	CAZ	CAT	CPM	CTR	CTX
1	321iII	E.coli	8mm\R	20mm	0mm\R	0mm\R	0mm\R
2	321iI	Unk	0mm	16mm	0mm	0mm	0mm
3	321iiI	E.coli	0mm\R	13mm	0mm\R	0mm\R	0mm\R
4	321iiII	E.coli	0mm\R	16mm	0mm\R	0mm\R	0mm\R
5	344iiIII	E.coli	0mm\R	12mm	0mm\R	0mm\R	0mm\R
6	276iiII	E.coli	0mm\R	14mm	0mm\R	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	Citrobact	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

Table 7: Shows the zone of inhibition for beta lactams tested by clinical isolates.

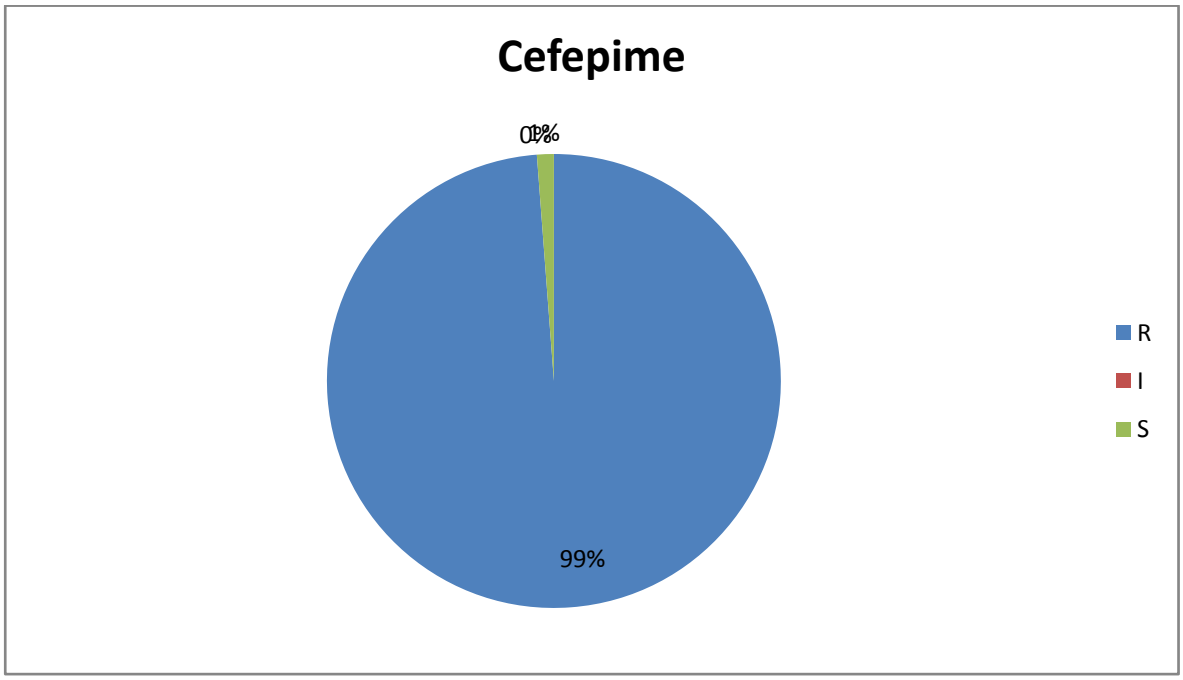
				Beta-lactams		
				CPM	CTX	CAZ
1	SF56LUT	IGMC	*E.coli*	17mm\R		
2	SF56SUT	IGMC	*E.coli*	18mm\R	15mm\R	13mm\R
3	L75SUTI	IGMC	*E.coli*	16mm\R		
4	PUSND2I	IGMC	*E.coli*	13mm\R	14mm\R	12mm\R
5	CKIII	IGMC	*E.coli*	15mm\R	10mm\R	15mm\R
6	CRIKIII"R	IGMC	*E.coli*			
7	SFUTIL	IGMC	*E.coli*	20mm\R	21mm\R	21mm\S
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 IGM	IGMC	*E.coli*	18mm\R	20mm\R	18mm\I
12	SF50LUT	IGMC	*Klebsiell*	15mm\R	19mm\R	15mm\R
13	SF50SUT	IGMC	*Klebsiell*	18mm\R	19mm\R	17mm\R
14	PUSNAV	IGMC	*Klebsiell*	15mm\R	18mm\R	11mm\R
15	NARANS	IGMC	*Klebsiell*	10mm\R	5mm\R	4mm\R
16	CKII		*Klebsiell*	20mm\R	20mm\R	19mm\I
17	CKI		*Klebsiell*	23mm\I	22mm\R	18mm\I
18	CRIKIII'		*Klebsiell*	20mm\R	19mm\R	19mm\I
19	1SVIII		*Klebsiell*	20mm\R	19mm\R	19mm\I
20	2LF		*Klebsiell*	18mm\R	17mm\R	10mm\R
21	1SVIw		*Klebsiell*	18mm\R	20mm\R	18mm\R
22	Pseudomonas.aer		*P. aerug*	30mm\S	26mm\S	25mm\S
23	CKIV	Kasauli	*Pseudon*	18mm\R	19mm\I	18mm\S
24	1SX	Shimla	*Pseudon*	15mm\R	12mm\R	15mm\I
25	13a	Shimla	*Pseudon*	7mm\R	11mm\R	6mm\R
26	PUSND1L	Shimla	*Shigella*	19mm\R	16mm\R	14mm\R
27	PUSND1S	Shimla	*Shigella*	29mm\S	30mm\S	4mm\R
28	Navdeep	Shimla	*Shigella*	22mm\I	24mm\I	21mm\S
29	1SIII	Shimla	*Shigella*	18mm\R	20mm\R	18mm\S
30	NARANLI	Shimla	*Proteus*	21mm\R	20mm\R	10mm\R
31	1SI	Shimla	*Proteus*	21mm\R	24mm\I	20mm\S
32	1SVIR	Shimla	*Proteus*	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

Different classes of beta-lactams tested showed different susceptibility patterns against the clinical isolates. Ceftazidime has shown high rate of sensitivity against the isolates i.e. 25%, ceftriaxone shown 14.20% sensitivity rate against the isolates. Cefepime had shown high rate of resistance against clinical isolates.

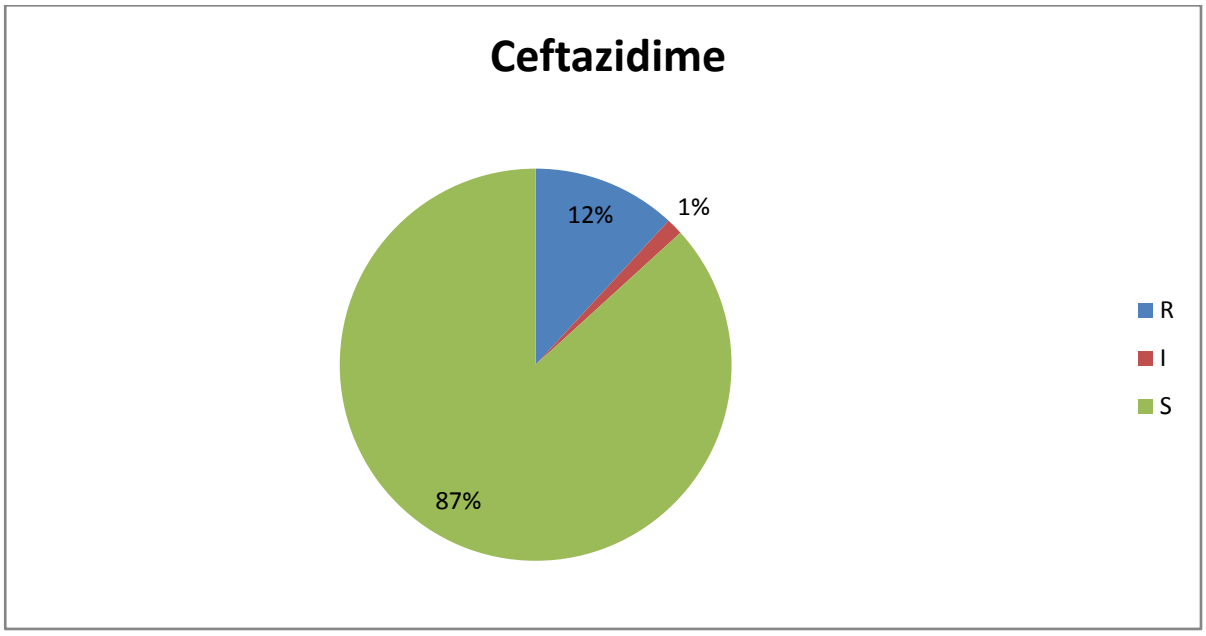
Figure 12: Shows the percentage distribution of antibiotic resistance for different beta-lactams tested



The percentage susceptibility of *E. coli* for Cefepime is 0% and shows 99% resistance against the antibiotic (b) depicts the percentage susceptibility for *E. coli* for Ceftazidime is 87% and shows 12% resistance against the antibiotic.



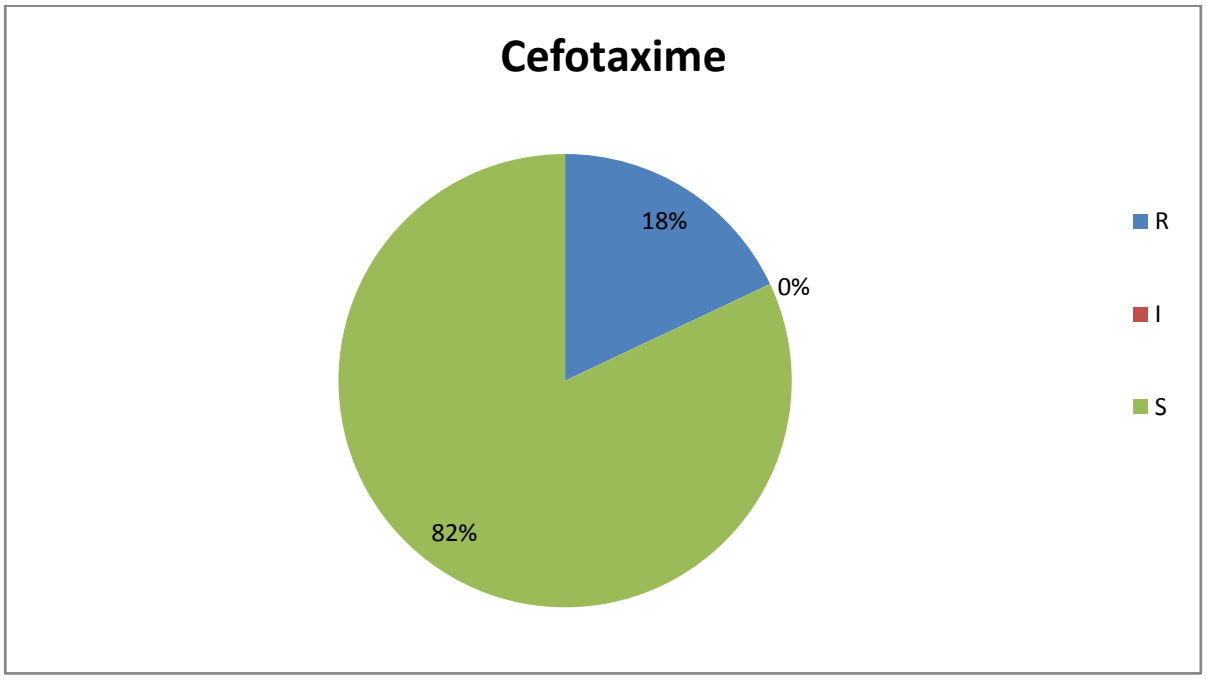
(a)



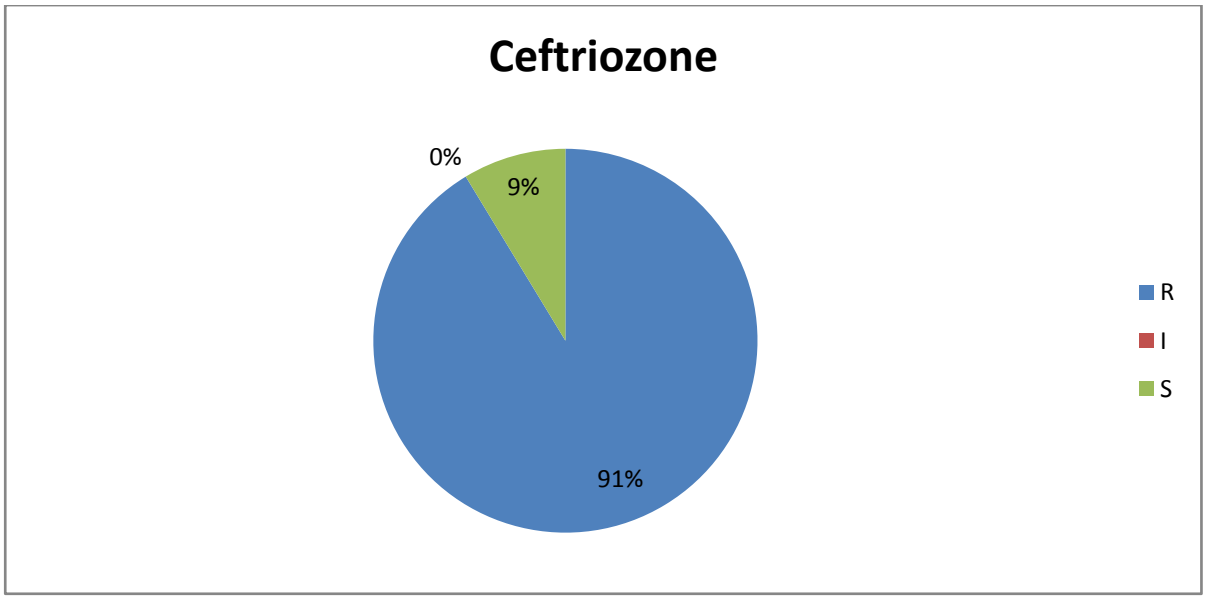
(b)

Figure 13- (a) and (b) depicts the percentage susceptibility of *E. coli* for Cefepime and Ceftazidime.

The percentage prevalence of *E.coli* for cefotaxime is 82% and 18% resistance against antibiotic and the percentage susceptibility of *E.coli* for ceftriaxone is 9% and 91% resistance against the antibiotic

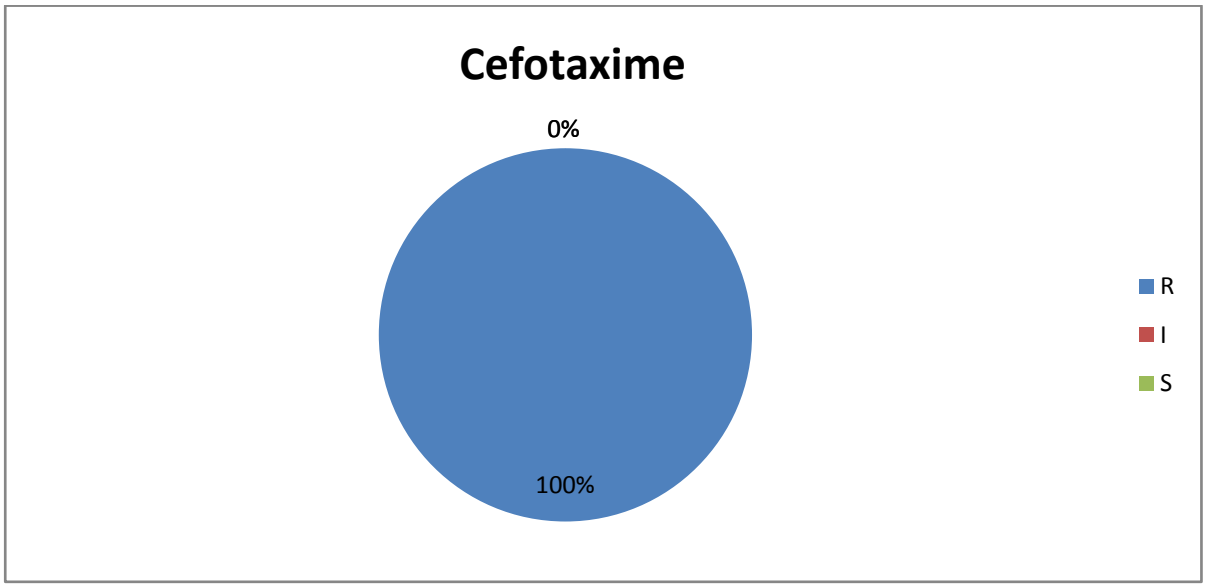


(a)

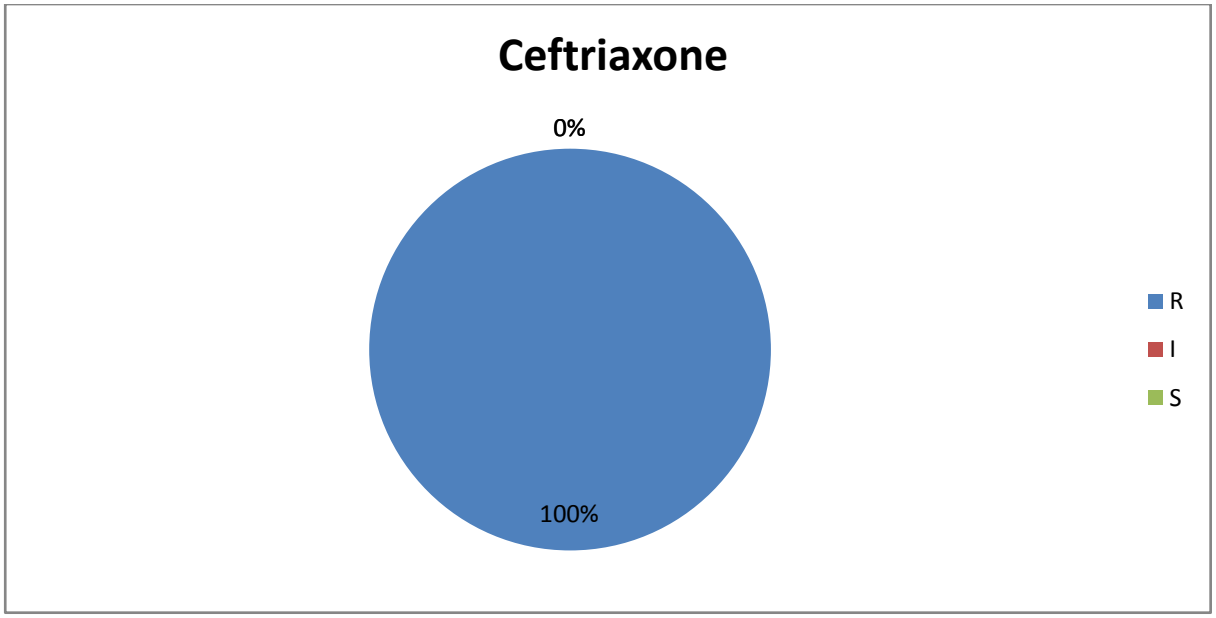


(b)Figure 14- (a) and (b) depicts the percentage prevalence of *E.coli* for cefotaxime and ceftriaxone

The percentage susceptibility of *Klebsiella spp* for cefotaxime is 0% and shows 100% resistance against the antibiotic and the percentage susceptibility of *Klebsiella spp* for cefotaxime is 0% and shows 100% resistance against the antibiotic.



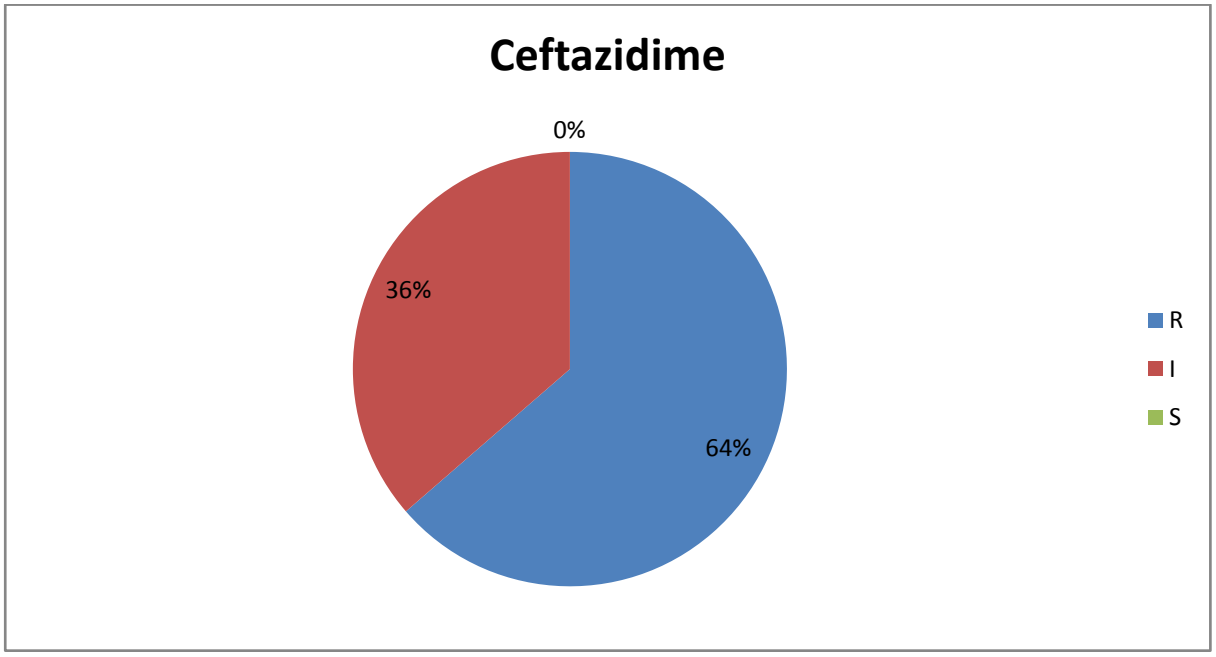
(a)



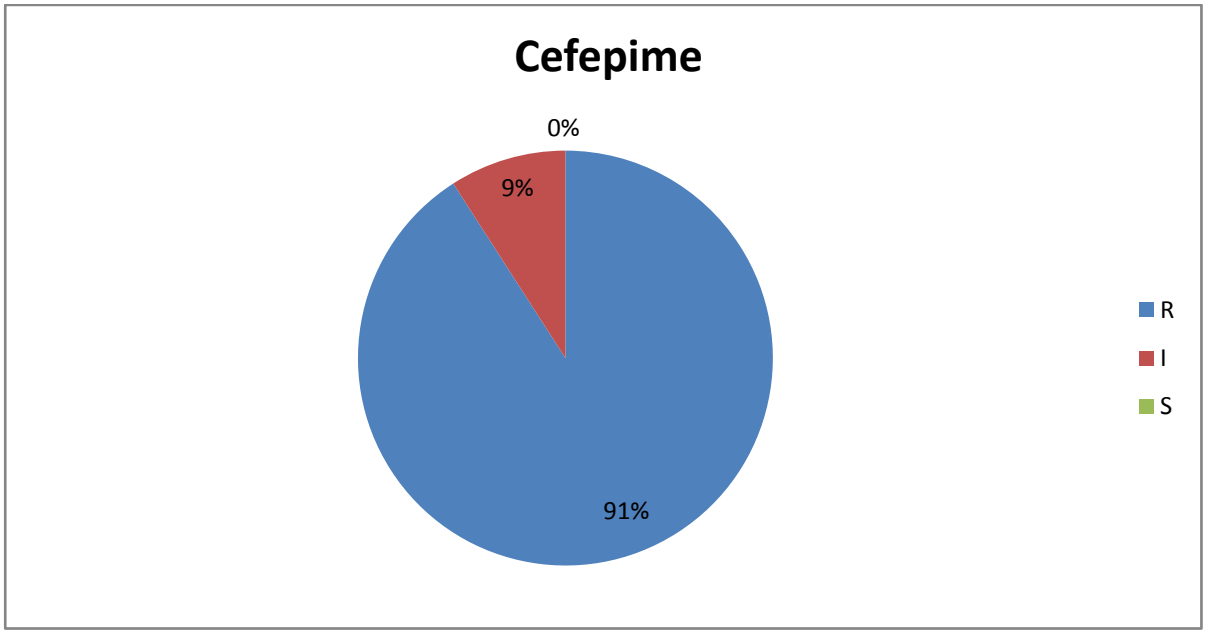
(b)

Figure 15- (a) and (b) depicts the percentage susceptibility of *Klebsiella spp* for cefotaxime and cefotaxime

The percentage susceptibility of *Klebsiella spp* for ceftazidime is 0% and shows 64% resistance against the antibiotic and the percentage susceptibility of *Klebsiella spp* for cefotaxime is 0% and shows 91% resistance against the antibiotic.



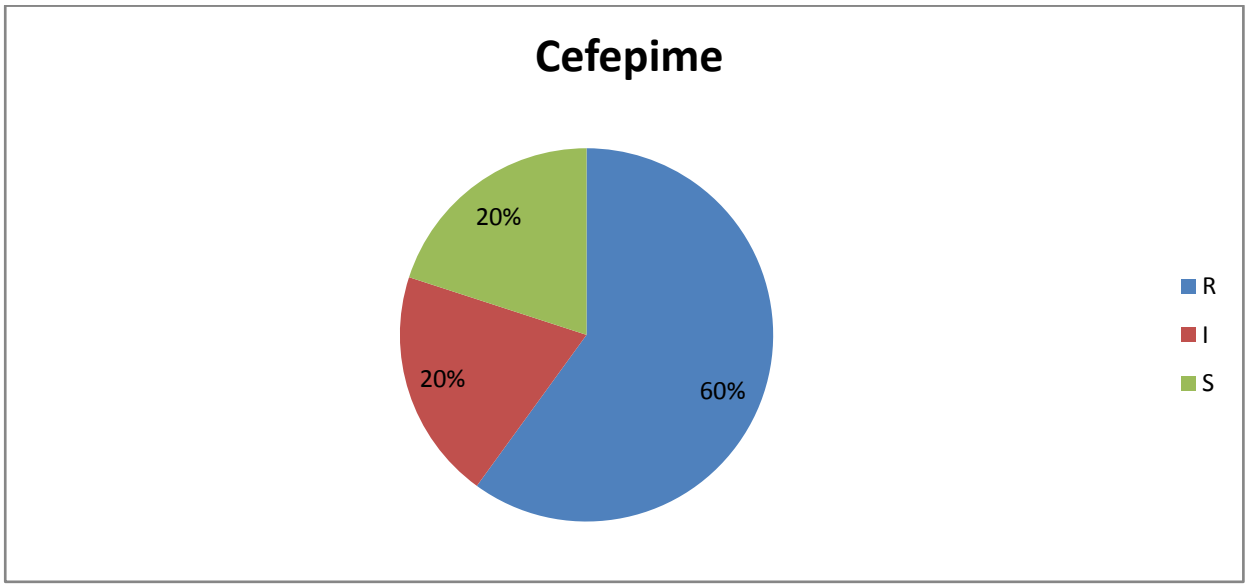
(a)



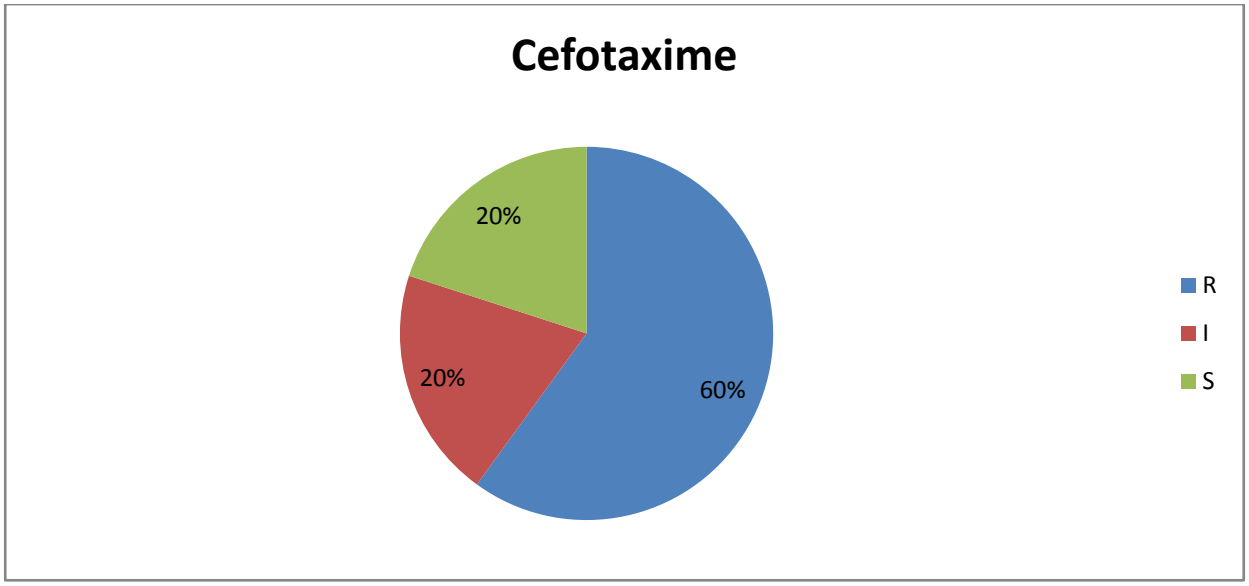
(b)

Figure 16- (a) and (b) depicts the percentage susceptibility of *Klebsiella spp* for ceftazidime and cefotaxime.

The percentage susceptibility of *Shigella spp*. for cefepime is 2 0% and shows 60% resistance against the antibiotic and (b) depicts the percentage susceptibility of *Shigella spp*. for cefotaxime is 2 0% and shows 60% resistance against the antibiotic.



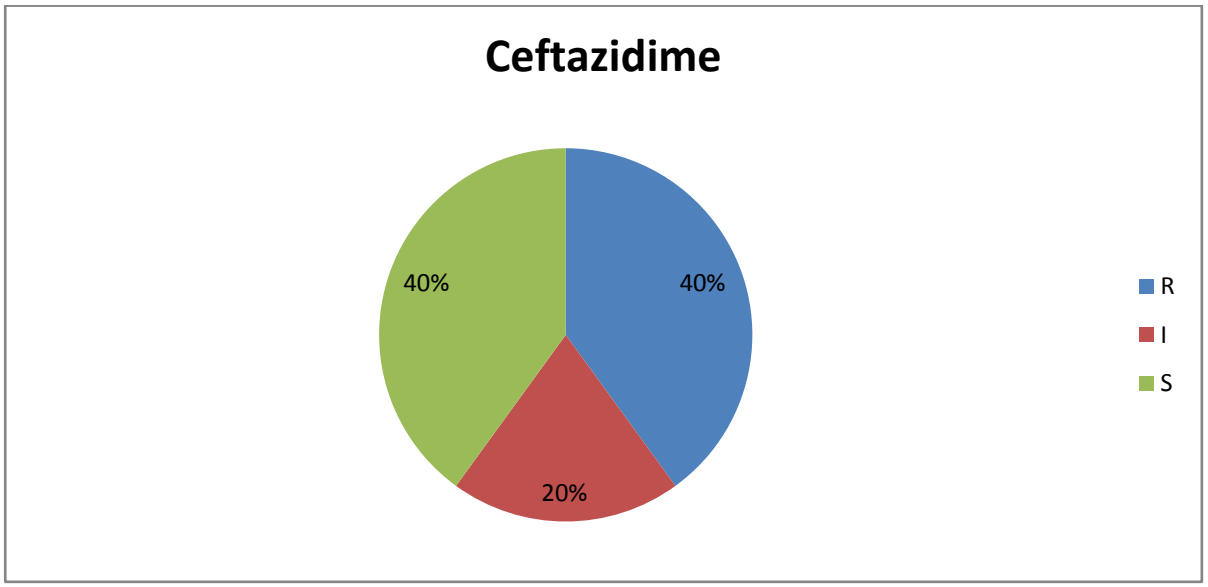
(a)



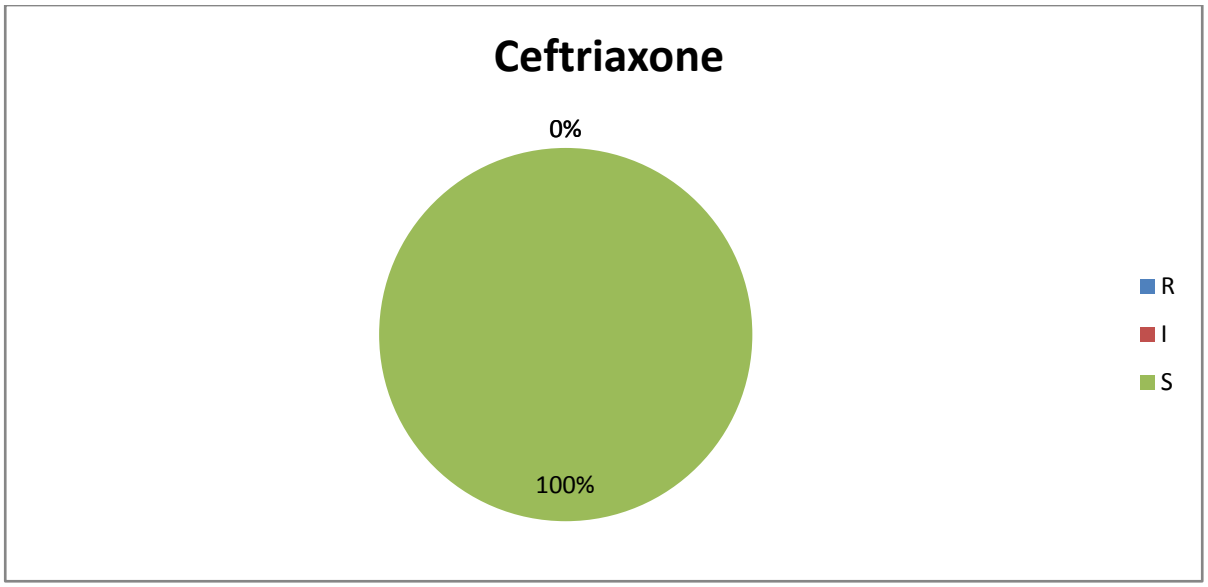
(b)

Figure 17 – (a) and (b) depicts the percentage susceptibility of *Shigella spp*. for cefepime and cefotaxime.

The percentage susceptibility of *Shigella spp*. for ceftazidime is 40% and shows 40% resistance against the antibiotic and the percentage susceptibility of *Shigella spp*. for cefotaxime is 100% and shows 0% resistance against the antibiotic.



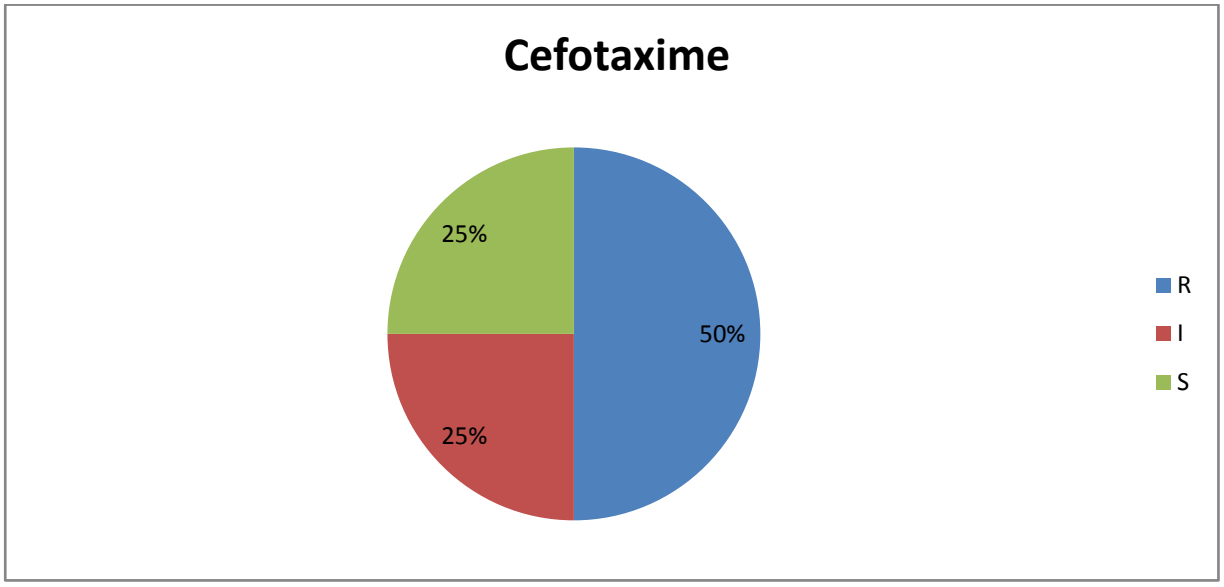
(a)



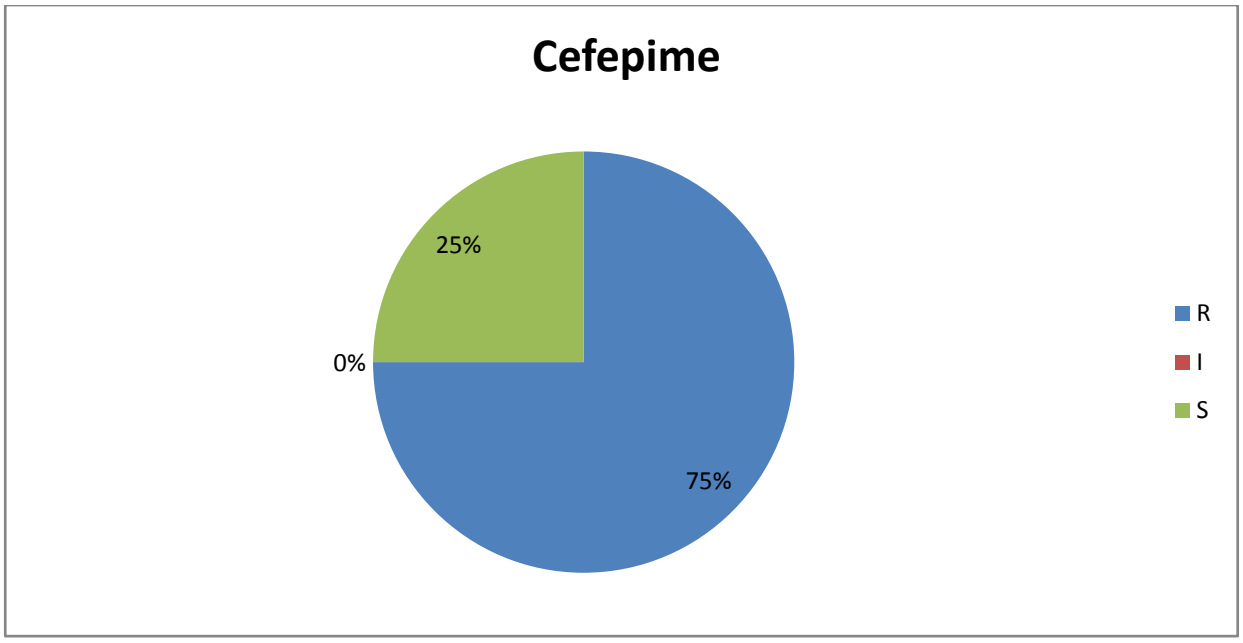
(b)

Figure 18-(a) and (b) depicts the percentage susceptibility of *Shigella spp*. for ceftazidime and cefotaxime i.e. 100% and shows 0% resistance against the antibiotic.

The percentage susceptibility of *Pseudomonas spp.*. for cefotaxime is 25% and shows 50% resistance against the antibiotic and the percentage susceptibility of *Pseudomonas spp.*. for cefepime is 25% and shows 75% resistance against the antibiotic.



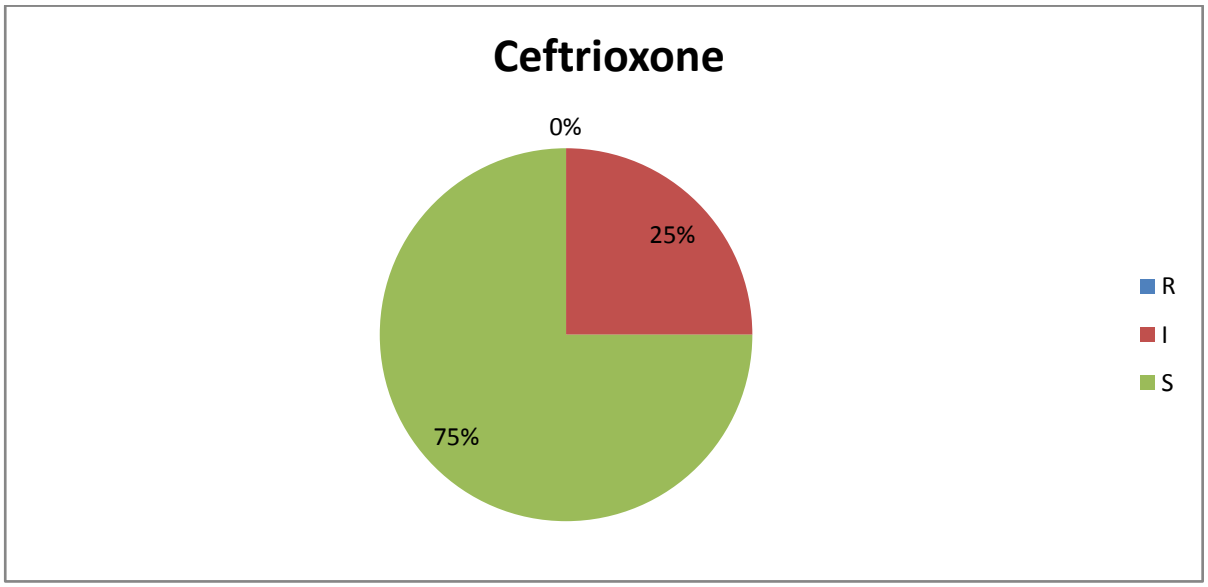
(a)



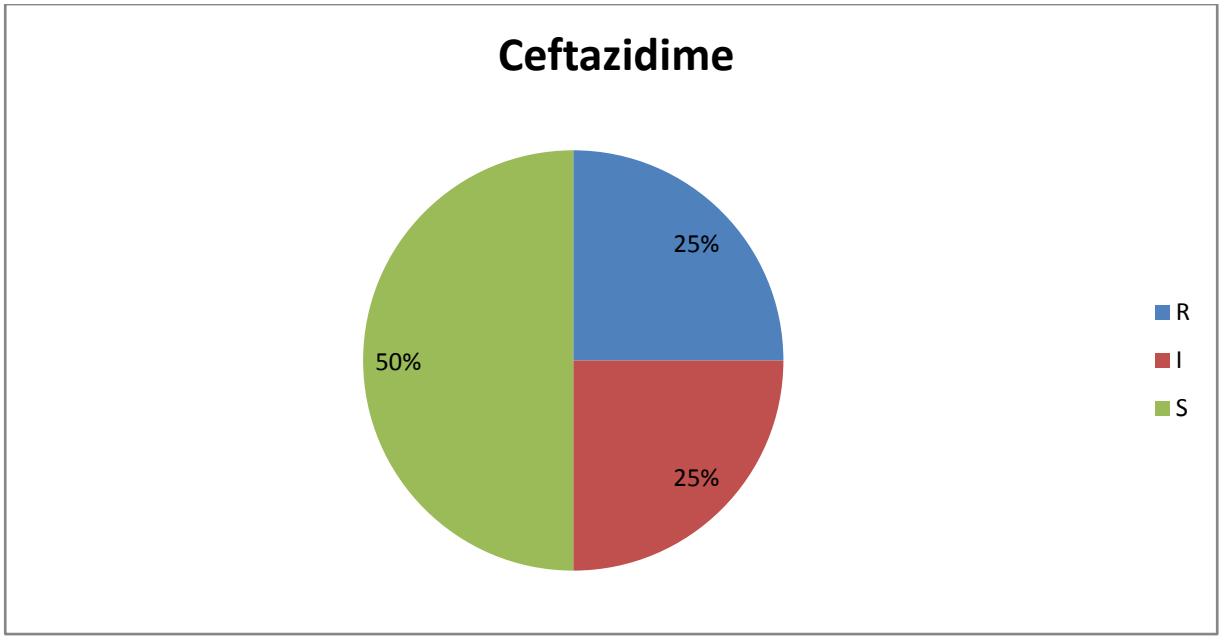
(b)

Figure 19-(a) and (b) depicts the percentage susceptibility of *Pseudomonas spp.*. for cefotaxime cefepime.

The percentage susceptibility of *Pseudomonas spp.*. for ceftriaxone is 25% and shows 0% resistance against the antibiotic and the percentage susceptibility of *Pseudomonas spp.*. for ceftazidime is 50% and shows 25% resistance against the antibiotic.



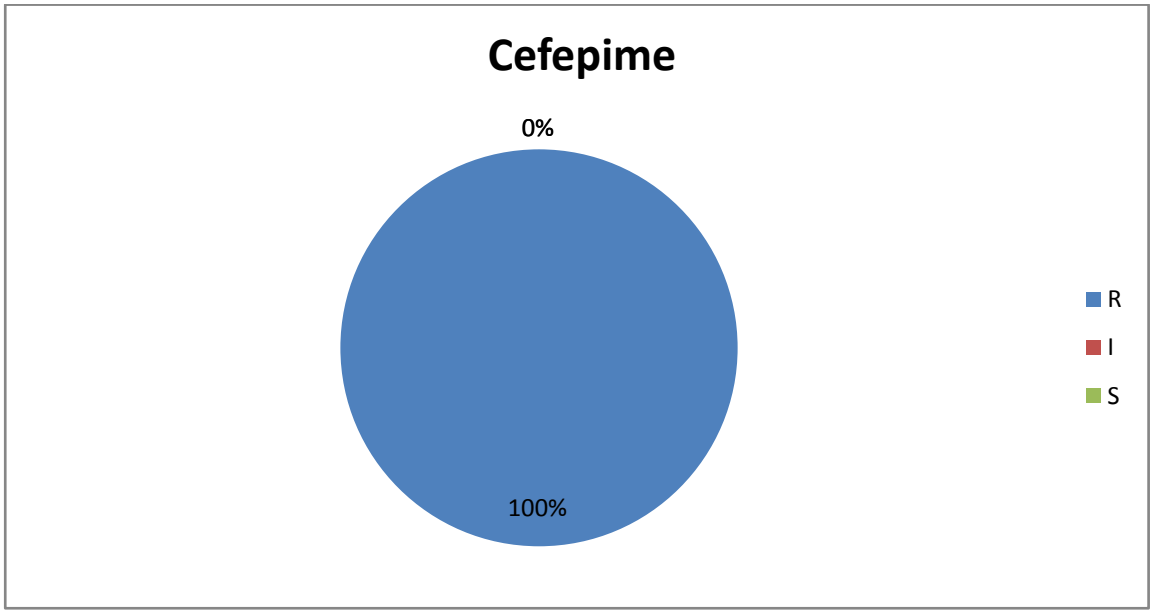
(a)



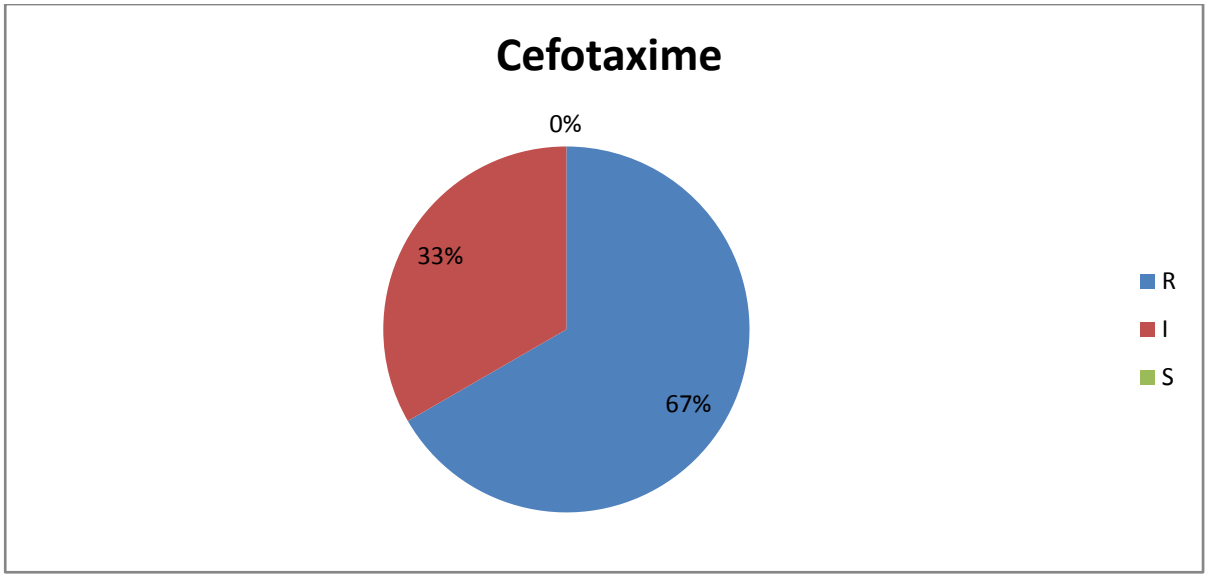
(b)

Figure 20- (a) and (b) depicts the percentage susceptibility of *Pseudomonas spp.*. for ceftriaxone and ceftazidime.

The percentage susceptibility of *Proteus spp*. for cefepime is 100% and shows 0% resistance against the antibiotic and the percentage susceptibility of *Proteus spp*. for cefotaxime is 0% and shows 67% resistance against the antibiotic.



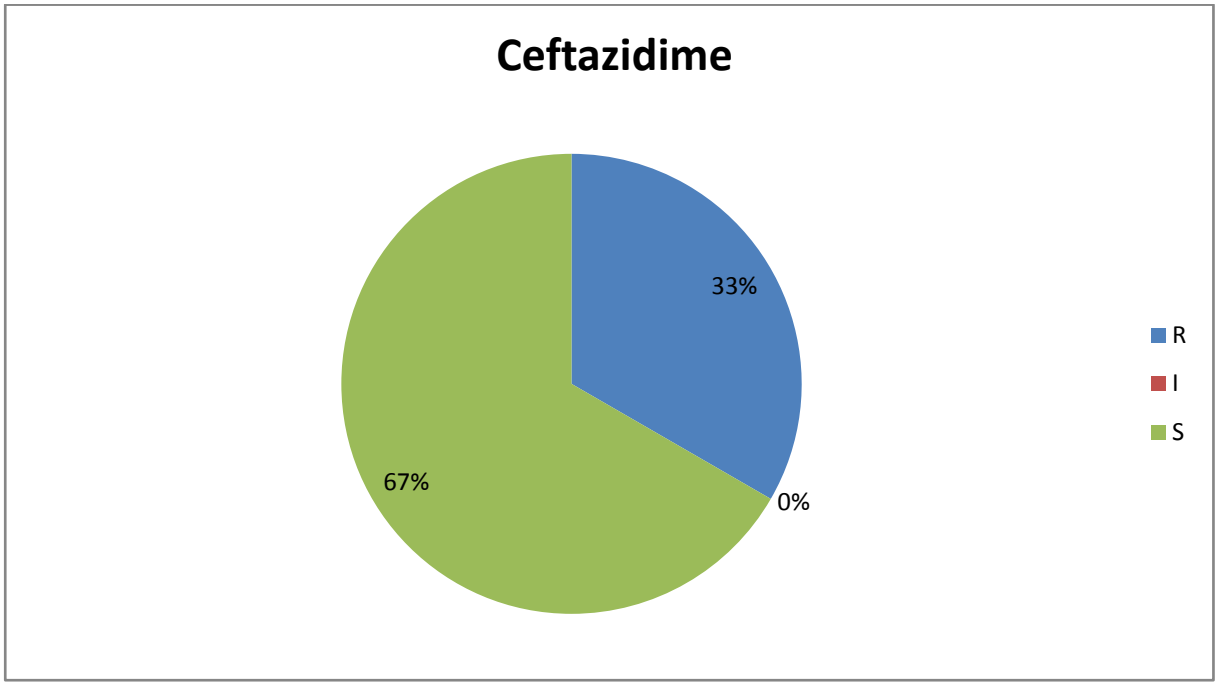
(a)



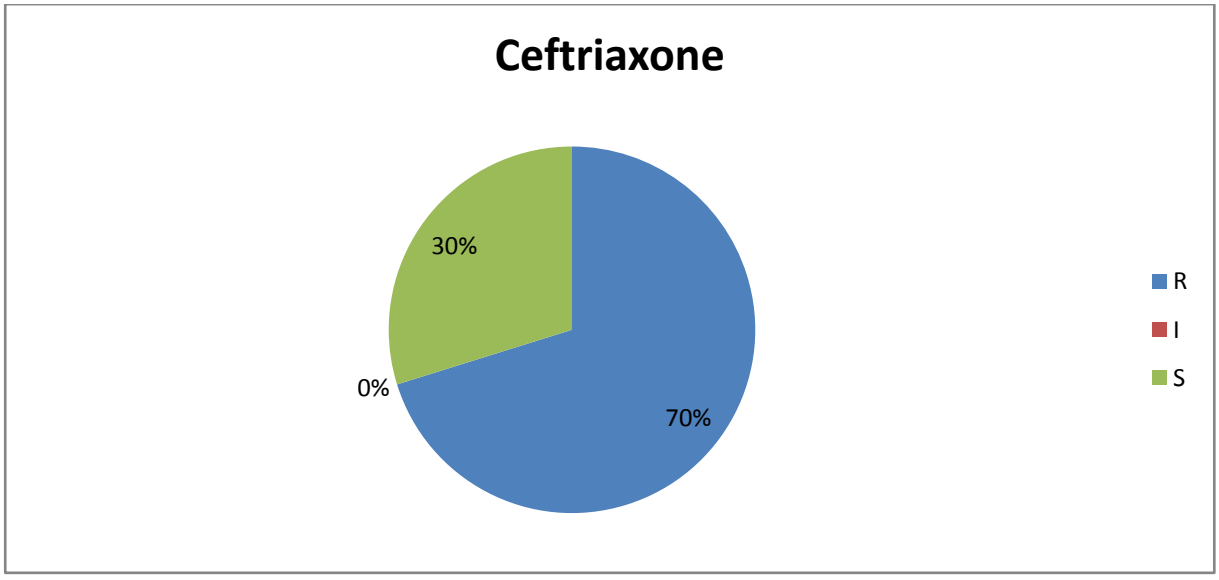
(b)

Figure 21- (a) and (b) depicts the percentage susceptibility of *Proteus spp*. for cefepime and cefotaxime

The percentage susceptibility of *Proteus spp*. for ceftazidime is 67% and shows 33% resistance against the antibiotic and the percentage susceptibility of *Proteus spp*. for ceftriaxone is 30% and shows 70% resistance against the antibiotic.



(a)



(b)

Figure 22- (a) and (b) depicts the percentage susceptibility of *Proteus spp*. for ceftazidime and ceftriaxone i.e. 30% and shows 70% resistance against the antibiotic.

Discussion:

Out of 73 samples tested the percentage prevalence of *E.coli* was predominant one among all the clinical isolates. Percentage distribution of *E.coli* was 47 % other isolates found were *Klebsiella* spp. (15%), *Proteus spp*. (4%), *Citrobacter spp*. (1%), *Pseudomonas spp.*. (6%), *Shigella spp.* (7%) and bacteria from unknown etiology (20%).

E.coli has shown 87% sensitive for ceftazidime and 82% sensitivity for cefotaxime while 12% resistance against ceftazidime and 18% resistance against cefotaxime. *E.coli* is highly resistance against cefepime showing 99% of resistance and 91% resistance against ceftriaxone. In the study published by Sumera sahid et al. in 2014 The *E. coli* were highly resistant to penicillin (100%), amoxicillin (100%) and cefotaxime (89.7%), followed by intermediate level of resistance to ceftazidime (73.8%), ceftriaxone (43.3%), imipenem (43.3%).

Klebsiella spp. isolated from the clinical samples has shown high resistance against all the beta-lactams tested. It has shown 100% resistance against cefotaxime and ceftriaxone. It has shown 36% and 9% intermediacy for ceftazidime and cefepime respectively while 64% and 91% of resistance against ceftazidime and cefepime respectively. Study by Islam MB et al has shown that among the 132 samples *Escherichia coli* had found in 103(78.0%) cases and Klebsiella spp. was found in 14(10.6%) cases. The percentage prevalence of *E.coli* in there was higher as compared to our. Out of 103 *E coli* 23(22.3%) cases was found as ESBL strain. On the other hand within 14 Klebsiella species, the ESBL strain was found in 5(35.7%) cases. Both E coli and Klebsiella species were 100% sensitive to imipenem. However, cephamycin was sensitive in 93.7% and 100% in E coli and *Klebsiella species* respectively. [45]

Proteus spp. has shown 100% resistance against cefepime and 67% resistance against cefotaxime. It has shown 33% intermediacy for cefotaxime. It is sensitive for ceftazidime and has shown 67% sensitivity and 33 % resistance against ceftazidime. On the other hand proteus spp has shown 30% sensitivity and 70% resistance against ceftriaxone.

Shigella spp. has shown 60% resistance against cefotaxime and cefepime while it has shown 40% and 0% resistance against ceftazidime and ceftriaxone respectively. It has shown 20% intermediacy cefepime, cefotaxime and ceftazidime. It is 20% sensitive for cefepime and cefotaxime while it has shown 40% and 100% sensitivity for ceftazidime and ceftriaxone respectively.

Pseudomonas spp.. has shown 25%, 75%, 50%, and 25% sensitivity for cefotaxime, ceftriaxone, ceftazidime and cefepime respectively. It has shown 50%, 0%, 25%, and 75% resistance against

cefotaxime, ceftriaxone, ceftazidime and cefepime respectively. It has shown 25% intermediacy for cefotaxime, ceftriaxone and ceftazidime.

MIC breakpoint determined for the Piperacillin antibiotic was observed at 10µg/ml in case of Pseudomonas isolates while in case of *E.coli* it was observed at 2.5µg/ml and in case of Shigella spp. it was observed at 20µg/ml. these concentrations determined were well above the ClSI recommended breakpoints concentrations.

The combination drugs provided by Venus medical research centre has slight activity against the clinical isolates. The activity shown by the drugs were more as compared to conventional drugs in some samples while they has similar activity like conventional drugs in other cases. The zone of inhibition was not increased in case of combination drugs.

Conclusion:

The beta lactams antibiotics are widely used for the empirical treatment of infections. Many generations of beta-lactams has been launched with the claims of higher sensitivity and less resistance but their sensitivity has decreased drastically over time. Thus the preference for beta-lactams especially cephalosporins as an empirical therapy among the prescribers was initially justified but the current sensitivity patterns do not support their empirical use in hospitals and community acquired infections.

In the study done on the clinical isolates collected from the regional hospital of Shimla (Himachal Pradesh) we have found that *E.coli* and *Klebsiella spp* are most resistant to the beta-lactams tested. On the other hand *Pseudomonas spp.* has shown only 6% of percent prevalence in the clinical isolates and is more sensitive towards the beta-lactams tested. In the 73 clinical isolates percentage prevalence of Citrobacter was 1%. Different generations of cephalosporins tested have shown high resistance patterns against the drugs.

This study conducted has given the beta-lactams resistance pattern of the different clinical isolates collected from the regional hospitals. Further it will help in sharing the information generated from the project regarding the drug resistance pattern in this area with the hospital department. It will also help in sharing of information with the pharmaceutical industries to produce the drug which is effective on particular pathogen. So, it is more important to look for trends in susceptibility profiles that may alert a clinical microbiology laboratory to a potential epidemiological problem in specific hospitals.

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Appendix

Appendix 1-zone of inhibition of isolates for Quinilones tested

				Quinilones			
	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

			Quinolnes	
			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	Pseudomonas.aeruginosa igmc		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 2- zone of inhibition shown by clinical isolates for Aminoglycosides tested

					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
Sample		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 3- zone of inhibition shown by clinical isolates for Glycopeptides tested

			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 4- zone of inhibition for VMRC antibiotics tested

				VMRC antibiotics			
				I	II	III	IV
1	SF56LUT	IGMC	*E.coli*	30mm	29mm	32mm	30mm
2	SF56SUT	IGMC	*E.coli*	30mm	35mm	34mm	30mm
3	L75SUTI	IGMC	*E.coli*				
4	PUSND2I	IGMC	*E.coli*	20mm	16mm	20mm	18mm
5	CKIII	IGMC	*E.coli*	19mm	24mm	22mm	21mm
6	CRIKIII"R	IGMC	*E.coli*	29mm	26mm	24mm	27mm
7	SFUTIL	IGMC	*E.coli*	28mm	34mm	30mm	26mm
8	11a	IGMC	*E.coli*	8mm	6mm	9mm	4mm
9	11b	IGMC	*E.coli*	31mm	32mm	29mm	30mm
10	13b	IGMC	*E.coli*	25mm	24mm	28mm	24mm
11	E.coli IGM	IGMC	*E.coli*	30mm	25mm	30mm	30mm
12	SF50LUT	IGMC	*Klebsiell*	32mm	38mm	30mm	29mm
13	SF50SUT	IGMC	*Klebsiell*				
14	PUSNAV	IGMC	*Klebsiell*	12mm	20mm	4mm	4mm
15	NARANS	IGMC	*Klebsiell*	12mm	10mm	14mm	10mm
16	CKII		*Klebsiell*	20mm	25mm	21mm	14mm
17	CKI		*Klebsiell*	26mm	26mm	24mm	27mm
18	CRIKIII'		*Klebsiell*	25mm	25mm	30mm	26mm
19	1SVIII		*Klebsiell*	30mm	28mm	24mm	27mm
20	2LF		*Klebsiell*	25mm	27mm	21mm	23mm
21	1SVIw		*Klebsiell*	26mm	26mm	22mm	20mm
22	Pseudomonas.aeru		*P. aerug*	19mm	21mm	15mm	16mm
23	CKIV	Kasauli	*Pseudon*	25mm	28mm	29mm	29mm
24	1SX	Shimla	*Pseudon*	17mm	18mm	20mm	22mm
25	13a	Shimla	*Pseudon*				
26	PUSND1L	Shimla	*Shigella*	36mm	35mm	30mm	29mm
27	PUSND1S	Shimla	*Shigella*	25mm	20mm	28mm	22mm
28	Navdeep	Shimla	*Shigella*	24mm	20mm	19mm	18mm
29	1SIII	Shimla	*Shigella*	22mm	23mm	22mm	12mm
30	NARANLI	Shimla	*Proteus*	25mm	26mm	29mm	25mm
31	1SI	Shimla	*Proteus*	24mm	26mm	25mm	21mm
32	1SVIR	Shimla	*Proteus*				
33	1NLFL	Shimla	*unk*				

34	1NLFs	Shimla	*unk*				
35	PUSND2s	Shimla	*unk*				
36	CRIKI"	Kasauli	*unk*	15mm	15mm	14mm	17mm
37	CRIKIII"w	Kasauli	*unk*	5mm	5mm	5mm	5mm
38	CRIKII"	Kasauli	*unk*	20mm	21mm	16mm	15mm
39	CRIKI'	Kasauli	*unk*	17mm	15mm	11mm	14mm
40	CRIKII'	Kasauli	*unk*	21mm	17mm	18mm	16mm
41	Pus 3s	Shimla	*unk*	22mm	23mm	24mm	22mm
42	1SII	Shimla	*unk*	24mm	24mm	26mm	24mm
43	NARANLs	Shimla	*unk*	5mm	5mm	5mm	17mm

Appendix 5- Zone of inhibition for plant extracts and VMRC antibiotics tested

			Plant extracts				VMRC Antibiotics			
	Sample	Organi	A	B	C	D	I	II	III	IV
1	321ill	E.coli	0mmR	0mmR	0mmR	0mmR	16mm	19mm	21mm	0mm
2	321il	Unk	0mm	0mm	0mm	0mm	12mm	16mm	15mm	0mm
3	321iil	E.coli					17mm	18mm	15mm	0mm
4	321iill	E.coli	0mmR	0mmR	0mmR	0mmR	15mm	12mm	18mm	0mm
5	344illl	E.coli	0mmR	0mmR	0mmR	0mmR	19mm	24mm	20mm	0mm
6	276iill	E.coli	0mmR	0mmR	0mmR	0mmR	14mm	20mm	15mm	0mm
7	275iill	E.coli	0mmR	0mmR	0mmR	0mmR	20mm	22mm	20mm	0mm
8	278ii	E.coli	0mmR	0mmR	0mmR	0mmR	15mm	20mm	21mm	0mm
9	299i	E.coli	0mmR	0mmR	0mmR	0mmR	14mm	16mm	14mm	0mm
10	344iil	E.coli					20mm	21mm	13mm	0mm
11	379iill	Citrobac	0mmR	0mmR	0mmR	0mmR	25mm	24mm	15mm	0mm
12	276iil	E.coli	0mmR	0mmR	0mmR	0mmR	26mm	27mm	26mm	26mm
13	299ii	E.coli	0mmR	0mmR	0mmR	0mmR	20mm	22mm	9mm	10mm
14	275i	E.coli	0mmR	0mmR	0mmR	0mmR	16mm	17mm	14mm	0mm
15	344ill	E.coli	0mmR	0mmR	0mmR	0mmR	17mm	22mm	25mm	0mm
16	379i	E.coli	0mmR	0mmR	0mmR-	0mmR	15mm	21mm	19mm	0mm
17	275iil	E.coli	0mmR	0mmR	0mm	0mmR	0mm	20mm	20mm	20mm
18	344il	E.coli	0mmR	0mmR	0mmR	0mmR	19mm	20mm	16mm	9mm
19	276i	E.coli	0mmR	0mmR	0mm	0mmR	18mm	15mm	15mm	0mm
20	379iill	E.coli	0mmR	0mmR	0mmR	0mmR	26mm	27mm	0mm	25mm
21	321iilll	Unk	0mm	0mm	0mm	0mm	12mm	18mm	18mm	0mm
22	369X	E.coli	0mmR	0mmR	0mmR	0mmR	11mm	14mm	12mm	0mm
23	369E	E.coli	0mmR	0mmR	0mmR	0mmR	15mm	17mm	16mm	0mm
24	278X	E.coli	0mmR	0mmR	0mmR	0mmR	12mm	15mm	13mm	0mm
25	329X	klebsiell	0mmR	0mmR	0mmR	0mmR	12mm	16mm	12mm	0mm
26	329E	E.coli	0mmR	0mmR	0mmR	0mmR	13mm	20mm	16mm	0mm
27	322X I	Shigella	0mmR	0mmR	0mmR	0mmR	25mm	21mm	20mm	19mm
28	322X II	unk	0mm	0mm	0mm	0mm	24mm	20mm	18mm	16mm
29	322X III	Unk	0mm	0mm	0mm	0mm	26mm	24mm	21mm	16mm
30	322 E II	E.coli	0mmR	0mmR	0mmR	0mmR	14mm	18mm	15mm	0mm