Study on Lactamase and Lactamase Inhibitors

Thesis report submitted in partial fulfilment of the requirement for the degree of

Bachelor of Technology in
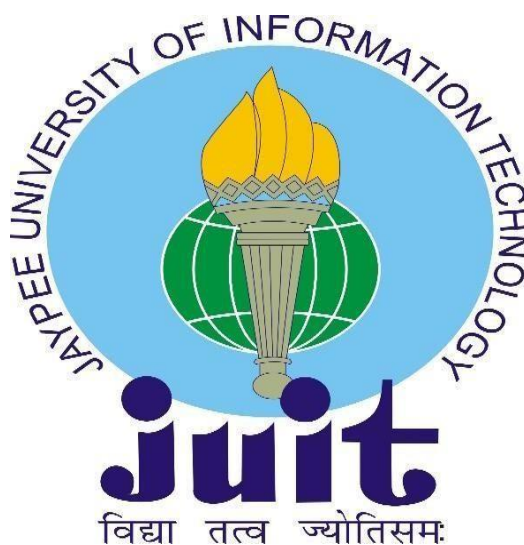
Biotechnology

by

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Under the supervision of

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To

Department of Biotechnology

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Candidate's Declaration

We now declare that the work contained in this report is our own original work entitled "**Studies on Lactamase and Lactamase Inhibitors**" in partial fulfilment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology submitted in the department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, H.P. is an authentic record of our own work over a period of time from July 2021 to May 2022 under the supervision of **Dr. Jitendraa Vashistt**, Associate Professor in the Department of Biotechnology.

The subject of the report has not been submitted for any other degree or diploma award.

Reetika Aggarwal (181841)

This is to confirm that the candidates' statements are true to the best of their knowledge.

(Supervisor Signature)

Dr. Jitendraa Vashistt

Associate Professor

CERTIFICATE

This is to certify that the work titled "Study on lactamase and lactamase inhibitors" submitted "Ms. Reetika Aggarwal" in partial fulfilment for the award of the degree of Btech. Biotechnology of Jaypee University of Information Technology, Waknaghat has been out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor …………   Reetika Aggarwal (181841)

Name of Supervisor   Dr. Jitendraa Vashishtt

Designation   Associate Professor

Date   19th May 2022

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Reetika Aggarwal (181841)

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ABSTRACT

The present study was focused on evaluation of lactamases and their inhibitors among *E. coli* isolates. Beta-lactam antibiotics are the most widely used antibiotics which come under a broad class of antibacterial compounds classified on the basis of chemical structure. Methodology followed to detect presence of lactamases among different strains of *E. coli*. Antibiotic susceptibility testing by using disc-diffusion method was done in order to check the susceptibility of clinical isolates of bacteria against the antibiotic Ceftazidime as well as combinational drug ceftazidime-clavulanate (along with lactamase inhibitor clavulanic acid). All three strains showed susceptibility against the antibiotic as well as the combinational drug used. DNA was isolated by using heat boiling method. PCR detection and amplification was also done to check the presence of lactamase gene in *E. coli*. *Oxa* was not present in any of the three strains and the bacteria were also found susceptible for lactam and combination of lactam and lactam inhibitors. *In silico* MSA for the detection of conserved region in *E. coli oxa* gene was also checked. The conserved sequences were depicted and highlighted among all the *E. coli* isolates. The present study may be useful for checking the scenario of resistance in bacteria. In future, the effect of other lactamases for resistance in other bacteria can be evaluated.

CHAPTER 1:   INTRODUCTION

Antibacterial compounds are a class of materials that fight pathogens that cause disease. It is frequently used interchangeably with the term antibiotic (s). Selman Waksman coined the name "antibiotics" in 1942. Thus, bacteria's harmful effect in biological contexts will be reduced by killing or lowering their metabolic activity.

Biomaterials with antibacterial properties are rapidly being developed and implemented in medical and dental treatment protocols. Developing goods with antibacterial chemicals or coatings with antibacterial properties has become an interesting research topic in dentistry. Implants, restorative materials, adhesives, denture-based products, and tissue conditioners all have antibacterial properties, and some have even been marketed.

Additionally, adding chlorhexidine and antibiotics to resin-based composites affected their mechanical properties because these chemicals leached out of the composites, causing the mechanical capabilities to deteriorate. Also, when exposed to heat, these organic antibacterial compounds were particularly sensitive to degradation. Beta-lactamase inhibitors, also known as antilactamase chemicals, block the activity of beta-lactamase enzymes, preventing the breakdown of beta-lactam medicines. On their own, they have limited antibacterial activity. They make beta-lactam antibacterials more effective against bacteria that manufacture beta-lactamase. These medicines block several plasmid-mediated and certain chromosomal beta-lactamases irreversibly.

Beta-Lactamases

The primary source of antibiotic resistance is the production of Beta-lactamases, enzymes that inactivate Beta-lactam antibiotics by shattering the amide link of the Beta-lactam ring. They've most likely evolved alongside bacteria as natural antibiotic resistance mechanisms over time. Antimicrobial therapy's widespread use in modern medicine may have imposed selection pressure on their development and dissemination.

Extended-Spectrum β-Lactamases

The introduction and global spread of ESBL, which can hydrolyze monobactam and broad-spectrum cephalosporins, was rapidly followed by the development of third-generation cephalosporins, which were initially resistant to the action of TEM- and SHV-type Beta-lactamases. Furthermore, increasing reports of carbapenemase formation and spread have raised worries regarding the efficiency of the current antibiotic arsenal against infections caused by multidrug-resistant gram-negative bacteria.

β-Lactamase

The most common mechanism of resistance in *Haemophilus* species is the development of lactamase. Ampicillin resistance caused by a change in protein binding is unusual, and diagnosis needs MIC or disc diffusion tests. The identification of -lactamase is the only test performed on N. gonorrhoeae isolates on a regular basis. Other types of testing should be sent to a specialized lab. There are three ways for detecting -lactamase, the most reliable of which is the cephalosporin (nitrocefin) approach. A color change to crimson after inoculation onto

nitrocefin-impregnated filter paper indicates -lactamase amide bond hydrolysis. This method can identify most Beta-lactamases from important isolates such as Haemophilus, N. gonorrhoeae, Moraxella, Enterococcus faecalis, and Bacteroides. Other methods do not identify all Beta-lactamases and have worse specificity than the nitrocefin assay.

Lactamase inhibitors attach to Beta-lactamases and render them inactive. Clavulanic acid, sulbactam, and tazobactam are examples of commercially available inhibitors. Beta-lactamase inhibitors have minimal direct antibacterial activity; nevertheless, when coupled with an antibiotic, they broaden the antibiotic's spectrum of activity and improve -lactamase stability. Unasyn is made up of ampicillin and sulbactam, while Augmentin is made up of amoxicillin and clavulanate. Tazocin and Zosyn are antibiotics that contain piperacillin and tazobactam in combination. Unfortunately, not all forms of Beta-lactamases are inhibited by the existing -lactamase inhibitors.

With the prevalence of carbapenem-resistant Acinetobacter, the use of -lactamase inhibitors as a treatment option should be evaluated based on susceptibility testing. Sulbactam has the highest activity of the Beta-lactamase inhibitors and is effective for invasive Acinetobacter infections such as pneumonia, bloodstream infections, when used alone or in conjunction with ampicillin, it can cause meningitis. In comparison to carbapenems, tigecycline, colistin, and polymyxin B, sulbactam is similarly effective in treating pneumonia and bacteremia. For most infections in adults, a dosage of at least 4 g of sulbactam per day in divided doses is suggested, while dosages as high as 9 g in divided doses have been used.

CHAPTER 2: REVIEW OF LITERATURE

➤ **Classification of Antibacterial Compounds**

- On the basis of type of action:

1. Bacteriostatic- Bacteriostatic antibacterial are those that prevent bacteria from growing. Tetracyclines with chloramphenicol, for example.

2. Bactericidal- Bactericidal drugs are those that destroy germs by attacking their cell walls.

- On the basis of source of antibacterial agents:

1. Natural Antibiotics- These are antimicrobial agents that are naturally occurring or derived substances that are chemically similar to those naturally occurring substances. These often exhibit high toxicity as compare to synthetic ones. E.g., cephamycin, gentamicin.

2. Synthetic Antibiotics- These are designed to have higher efficacy and lower toxicity, and thus are preferred over natural antibiotics because bacteria are not exposed to the chemicals until they are released. Ampicillin, for example.

- On the basis of spectrum of activity:

1. Narrow spectrum- Those which can work on a narrow range of microorganisms, i.e., they can

2. specifically work against gram-positive only or gram-negative bacteria only. E.g., cephalosporins.

3. Broad spectrum- The broad antimicrobial affects both gram-positive and gram-negative harmful microorganisms. Quinolones and penicillin, for example.

- On the basis of chemical structure:

1. Beta-Lactams- These are a well-known medication class with a four-membered lactam ring known as the B-lactam ring. They differ, however, in terms of side chains and additional cycles. This class includes penicillin derivatives, cephalosporins, monobactams, and carbepenems, such as imipenems.

2. Aminoglycoside- Two amino sugars are connected to an aminocyclitol by a glycosidic bond in this group. Example: kanamycin and neomycin.

➢ BETA-LACTAMS
Chemical structure, mode of action and mechanisms of resistance;
Beta Lactamases

It's a cyclic amide, and beta-lactams are termed so because the Nitrogen atom is attached to the Beta- Carbon atom relative to the carbonyl. The simplest Beta- lactam possible is 2-azetidinone.

Historical Data

Penicillin's antibacterial capabilities were discovered by Alexander Fleming in 1928. While working on another bacteriological problem, Fleming noticed a contaminated culture of Staphylococcus aureus with the mould Penicillium notatum. Fleming foresaw the potential of this terrible circumstance wonderfully. Stopping what he was doing allowed him to characterise the compound around the mould and isolate it. Penicillin was his term for it, and he published his findings along with several penicillin applications. In a mouse model of streptococci infection, the first treatment experiment with penicillin was not carried out until 1940. At the time, the first Beta-lactam antibiotic was found.

The reactive B-lactam ring structure, which is a highly strained and reactive cyclic amide, is found in all currently known Beta-lactam antibiotics. The five-ring systems penam, penem, carbapenem, cefem, monobactam, and Clavams are important.

1. Penams- Penicillins are part of a wide group of Beta-lactams known as penams. Penicillins have a bicyclic structure known as 6-aminopenicillanic acid, or 6-APA. The thiazolidinic ring and the lactam ring are generated via condensation of L-cysteine and D-valine, resulting in an enclosed dipeptide. Penicillins (penams) and similar chemicals are susceptible to a range of degradative processes due to the reactive nature of the Beta-lactam ring structure.

2. Cephems- Since the 1970s, cephalosporins, the most prevalent representative type of cephalosporins, have been one of the most effective and widely used anti-infective medications. They're well tolerated, and their development has paralleled that of penicillins. Cephalosporins took a long time to develop a therapeutically relevant medicine, unlike penicillins, where the first agent in the series was launched with little development of the structure-activity relationship.

Chemical Classification

Cephems are divided chemically into five groups: cephalosporins, cephamycins, *oxa*-1-cephems, carba-1-cephems, and miscellaneous.
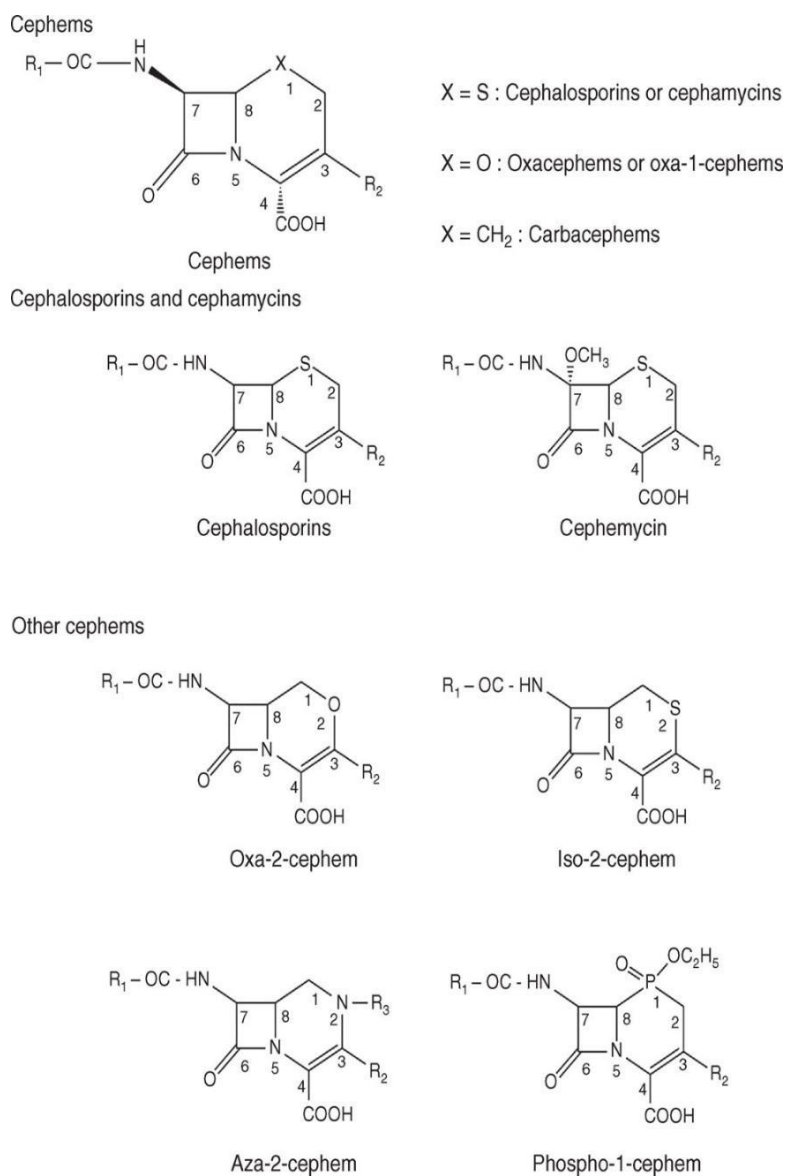

Figure 3.1 depicts different chemical structure of cephems

Microbiological Classification

First-generation, second-generation, third-generation, fourth-generation, and fifth-generation cephalosporins were previously categorised as antibacterial agents. There are differences in antimicrobial spectrum, -lactamase stability, absorption, metabolism, stability, and side effects. First-generation members have less activity than third-generation, fourth-generation, or fifth-generation broader spectrum cephalosporins. Penicillins have similar structure-activity features to cephalosporins, which are responsible for a variety of qualities (oral action, Beta-lactamase stability, and so on.

Table 3.1: depicts major group of cephalosporins according to their antimicrobial activity

First Generation	Second Generation	Third Generation	Fourth Generation	Fifth Generation
Cephalothin	Cefamandole	Cefotaxime	Cefepime	Ceftobiprole
Cephapirin	Cefuroxime	Ceftizoxime	Cefpirome	Ceftaroline
Cefazolin	Cefonicid	Ceftriaxone		
Cephalexin[a]	Ceforanid	Ceftazidime		
Cephradine[a]	Cefoxitin[b]	Cefoperazone		
Cephadroxil[a]	Cefmetazole[b]	Cefixime[a]		
	Cefminox[b]	Ceftibuten[a]		
	Cefotetan[b]	Cefdinir[a]		

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Ceftazidime- belongs to the cephalosporin antibiotics class. It's a cephalosporin of third generation (table 3.1).

Infections caused by Pseudomonas aeruginosa, gram-negative aerobic infections, and neutropenia are all treated.

The following are some clinically important infections' MIC susceptibility data:

0.015 g/mL – 512 g/mL Escherichia coli

0.03 g/mL – 1024 g/mL Pseudomonas aeruginosa

3. Monobactams- Because they have a monocyclic -lactam ring, monobactams are resistant to Beta-lactamases. Gram-negative bacteria are affected, but Gram-positive bacteria are unaffected. The first medicine found was aztreonam. Patients with penicillin allergy caused by immunoglobulin E can use it without reaction, and no major side effects have been documented other than skin rashes and minor aminotransferase abnormalities. Staphylococci and enterococci are bacteria that can cause a variety of ailments.

4. Carbapenems- These antibiotics have a similar structure to -lactam antibiotics. Imipenem, the first of its kind, is effective against a wide range of Gram-negative rods, Gram-positive bacteria, and anaerobes. It is resistant to certain Beta-lactamases, however dihydropeptidases in renal tubules inactivate it. As a result, it's controlled in conjunction with a peptidase inhibitor like cilastatin.

5. Clavams- Clavams is a Streptomyces clavuligerus-derived antibiotic. Clavam is a novel -lactam antibiotic that is being developed. The clavulanic acid class and the 5S clavams class make up this category. Clavulanic acid is an antibiotic with a broad spectrum of action, and 5S clavams may have antifungal characteristics. They're identical to penams, but instead of sulphur, they have oxygen. *Oxa*penams is another name for them. The compound clavulanic acid, from which this compound class gets its name, is an example.

Clavulanic acid inhibits the beta-lactamase enzyme. Clavulanic acid has a beta-lactam ring that binds to beta-active lactamase's site and inactivates the enzyme, enhancing the antibacterial activity of beta-lactam antibiotics like amoxicillin.

Antimicrobial resistance to β-lactam

Since Alexander Flemming discovered penicillin, the first antibiotic, in 1928, a great deal has changed in this field. To begin with, the use of antibiotics in the treatment of infectious diseases was an unprecedented medical breakthrough. Despite this, a considerable number of bacteria with acquired resistance emerged quickly, resulting in therapeutic failures. For example, six years after benzylpenicillin was introduced to the market, the prevalence of staphylococci resistance in British hospitals increased from less than 10% to 60%, and it is now over 90% globally.

Antibiotic mode of action and resistance

Beta-Lactams are an antibiotic class with bacterial specificity. Bacteria are prokaryotic, which means they have vast structural and metabolic differences from eukaryotic cells like the animal or human host. Antibiotics can target a number of different things. Antibiotics work in five ways: cell wall inhibition, cytoplasmic membrane impairment, nucleic acid synthesis inhibition, protein synthesis inhibition, and metabolic antagonist impact. In general, there are four basic mechanisms by which bacteria develop drug resistance: modification of the antimicrobial target, which can be due to a complete loss of affinity or a simple reduction of it, reduction in the amount of antimicrobial that reaches the target due to entrance reduction caused by decreased permeability due to porin mutation or exit increase caused by efflux transporter pumping out, and the presence of an enzymatic mechanism that causes resistance.

Beta Lactamases

Bacterial enzymes that open the beta-lactam ring and render the beta lactam antibiotic ineffective. Beta-lactamases are encoded on chromosomes and mobile genetic elements, respectively (e.g., Plasmids).

Because they are not all active against all beta-lactam antibiotics, they are categorised into five basic classes based on their affinity for specific beta-lactam antibiotics:

- AmpC beta lactamases are therapeutically important cephalosporinases found on the chromosomes of many Enterobacteriaceae and a few other bacteria, causing resistance to cephalothin, cephazolin, cefoxitin, most penicillins, and beta-lactamase inhibitor-beta-lactam combinations. Penicillins, cephalosporins, cephamycins, and monobactams are examples of antibiotics.

- ESBLs are plasmid-carried beta-lactamases produced by Klebsiella species, *E. coli*, and other Enterobacteriaceae bacteria. Resistance is conferred by beta-lactam antibiotics such as penicillins (e.g., piperacillin), cephalosporins, and the monobactam aztreonam.

- Metallo-beta-lactamases are a group of enzymes that catalyse the hydrolysis of a wide variety of Beta-lactam antibiotics, including carbapenems. In certain organisms, such as stenotrophomonas maltophilia, they are chromosomally encoded, but in others, such as gram-negative bacteria, they are acquired.

- taphylococci, such as Staphylococcus aureus, produce penicillinases, which are enzymes generated by a variety of organisms.

- Serine carbapenemases (plasmid mediated) are most commonly generated by K. pneumoniae, although other Enterobacteriaceae have been observed as well. *Oxa*-lactamase, for example.

β-Lactams' target alteration

The most well-known example of Beta-lactam target alteration is MRSA. Methicillin resistance is caused by the presence of the mecA gene in staphylococci by definition. By default, S. aureus has four PBPs (PBP 1, 2, 3 and 4). The mecA gene encodes PBP2a or PBP2', a peptidoglycan transpeptidase with a molecular weight of 78 kDa that differs from S. aureus endogenous PBP. Peptidoglycan transpeptidase PBP2a continues to function normally. The PBP2a, on the other hand, is unusual in that the Beta-lactam recognition site has been altered. With the exception of PBP2a, which is insensitive to a number of Beta-lactams, including methicillin, when PBPs are coupled to Beta-lactams, they become inactive. Despite its association with methicillin, PBP2a can also increase cell wall production. An operon processes and mediates the regulation of mecA expression and, as a result, the synthesis of PBP2a, which confers methicillin resistance in MRSA. The complex regulator of the mecA gene is made up of three genes: MecR1, MecA, and MecI. The protein's outer domain is a MecR1 PBP as well. Transmembrane signalling is triggered when the Beta-lactam binds covalently to the PBP domain, in this case the fragment MecR1, resulting in the release of a cytoplasmic fragment containing MecR2. This fragment of MecR2 will then split the protein into two fragments, each with intact MecI, allowing PBP2a to be generated and relieving the repression of the mecA gene.

Permeability changes to β-lactams

One of the mechanisms of resistance to Beta-lactam is a change in permeability in the outer membrane. This shift in permeability might be caused by the presence of efflux proteins or the alteration or removal of porins. Efflux proteins have been connected to the pumping of unrelated chemicals such as antibiotics, organic solvents, dyes, and detergents through the cell walls of Gram-negative and Gram-positive bacteria. MDR exporters, also known as MDR efflux pumps, are a class of structurally diverse compounds that have been identified as MDR exporters or MDR efflux pumps.

The two primary types of efflux pumps are ATP-dependent efflux pumps and secondary transporters driven by proton motive force (PMF). The four basic families of PMF transporters are the resistance nodulation division (RND), the major facilitator superfamily, the small MDR family, and the MDR and toxic compound extrusion (MATE) family.

Proton and sodium ion gradients have been revealed to be the energy source for substrate transport in MATE family transporters. Another significant category of MDR pumps is the ABC family, which is ATP-dependent and not driven by PMF. The ABC transporters are more important for clinical resistance to chemotherapy in eukaryotic cells, such as tumour cells, parasites, and opportunistic fungi.

The RND-type pump has been studied the most in Gram-negative bacteria. It's present in the cytoplasmic membrane of bacteria, where it works with a membrane fusion protein (MFP) that connects the periplasmic region to the outer membrane efflux protein (OEP). RND–MFP–OEP form a complex capable of transporting a substrate (such as an antibiotic) from the inside of the bacteria to the outside. In *E. coli*, the AcrAB–TolC complex, in which AcrB is the RND, AcrA is the MFP, and TolC is the OEP, is the most well-studied.

Multiple antibiotic resistance (mar) locus expression has been connected to the presence of different efflux pumps, such as AcrB and porin losses. The oxidative stress apparatus, which includes superoxide dismutase (soxSlocus) and Rob-binding proteins that serve as transcriptional regulators, SdiA, and AcrR, appears to be complex in its genetic regulation of the AcrAB–TolC system.

The AcrAB–TolC system has also been discovered in Gram-negative bacilli with clinical significance. The AcrAB–TolC system has recently been discovered to play a function in Salmonella enterica pathogenesis. Because it functions in the intracellular regulation of coenzyme A levels in *E. coli*, AcrAB has been linked to cell basic metabolism.

Mutations in the TolC or AcrA/B proteins cause hypersensitivity to antibiotics such quinolones, tetracyclines, tigecycline, eritromycin, and novobiocin in *E. coli* and other Enterobacteriaceae strains. A similar pump (MexEF-OprN) in P. aeruginosa has been demonstrated to have specific Beta-lactamase inhibitors as substrates (clavulanate, cl*oxa*cillin and BRL42715). However, there has been little evidence that AcrAB–TolC or other *E. coli* efflux pathways are implicated in B-lactam resistance to date.

E. coli Oxa-β-lactamases

The most basic classification approach for -lactamases is based on molecular structure; there are four major classes in this scheme. Both chromosomally encoded and plasmid-mediated enzymes of Classes A through C have been well established. Class D -lactamases have proven to be far more difficult to find, with most being identified primarily as plasmid-encoded Beta-lactamases in Gram-negative bacteria [1]. These early enzymes were essentially penicillinases, which, unlike class A-lactamases, were capable of hydrolyzing and conferring resistance to both penicillin and *oxa*cillin, hence the term *oxa*cillinases and the prefix *OXA* [2]. When Bush et alscheme considered the substrate profiles of the early *OXA* enzymes, these enzymes were given the name 2d [3].

EARLY *OXA* B-LACTAMASES

Gram-negative bacilli had two types of plasmid-encoded -lactamases, according to Hedges et al.: the ubiquitous TEM enzymes and a smaller group that could hydrolyze *oxa*cillin. Unlike the TEM enzymes, this group possessed a wide range of substrates and was encoded by a smaller number of plasmids. These Beta-lactamases shared features with R1818, a plasmid identified by Datta and Kontomichalou and later renamed R46. These enzymes had a lower specific activity against penicillin than TEM-lactamases [4]. On the other hand, they demonstrated significantly higher action against *oxa*cillin and methicillin. The widespread use of flucl*oxa*cillin and methicillin to treat staphylococcal infections is likely to have corresponded with their introduction [5].

The isoelectric points of closely related -lactamases were once used to identify them. *OXA*-1, *OXA*-2, and *OXA*-3 are the three different *oxa*cillinases identified by Sykes and Matthew [6]. The first two shared a trait with the majority of the others in this class: they were inhibited by chloride ions [7]. The first gene sequenced was bla*OXA*-2, which came from plasmid R46. A transposon carried on plasmid RGN238 was used to sequence the first bla*OXA*-1 gene [8]. Only about 48% homology was found when compared to the *OXA*-2 -lactamase [9]. The active-site serine at position 71 and a leucine at position 179, however, showed some conservation [10].

These were the first patterns found in *OXA* enzymes, and they've since become a major aspect in their research [11]. The bla*OXA*-1 gene was discovered between the aminoglycoside resistance gene and its promoter in a Tn21-derived transposon [12]. Surprisingly, the bla*OXA*-2 gene was identified in the same place, showing a considerable evolutionary push for both their presence in the bacterial cell and their carriage within this specific transposon [13]. Following *OXA* enzymes were assigned a unique number based on the order in which they were discovered, and these numbers were later confirmed using the Lahey Clinic's unique amino acid sequence [14].

The five enzymes tested by this study exhibited a lot of variation, which suggested that they came from different places and evolved from an ancestral gene a long time ago [15]. It was then proposed that PSE-2 be reclassified as *OXA*-10 due to its structural similarities to *OXA*-lactamases [16]. Because of the high conjugation rate of P*oxa*-48a, *Oxa*-48 genes have been discovered in a variety of Enterobacterales, including *E. coli* [17].

➤ **Escherechia coli** (*E. coli*)

Bacteria can be found in the environment, foods, and people's and animals' intestines. They are a big and diverse bacterial group. Although the majority of *E. coli* strains are safe, some can cause illness. Some strains of *E. coli* cause diarrhoea, while others cause infections of the urinary system, pneumonia, and other disorders [18].

- The species Escherichia coli comprises several pathotypes that cause a range of diseases, in addition to being a key member of the normal intestinal microflora of humans and other mammals. Enteric disease, such as diarrhoea or dysentery, is caused by at least six different pathotypes, and extra-intestinal infections, such as urinary tract infections and meningitis, are caused by other pathotypes [19].

- *E. coli* virulence factors can alter a variety of cellular functions in eukaryotes, including cell signalling, ion secretion, protein synthesis, mitosis, cytoskeletal function, and mitochondrial function [20].

- Pathogenic *E. coli* virulence factors are typically encoded on genetic elements such as plasmids, bacteriophage, transposons, and pathogenicity islands, which can be mobilised into various strains to form novel virulence factor combinations.

- The genomic organisation of the *E. coli* pathotypes sequenced so far shows a stunning mosaic pattern, with 2,000 genes in 247 islands in one pathotype that are not present in K-12. In pathogenic *E. coli*, up to 0.53 MB of DNA contained in K-12 can be missing.

- Genes that encode virulence factors of pathogenic *E. coli* are regulated by both pathotype-specific regulators that are absent from commensal *E. coli*, and by 'housekeeping' regulators that are present in commensal *E. coli*.

- ### *E. coli* DH5α strain:

  The strain was developed by D. Hanahan as a cloning strain with many mutations that enable high-efficiency transformations. The genome of this strain is made up of 4,686,137 nucleotides, 4359 genes, and 4128 protein-coding genes on a single circular chromosome. This strain also contains plasmids and is quite good at receiving plasmid insertion.*E. coli* is a Gram-negative bacillus bacterium. With a generation time of roughly 30 minutes and a growth temperature of 37 degrees Celsius, they replicate via consecutive binary fission. If oxygen is not present, *E. coli* may make ATP by both aerobic respiration and fermentation.This strain can be identified and distinguished from other *E. coli* strains using the genetic sequence of its 16s small ribosomal subunit, which has been fully sequenced.

- ### ATCC 25922

  ATCC 25922, an extensively used quality control strain, was originally isolated from a human clinical sample collected in Seattle and Washington (1946). It's biotype 1 and serotype O6.

- ### Polymerase Chain Reaction (PCR)

  Kary Mullis and his colleagues invented PCR in 1983. He was awarded the Nobel Prize in Chemistry in 1993. It has become the gold standard for amplification operations in diagnostics and research, as well as the most extensively used nucleic acid amplification technique.

It's a technique for amplification of specific DNA sequences in vitro employing the temperature-mediated DNA polymerase enzyme by simultaneously extending complementary strands of DNA.

A "target" DNA sequence can be amplified several million-fold in a matter of hours using PCR, a DNA replication test tube technology.

Applications:

1. To detect gene of interest.
2. To modify DNA fragments.
3. DNA analysis of different specimens.
4. Diagnostics and genetioc Testing.

CHAPTER 3: METHODOLOGY

Chemicals Used:

Nutrient Agar, Nutrient Broth, Ethanol, Crystal Violet Stain, Gram's Iodine, Safranin, Ethidium Bromide, Agarose gel, TAE Buffer, Nuclease free water, Taq DNA Polymerase, Primers, PCR Grade Water, Primers, Template DNA, Master mix (5μL), CAZ3O(Ceftazidime), CAC30/10(Clavulanic acid).

❖ GRAM STAINING:

1. Three strains were taken for gram staining, *E. coli* DH5α, ATCC 25922 and clinical isolate.
2. Colonies were taken from each strain from a 24-hour grown culture.
3. Three slides were prepared.
4. One drop of distilled water was put on each glass slide. Then bacterial sample was taken on that slide. Heat fixing of bacteria was done with the help of Bunsen burner three times. Let the slide dry.
5. Using a dropper, apply the primary stain (crystal violet) on the slide and let it sit for 1 minute. The slide was then carefully cleaned with water for 5 seconds to remove any remaining stain.
6. A dropper was used to apply Gram's iodine on the slide in order to adhere the crystal violet to the cell wall. Allow it to sit for one minute.
7. After that, I cleaned the slide with ethanol for 3 seconds before gently rinsing it with water.
8. Next, apply the safranin secondary stain and let it sit for 1 minute. 5 seconds of gentle water rinsing Gram-negative cells should be stained red or pink, while gram-positive cells should remain purple or blue in color.
9. Finally, we viewed all the three slides using a Compound Microscope.

❖ Antibiotic Susceptibility Testing (AST) by Disc-Diffusion Method:

1. 3 Agar plates were taken and all 3 strains of *E. coli* were taken. Bacterial inoculum was prepared. With the help of a pipette, strain was taken and diluted to a Nutrient Broth.

2. As if it were a gram-negative bacterium, the organism's broth culture is collected with a sterile swab, and excess liquid is removed from the swab by gently pressing or rotating it against the inside of the tube.

3. A bacterial lawn is formed by streaking the swab across an agar plate.

4. It was streaked in one direction with a swab, then rotated 120 degrees and streaked again, then rotated 120 degrees again and streaked again.

5. A disc containing antibiotics CAZ30 (Ceftazidime) and a lactamase inhibitor CAC30/10 (Clavulanic acid) was administered to the plate using an antibiotic disc dispenser.

6. Flame-sterilized discs were gently pressed onto the agar to ensure they were adhered.

7. After that, the plates were incubated overnight.

❖ DNA ISOLATION BY HEAT BOILING METHOD:

1. Gel was prepared. 0.64 g Agarose gel was poured in 80mL TAE.

2. Gel was put in a microwave for boiling.

3. 8 microliters ETBR was poured into the gel.

4. Casting tray was prepared.

5. Supernatant was prepared by using all the 3 strains which was mixed with Nuclease free Water.

6. Vials were placed in a water bath at a temperature of 95 degrees C and current 28.4 A.

7. 13 well plate comb was placed in a casting tray on which DNA gel was poured.

8. 3 vials were centrifuged for 3 mins at 10,000 rpm.

9. 20 microliters from each vial were taken and put to new vial with a help of a pipette.

10. Once the gel got solidified, loading was done.

11. TAE Buffer was poured.

❖ POLYMERASE CHAIN REACTION:

6 samples were taken (2 of which were negative).

The following components were added:

Table 4.1: Depicts different components added during PCR

Components	Amount (ul)
Nuclease free Water	4.1
Template DNA	0.5
Primer (16 S *E. coli* Primer and *Oxa* Primer)	0.2 F + 0.2R
Master mix	5

Total: 10 µl

The Tm of *E. coli 16 S* gene is 57-degree Celsius.

The Tm of *Oxa* gene is 55-degree Celsius.

- **Agarose Gel Electrophoresis**

  -Preparing 1 litre of 1 X Stock of TAE—

  4.84 g of Tris base was added to a flask. Then 1.14 ml of acetate (acetic acid) and 2 ml 0.01M

  sodium EDTA were added to a same flask.

  To make 1 litre 1X TAE volume makeup to1000 ml in distilled water.

-Preparation of 1.5% agarose gel—

1.5% agarose was made in 40 ml of TAE. i.e., 0.6 g of agarose was weighed and added into a microwavable flask containing 40 ml of TAE. The mixture was boiled for approximately 5-7 mins with constant swirling after every 30 secs. After the mixture became clear, which was originally foggy, the heating was stopped to avoid over-heating of solution to avoid evaporation of the buffer leading to change in the final percentage of Agarose. The agarose was dissolved by heating and EtBr (Ethidium Bromide) was added to a concentration of 0.5 µg/ml.

-Loading of Sample and Running on Agarose Gel---

The Agarose was poured into a gel tray with a comb in place. It was left for 15-20 mins to solidify. The gel was then placed in the gel box and the box was filled with 1X TAE. The molecular weight ladder was added. Loading buffer was added to the samples and were loaded into the gel. The electrodes were attached (black for negative and red for positive). Six samples were loaded and run at 50 V till the dye line reached the bottom of the gel. It took about 1-1.5 hrs. After that, the electrodes were removed, the gel was taken out of the gel box. The PCR products were analyzed under UV light and compared with the ladder.

❖ MULTIPLE SEQUENCE ALIGNMENT(MSA):

MSA is a bioinformatics method that aligns one or more biological sequences of equal length, which can be proteins or nucleic acids. Homology can be anticipated from the output, and evolutionary links can be studied.

1. Following 13 sequences of *OXA* gene of *E. coli* were collected from NCBI.

>HM063063.1 Escherichia coli strain *OXA*-E18 beta-lactamase-like (*OXA*) gene

>HM063062.1 *E. coli* strain *OXA*-E2 beta-lactamase-like (*OXA*) gene

>HM063061.1 *E. coli* strain *OXA*-B22 beta-lactamase-like (*OXA*) gene

>HM063060.1 *E. coli* strain *OXA*-B18 beta-lactamase-like (*OXA*) gene

>HM063059.1 *E. coli* strain *OXA*-B1 beta-lactamase-like (*OXA*) gene

>HM063058.1 *E. coli* strain *OXA*-A34 beta-lactamase-like (*OXA*) gene

>HM063057.1 *E. coli* strain *OXA*-A26 beta-lactamase-like (*OXA*) gene

>HM063056.1 *E. coli* strain *OXA*-A23 beta-lactamase-like (*OXA*) gene

>HM063055.1 *E. coli* strain *OXA*-A18 beta-lactamase-like (*OXA*) gene

>HM063052.1 *E. coli* strain *OXA*-4 beta-lactamase-like (*OXA*) gene

>HM063051.1 *E. coli* strain *OXA*-1 beta-lactamase-like (*OXA*) gene

>HM063053.1 *E. coli* strain *OXA*-A3 beta-lactamase-like (*OXA*) gene

>HM063054.1 *E. coli* strain *OXA*-A5 beta-lactamase-like (*OXA*) gene

2. The multiple sequence alignment was performed using CLUSTAL Ш.

3. Conserved regions were highlighted and phylogenetic tree was generated.

4. Hotspot regions were observed.

5. Their catalytic domain with respect to lactamase was checked.

CHAPTER 4: RESULTS AND DISCUSSION

- <u>GRAM STAINING:</u>

Hans Christian Gram invented Gram staining in 1884. Because it distinguishes between Gram-positive and Gram-negative bacteria, this stain is known as a differential stain. Gram-positive bacteria produce a purple colour when stained, whereas Gram-negative bacteria produce a pink color. These words are used to distinguish two morphological categories of bacteria and have nothing to do with electrical charge. Because of the changes in the structure of their cell walls, they stain differently. Took 3 different strains of bacteria and when they were viewed under a microscope, they appeared pink in colour which shows that it's a gram-negative bacterium which was *E. coli*. 3 strains were DH5α, ATCC 25922 and Clinical Isolate. They were rod-shaped bacteria.
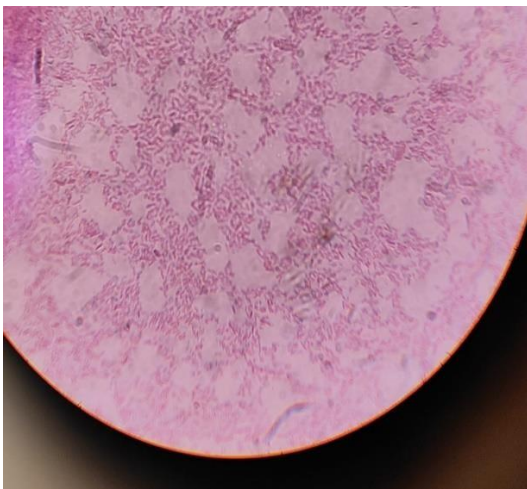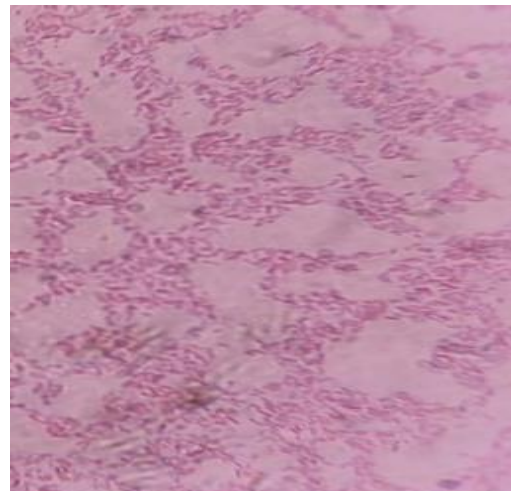
a   b

Figure 5.1 (a and b): gram staining of *E. coli* at 100 x respectively

- <u>Antibiotic Susceptibility Testing (AST) by Disc-Diffusion Method:</u>

A pure bacterial culture is swabbed uniformly on an agar plate after being suspended in saline and having its turbidity standardized. The agar is subsequently covered with an antibiotic- or extract-impregnated filter paper disc. The disc constituent(s) diffuse into the agar via the filter paper. The concentration of these ingredients is highest close to the disc and decreases as the distance from the disc grows. If an antibiotic or extract is efficient against bacteria at a specific concentration, no colonies will form in agar with a concentration greater than or equal to the effective concentration. This is the inhibitory zone.

In general, bigger zones of inhibition correspond to lower antibiotic or extract minimum inhibitory concentrations (MICs) for that bacterial strain. The exception is when the antibiotic or extract molecules are big or hydrophobic, as these diffuse slowly through the agar.

Table 5.1: Depicts different zone of inhibitions of all the three strains against the antibiotic and the lactamase inhibitor (combinational drug)

Culture	Antibiotic (x)	Lactamase Inhibitor (in combination) (y)	Zone of Inhibition Diameter (in mm) x- Antibiotic diameter y- Lactamase diameter
ATCC 25922	CAZ 30 (Ceftazidime)	CAC 30/10 (Clavulanic acid)	x-38 mm y-43 mm
Clinical Isolate	CAZ 30 (Ceftazidime)	CAC 30/10 (Clavulanic acid)	x- 35 mm y- 41 mm
DH5α	CAZ 30 (Ceftazidime)	CAC 30/10 (Clavulanic acid)	x- 41 mm y- 5 mm

As from the above table, we interpreted that zone of inhibition of lactamase inhibitor is larger than zone of inhibition of normal antibiotic. Thus, lactamase was present in the plate which shows that microorganism is sensitive to the lactamase inhibitor which is Clavulanic Acid.
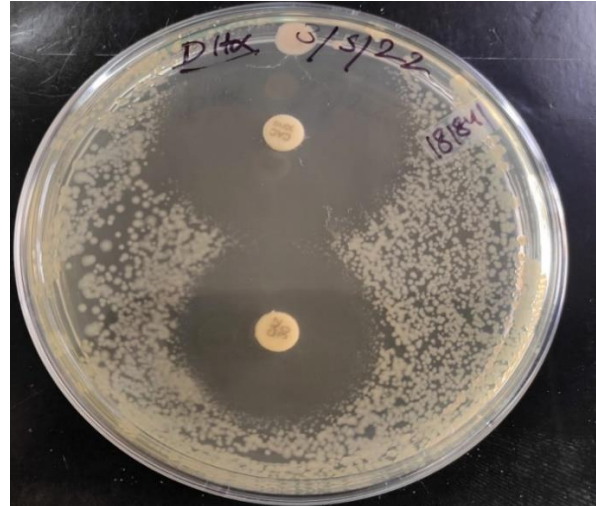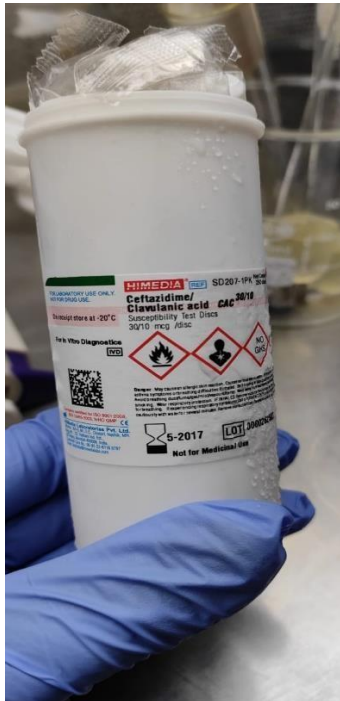

Figure 5.2 a: AST of ATCC 25922

Figure 5.2 b: AST of DH5α

Figure 5.2 c: 3 strains of *E. coli (Clinical Isolate, DH5α and ATCC 25922)* showing AST

Figure 5.2 d: combinational drug

Ceftazidime

 (Lactamase and lactamase inhibitor)

Figure 5.2 e: antibiotic

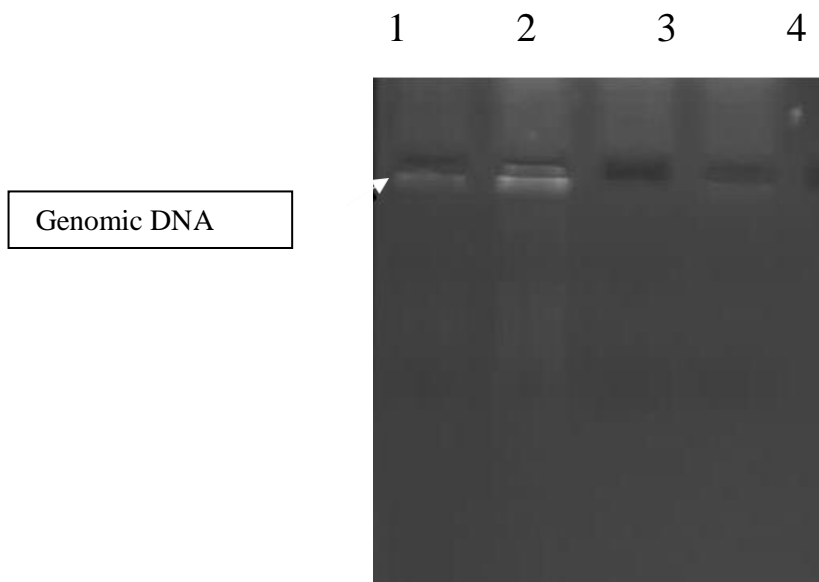- ## DNA EXTRACTION BY HEAT BOILING METHOD:

1 2 3 4

Genomic DNA

Figure 5.2 f: Genomic DNA OF *E. coli* extracted using heat boiling method and separated on 0.8% Agarose gel

DNA isolation was done by using heat boiling method and DNA was separated on 0.8% Agarose gel. It was found out that *Oxa* was absent in the sample. Thus, it showed that bacteria were susceptible towards the antibiotic and lactam ring will not be degraded.

- <u>POLYMERASE CHAIN REACTION:</u>

Figure 5.2 g: shows a picture of pipetting EtBr to the DNA sample

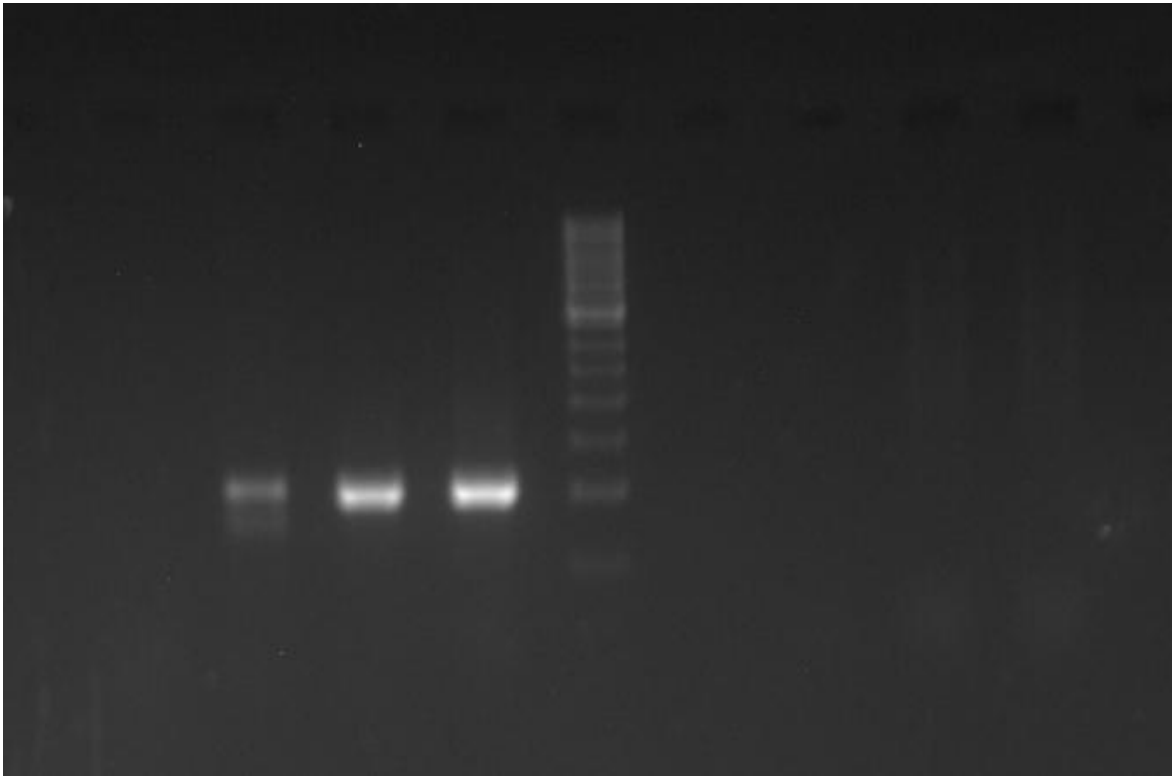**Figure 5.2 h: depicts a photograph of loading Gel**

Figure 5.11: 9 wells showing PCR products separated on 1.2% Agarose gel

1 was negative control of *E. coli*; 2 was *E. coli 16 S* which was 508 bp product; 3 was *E. coli 16 S*; 4 was *E. coli 16 S*; 5 was 250 bp ladder; 6 was negative control of *Oxa 1*; 7,8 and 9 were *Oxa* only which were not get amplified. This means that *Oxa* gene was not present because if gene was present, it should have gotten amplified but it didn't. So, it was found out that *Oxa* was not present in all the three strains and bacteria were resistant.

All the three strains of *E. coli* were highly resistant against the antibiotic as well as the combinational drug (lactamase inhibitor) which was found out by AST and thus when *E. coli* and *Oxa* were amplified then it was observed that *Oxa* gene was not amplified whereas *E. coli* was showing amplification. This means that *Oxa* gene was not present in the sample and thus bacteria will not degrade the lactam ring as it was highly resistant.
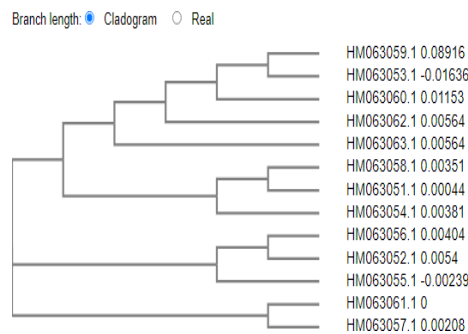
- ## MULTIPLE SEQUENCE ALIGNMENT:
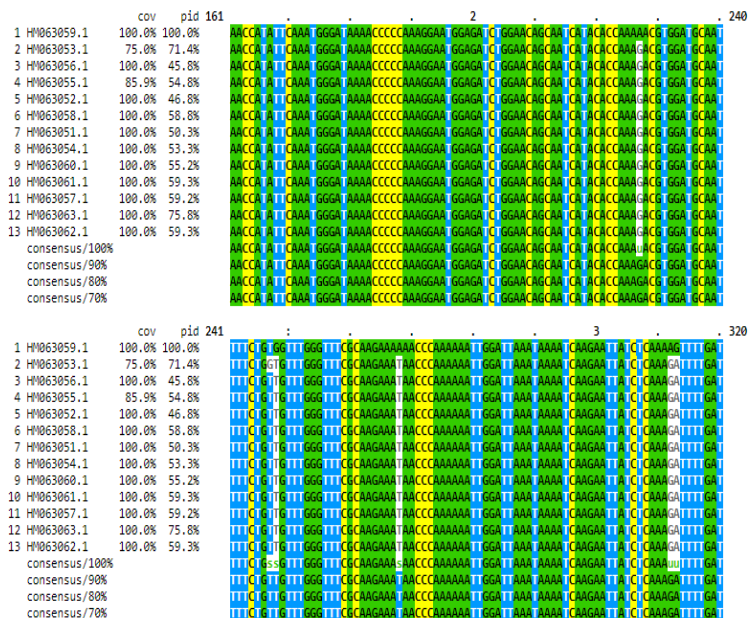

Figure 5.12: phylogenetic tree

Figure 5.13: highly conserved regions of multiple sequence alignment of *E. coli oxa*

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Oxa sequences were obtained from NCBI and they were analyzed by doing Multiple Sequence Alignment.

Above are the highly conserved regions in *Oxa* sequences from 161 to 240. Similarity is between 161 to 320.

161 to 320 are also showing the conserved sequences.

CHAPTER 5: CONCLUSION

The present report discussed beta-lactam antibiotics and their sensitivity towards *E. coli* bacteria which is a gram-negative bacterium. For analyzing this, 3 different strains of *E. coli* were taken and were analyzed. For the first step, Gram-staining was done in order to detect the gram-negative bacterium which was *E. coli.*

Antibiotic susceptibility by disc diffusion method was determined in order to check the susceptibility of the microorganism towards beta-lactam. DNA Isolation was carried out with the bacterial isolates. Further PCR was done in order to amplify *16srRNA* gene and *Oxa* gene in *E. coli*.   It was observed that *Oxa* gene showed no amplification which signifies that lactamase was absent in the bacterial isolates. Then Multiple Sequence Alignment was performed and it was observed that most of the *E. coli Oxa* sequences were highly conserved. Phylogenetic tree was generated and analyzed. The conserved sequences were depicted and highlighted among all the *E. coli* isolates. In future, the effect of other lactamases for resistance in *E. coli* can be evaluated.

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