Mapping and Molecular Characterization of Antibiotic Resistant Gram-negative Bacteria in Pakistan

By

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28-FBAS/PHDNS/F16

Sulaiman Bin Abdullah Aba Al-Khail – Centre for Interdisciplinary Research in Basic Sciences (SA-CIRBS)

Faculty of Sciences

International Islamic University, Islamabad

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Biochemistry
Antibiotic
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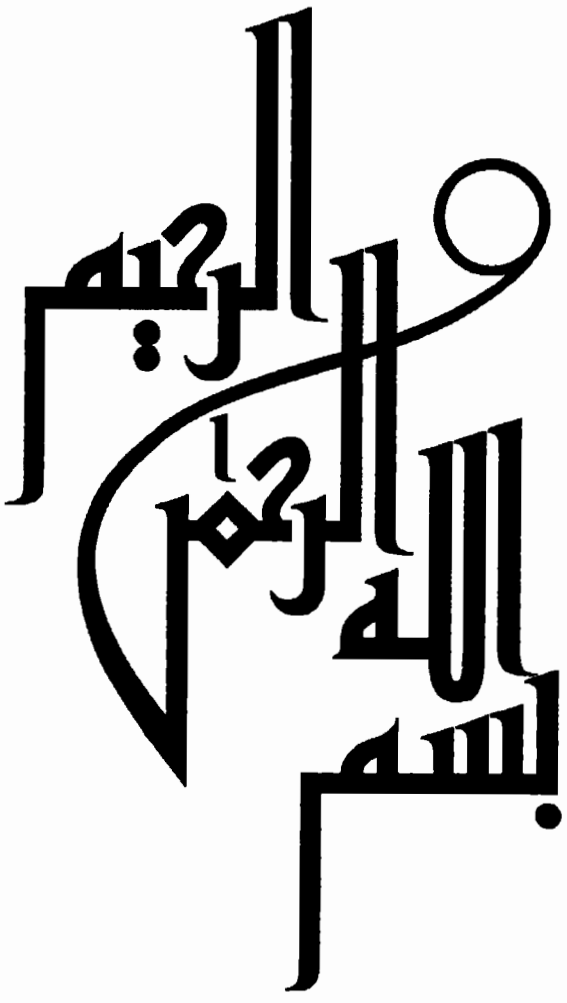
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FINAL APPROVAL

It is certified that we have read the thesis submitted by *Ms. Bibi Jamila,* and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biosciences.

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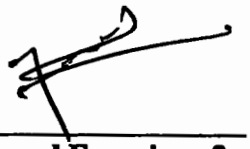

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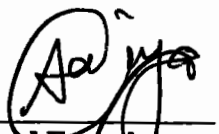

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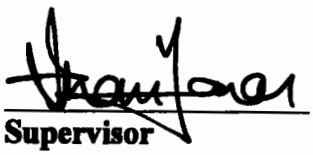

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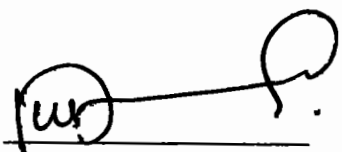

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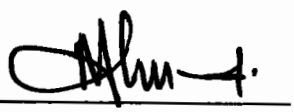

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DEDICATION

I dedicate this thesis

to my

Aday, Baba

and

my supervisor

Dr. Farhan Younas

DECLARATION

I, Bibi Jamila, hereby declare, that I am aware of consequences of a deliberately or negligently wrongly submitted affidavit. The work presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date _____

Bibi Jamila

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In the name of ALLAH, the most gracious and merciful, the one only the creator, Who is perfect in all Characteristics, Worthy for worship. Countless salutation to the centre of knowledge, The Holy Prophet Muhammad (Peace be upon him), Who declared it an obligatory duty of every Muslim to seek and acquire knowledge and Whose teaching has served as a beacon of light for the humanity in the hours of despair and darkness.

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LIST OF ABBREVIATIONS

16S rRNA	16S Ribosomal Ribonucleic Acid
AK	Amikacin
AML	Amoxicillin
AMP	Ampicillin
ARB	Antibiotic Resistant Bacteria
ARGs	Antibiotic-Resistance Genes
ATM	Aztreonam
BLAST	Basic Local Alignment Search Tool
CAZ	Ceftazidime
CE	Cephradine
CEF	Cefixime
CI	Clinical isolate
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
CP-NS	Carbapenem Non-Susceptible
CRO	Ceftriaxone
CTX	Cefotaxime
DNA	Deoxy-Ribonucleic Acid
DO	Doxycycline
DOR	Doripenem
EDTA	Ethylenediamine tetra acetic acid
ERM	Erythromycin Ribosome Methylase
ESBL	Extended Spectrum B-Lactamase
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
FEP	Cefepime
FOS	Fosfomycin
I	Intermediate
ICT	Islamabad Capital Territory
InPEC	Intestinal Pathogenic *E. coli*
IPM	Imipenem
kb	Kilobase Pairs
kD	Kilodalton
KF	Cephalothin
LEV	Levofloxacin
MAR	Multiple Antibiotic Resistance
MDR	Multi Drug Resistance/ Multi Drug Resistant
MEM	Meropenem
MHA	Mueller Hinton Agar

Mm	Millimetre
NA	Nalidixic Acid
NMEC	Neonatal Meningitis
NOR	Norfloxacin
OCC	Outer Membrane Carboxylate Channels
OMP	Outer Membrane Protein (
PBPs	Penicillin Binding Proteins
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
R	Resistant
rpm	Revolutions per minutes
RT-qPCR	Quantitative Real-Time Reverse Transcription PCR
S	Sensitive
SCF	Sulbactam+Cefoperazone
SXT	Cotrimoxazole
TIC	Ticarcillin
TMP-SMX	Trimethoprim-Sulfamethoxazole
TZP	Pipra-Tazobactam
UPEC	Uropathogenic *E. coli*
UTIs	Urinary Tract Infection
WHO	World Health Organization
WT	Wild type
Z	Cefazolin
ZOI	Zone of inhibition

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Abstract

Multidrug resistance (MDR) is the resistance shown against at least one antibiotic from three or more antibiotic categories. Resistance to antibiotics is a growing crisis in clinical medicine as resistant organisms enhance morbidity and mortality. The number of microorganisms exhibiting antibiotic resistance, especially resistance to multiple antibiotics, is increasing continuously. Thus, disease causing agents which were once susceptible to antibiotics are showing resistance to these therapies. Gram-negative bacteria accounts for a significant proportion of the list of bacteria which are developing resistance at an alarming rate. Percentage of resistant bacteria can vary from one part of the world to another. No local data on resistance patterns is available in many health facilities especially in developing countries such as Pakistan. Precise and demographic specific statistics on antimicrobial resistance profiles of antibiotics is need of the hour. The study aims at developing a national level picture of the antibiotic resistant Gram-negative bacteria in health care facilities across Pakistan and also to identify the molecular determinants of the resistance. In this research, clinical isolates of two Gram-negative bacterial species, *Pseudomonas aeruginosa and Escherichia coli* were sampled from major cities of four provinces of Pakistan. The isolates were re-cultured in lab and antibiotics from different classes were used to find out their resistance against commonly used antibiotics. Our results revealed meropenem, imipenem and doripenem as the most effective antibiotics against all *E. coli* with ≥60 % sensitivity percentage. Amikacin (72 %), imipenem (80 %) and meropenem (84 %) were the most effective antibiotics against *P. aeruginosa* strains. The province wise result showed that, *E. coli* from Balochistan province showed highest percentage (71.43 %) of resistance, then KPK (66.67 %), Punjab (60 %) provinces were placed while Sindh samples showed least resistance (53.06 %). KPK had the highest rate of resistance (69.23) against antibiotics in *P. aeruginosa* species, followed by Balochistan (63.46 %), Sindh (35.9 %) and Punjab/ICT (34.62 %). All except one of the *E. coli* samples (96.15 %) in our study were MDR and showed resistance against at least five antibiotics of different antibiotic groups. 76 % (n=19) of *P. aeruginosa* samples were MDR in our study. In our study, a Multiple Antibiotic Resistance Index (MARI) value larger than 0.25 was seen in 96.15 % (n=25) of the *E. coli* isolates, and 68 % (n=17) of *P. aeruginosa* isolates that indicates overuse of antibiotics in Pakistan. DNA extraction of these isolates was performed by using commercial DNA kits. It was

quantitatively analysed by doing Nanodrop and qualitatively analysed by gel electrophoresis along with gel doc. To amplify specific gene by PCR, reverse and forward primers for OmpC, OmpF, OmpW, OprD and OpdK were designed. After performing amplification this amplified gene was sent for sequencing with the same forward primer (used in PCR) to know about various mutations. Different mutations were seen in the amino acid sequences of OmpC, OmpF, OprD and OpdK. 3D structure of OmpC and OprD porins were predicted by Phyre-2 program and mutations were highlighted using UCST Chimera program. In conclusion, resistance to antibiotics is increasing in *E. coli* and *P. aeruginosa* which is a matter of concern and needs proper management. The uncontrollable spread of MDR genes among clinical isolates of Gram-negative bacteria in the local hospitals calls for rapid government intervention. These kind of monitoring studies must be performed on regular basis in order to monitor the stats of infectious diseases and control the spread of these infectious diseases. To stop the future spread of resistant, virulent, and genetically varied strains of various Gram-negative bacteria, it is essential to implement effective infection control procedures, adhere to rigorous hygiene standards, and administer antibiotics as needed.

Chapter 1
Introduction

1 Introduction

According to Davies and Davies (2010), antibiotics are chemical substances derived from microorganisms that either kill or stop the growth of other germs. The majority of antibiotics currently in use come from the phylum Actinobacteria, and over 80 % of antibiotics obtained from actinobacteria are expressed by bacteria found in soil belonging to the genus Streptomyces (Barka *et al.*, 2015). Before the discovery of natural antibiotics, synthetic substances such as salvarsan, sulfa medicines, and quinolones were frequently utilized as chemotherapeutic agents (Aminov, 2010). Since Fleming's discovery of penicillin in 1929, a wide range of antibacterial medicines have been created, having a significant impact on global life expectancy rates and human health (Coico, 2006). It can be said confidently that antibiotic action is one of the most important treatment options of modern medical drugs that is used to treat diseases.

1.1 History of antibiotics

Infections were the primary cause of death throughout the course of 19th century. The development of antibiotics made it possible to treat hospital-acquired illnesses and significantly lowered the rate of morbidity and mortality brought on by infections. Arsphenamine (Salvarsan), an antibacterial drug for the successful treatment of syphilis, was created for the first time by Paul Ehrlich in 1910. It was the most popular drug at the time and was produced in massive quantities (Schwartz, 2004). Prontosil (sulfamidochrysoidine), a sulfa medication, was created by the Bayer Company with the help of two highly qualified chemists, Fritz Mietzsch and Josef Klarer. Gerhard Domagk's introduction of the antibiotic as an antibacterial in 1932 marked a significant advancement in the medical field (Apaydın & Török, 2019).

Numerous businesses began the bulk manufacture of its derivatives since sulfanilamide was an inexpensive, off-patent medicine (Schwartz, 2004; Domagk, 1935). The first person to get the antibiotic penicillin from the soil-dwelling fungus Penicillium notatum was the British bacteriologist Alexander Fleming in September 1928. However, the discovery of penicillin, which represented a significant advance in medicine, was initially announced in 1929, and the first human clinical trials were carried out in 1940 (Schlegel, 2003, Aminov, 2010).

An important advancement in managing and combating bacterial illnesses was made with the discovery of penicillin and its adoption into the human health care system. However, the issue with antibiotic use is that while they are antagonistic to pathogenic bacteria, they are also antagonistic to the natural microbiota in the human body (Walsh, 2003). As a result, it is advised that before prescribing any antibiotic, one should think about the drug's intended benefits and negative effects. Therefore, prior to introducing an antimicrobial into the healthcare system, it is essential to understand its mode of action (Etebu & Arikekpar, 2016).

1.2 Classification of antibiotics

Antibiotics are classified into five major groups i.e., on the basis of action, source, spectrum, chemical structure and function.

1.2.1 Classification based on Action

Antibiotics are classified based on action into two major groups i.e., bactericidal and bacteriostatic antibiotics. Antibiotics that target the bacterial cell membrane or cell wall causing its disruption and those that inhibit or slow down the bacterial growth are known as bactericidal and bacteriostatic antibiotics respectively (Ullah & Ali, 2017). Bacteriostatic antibiotics inhibit some metabolic pathways and synthesis of protein. Under high concentration, few bacteriostatic drugs may act as bactericidal drugs (Aminov, 2010). Some common bacteriostatic and bactericidal drugs are Sulphonamides, Amphenicols, Spectinomycin, Trimethoprim, Tetracycline, Erythromycin and Penicillin, Carbapenems, Cephalosporins, Aminoglycosides, Quinolones, Vancomycin, Fluoroquinolones, Polymyxins respectively.

1.2.2 Classification on the basis of source

Antibiotics are derived from three major sources i.e., fungal, synthetic and semi- synthetic. Cephalosporins, benzylpenicillin and cefamycins are obtained from natural sources. But their toxicity is higher as compared to synthetic antibiotics. Ampicillin is considered as semi-syntheic antibiotic, with lowered toxicity and increased efficacy. Norfloxacin and moxifloxacin are well-known synthetic antibiotics with increased efficacy and reduced toxicity (Ullah & Ali, 2017).

1.2.3 Classification on the basis of Spectrum

Antibiotics are classified on the basis of their target in two categories i.e., broad spectrum antibiotics and narrow spectrum antibiotics. Broad spectrum antibiotics target pathogenic bacteria which includes Gram-negative and Gram-positive bacteria. Narrow spectrum antibiotics target the specific group of microorganisms i.e., either Gram-negative or Gram-positive bacteria. These antibiotics do not harm the body's natural microorganisms and are unable to cause superinfections. They also have a reduced rate of resistance because they only target specific bacteria. Some well-known examples of narrow and broad-spectrum antibiotics are β-lactam, Cloxicillin, Methicillin, Cephalosporins (generation one and two), Vancomycin, Rifampin and Ampicillin, Amoxicillin, Quinolones, Aminoglycosides, Carbapenems, Cephalosporins (generation three, four and five), Macrolides, Tetracycline, Chloramphenicol, Tircarcillin, Rifamycin respectively (Ullah & Ali, 2017).

1.2.4 Chemical Structure based Classification

Classification on the basis of chemical structure is very significant because same structures possess same level of efficacy, toxicity and many other properties. The antibiotics are categorized into eight classes i.e., β-lactam, macrolides, aminoglycoside, quinolones and fluoroquinolones, sulfonamides, nitroimidazoles and tetracyclines.

β-Lactams consist of four membered lactam ring varying in attached side chains and extra cycles. Derivatives of penicillin, monobactams, carbapenems and cephalosporins fall in this category of antibiotics. In Cephalosporins, mostly changes occur on position number 3 and 7 (Adzitey, 2015).

Macrolides are derived from natural products mainly through polyketide group consisting of 14-, 15-, or 16- unit macrocyclic lactone ring with attached deoxy sugars. Common macrolides are roxithromycin and erythromycin. Some studies showed that the certain specified substitution on carbon number 6, 9, 11, or 12 resulted in improved in-vitro activity against Mycobacterium tuberculosis (Zhu *et al.*, 2008).

Aminoglycosides comprise of two amino sugars attached to an aminocyclitol with the glycosidic bond. They obstruct the protein synthesis crucial for bacterial growth. Studies have revealed that

the modification in location and number of functional groups attached to the aminoglycosides effects the biological activity like protein synthesis inhibition. They also showed that protein synthesis inhibition is more effective while the compound has two amino groups compared to those who have one amino group. Some common examples of this group of antibiotics are kanamycin, streptomycin, neomycin and spectinomycin (Ullah & Ali, 2017).

Quinolones are synthetic antibiotic agents derived from quinine. Fluoroquinolones are the derivatives of quinolones by adding fluorine group at 6th position. They inhibit DNA gyrase to prevent multiplication of bacteria. Studies showed that the modifications at 1-, 5-, 6-, 7-, and 8th position of this bicyclic ring results in the improved efficacy of pharmacokinetics and antibacterial activity. Although some adverse effects are also associated with these modifications. Various examples are, nalidixic acid (NA), ciprofloxacin (CIP), levofloxacin (LEV) (Adzitey, 2015).

Sulphonamides are the major class of antibiotics having the functional group (R1- SO2-NR2R3) (Ullah & Ali, 2017). They inhibit growth and bacterial replication by inhibiting the synthesis of RNA and DNA. They are potent against the pathogens of the broad spectrum i.e., *Escherichia coli* Staphylococcus, Salmonella and Streptococcus species. The most common sulphonamides are sulfadiazine, sulfamethizole and trimethoprim (Adzitey, 2015). Streptogramins are the class of antibiotics which were obtained by several strains of Streptomyces. They are involved in the inhibition of synthesis of protein by impeding the formation of peptide bond and hinders the exit tunnel of peptide present in large subunit ribosome (50S), resulting in the prevention of polypeptide chain extension (Noeske *et al.*, 2014). This group of antibiotics are divided into two sub-groups i.e., group A and group B. Dalfopristin and Quinupristin belong to group A and group B respectively. Some other examples are vernamycin, staphylomycin, ostrogrycin and pristinamycin (Vazquez, 1967).

Imidazole ring containing compounds are known as nitroimidazoles. The position of nitro functional group determines different nitroimidazole drugs such as, when nitro group is present at position number 2, it is called as benznidazole and if it is present at position number 6, it is called as metronidazole. Compounds having four ring hydrocarbons are classified as tetracyclines having a skeleton of octahydrotetracene-2-carboxamide and a polyketide's subclass. The new derivatives of tetracyclines are semi-synthetic in nature but initially derived through bacterial specie

Streptomyces. The common examples are doxycycline and oxytetracycline.

1.2.5 Classification based on the function of antibiotics

The major factor of antibiotics is their mode of action. There are different processes such as synthesis of cell wall, function of cell membrane, synthesis of protein, synthesis of nucleic acid and many other that help in the growth of bacterial cell. Different antibiotics target and inhibit these processes.

1.2.5.1 Inhibition of cell wall synthesis

The cell wall of bacteria is made up of peptidoglycan layer. Antibiotics target this peptidoglycan layer and inhibit the synthesis of bacterial cell wall. β-lactam antibiotics are the major inhibitors of cell wall synthesis such as derivatives of penicillin, carbapenems, cephalosporins and monobactams. These β-lactam antibiotics imitate as a false compound and inhibit the reaction of transpeptidation and synthesis of peptidoglycan, resulting in the activation of lytic enzymes. β-lactam antibiotics cannot reach the cell wall of Gram-negative bacteria because of the blockade caused by their outer membrane. The bacterial cell resists this class of antibiotics by producing β-lactamase enzyme (Ullah & Ali, 2017).

1.2.5.2 Membrane function inhibitors

Bacterial cytoplasmic membrane works as a barrier for particular agents to pass through it and regulates its internal structure. Any interruption in these functions results in the destruction of the cell or causes cell death. An antibacterial agent known as polymyxins, are cyclic peptides with a long hydrophobic tail, are found in A, B, C, D, and E forms. However, form B and E are used in therapeutics. Polymyxins have been found as toxic agents for Gram-negative strains of bacteria because of their higher specificity against polysaccharides. Systematically, when these polymyxins bind with the substrate of lipopolysaccharide, it results in the structural change of membrane and increase its permeability, which ultimately disturbs the osmotic balance. Moreover, some changes in the membrane like respiratory inhibition, water uptake increased, and molecules discharged from the internal structure of the cell resulted in cell death are also observed. These antibacterial agents have minimum or null effect on Gram-positive bacterial strains because of their thick cell

wall which refrains the molecules to enter the cell membrane (Newton, 1965).

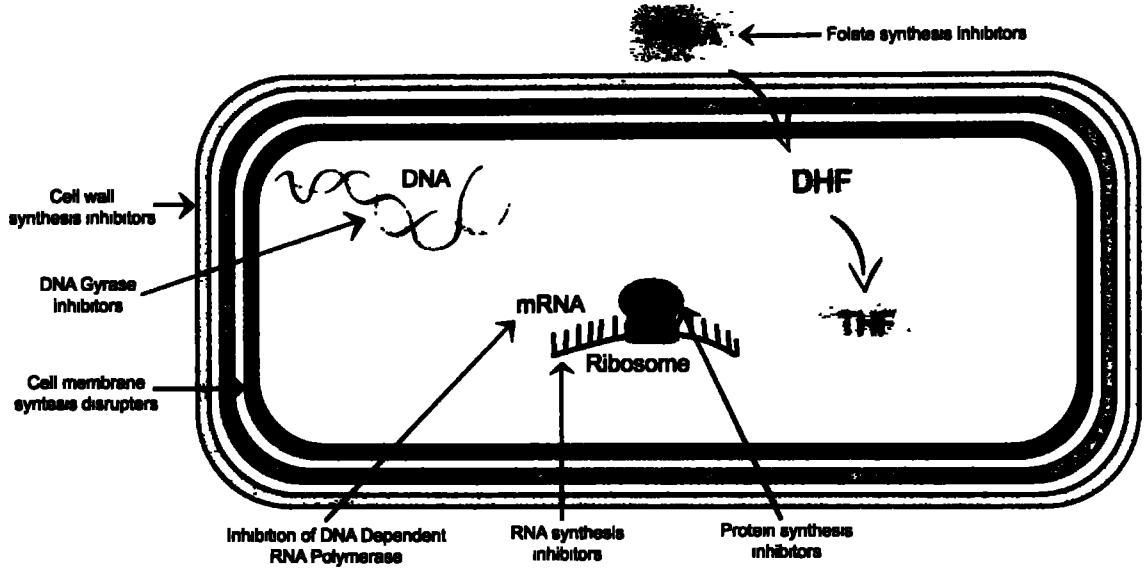

Figure 1.1 Classification of antibiotics which also shows their mode of action (Adapted from Kapoor et al., 2017).

1.2.5.3 Inhibitors of protein synthesis

Synthesis of protein is an important phenomenon in both humans and bacterial cells. So, antibiotics are developed as inhibitors of protein synthesis which disrupts any phase of protein synthesis i.e., initiation, elongation and termination steps (Kohanski *et al.*, 2010). Antibiotics bind with the two ribosomal subunits named 30Sand 50S, which affects the synthesis of protein. For example, macrolides, streptogramins, phenicols, oxazolidinone and lincosamide are observed as 50S inhibitors. These antibiotics generally restricts the initiation of translation or impedes the peptidyl transferase reaction preventing the elongation of peptide chain (Patel *et al.*, 2001). Aminoglycosides and tetracyclines are considered as 30S inhibitors. Aminoglycosides effects the protein synthesis steps and causes mistranslation which ultimately refrains the synthesis of proteinand damage the cytoplasmic membrane resulting in cell death. Tetracyclines antibiotics generally inhibit the translation step by blocking the access of aminoacyl tRNA to ribosomes.

1.2.5.4 Inhibitors of nucleic acid synthesis

The antibiotics that interfere with the DNA and RNA synthesis processes are called inhibitors of nucleic acid synthesis. Antibiotic rifampin is known as RNA synthesis inhibitor. It works by

inhibiting the RNA elongation by binding with the DNA- dependent RNA polymerase, preventing the transcription of gene leads to cell death. Quinolones such as CIP and nA act as DNA synthesis inhibitors. They work by binding with the DNA gyrase which inhibits the opening of supercoiled double stranded DNA, resulting in halting the replication of DNA leading the cell damage (Ullah & Ali, 2017).

1.3 Antibiotic resistance

The period from 1930s to 1960s can be called as "golden era" of antibiotics as it gave rise to many medicinally important antibiotics (Wright, 2014). Unfortunately, this period was very short, and this golden period ended abruptly because emerging resistant pathogens became very resistant against these antibiotics and researchers were not very efficient to cope up with the pace of antibiotic resistance development. Constant failure to engineer new antibiotics and imprudent usage of antibiotics are the major reasons behind the rise of antibiotic resistance (Sengupta *et al.*, 2013). Irresponsible use and widespread excessive supply of antibiotics caused the emergence of resistant bacterial species.

The set of all antibiotic resistance genes is termed as antibiotic resistome. It usually includes those associated with pathogenic bacteria which have been isolated in the clinics. Also, antibiotic producing non-pathogenic bacteria and other resistance genes are included in it. The resistome can also refer to an inherited set of genes that is used to resist infections. Antibiotic resistance is an old process and the "resistome" is an ever changing and rising problem. The major reasons of the resistome are global over-population, poor sanitation, increased relocation around the world, higher use of antibiotics in medical practices, and in agriculture and poor disposal system of sewerage (Golkar *et al.*, 2014, Gould & Bal,2013).

Antibiotics revolutionized medicine in the twentieth century because of their ability to prevent bacterial infections and to treat these infections. However, the increased use of antibiotics impelled bacteria to grow into resistant strains. The ability to treat infectious diseases was thus confronted by the rise of antibiotic resistant bacteria (ARB) and subsequently antibiotic-resistance genes (ARGs) (Wright, 2010). The situation is so serious that the World Health Organization has classified antibiotic-resistance as a "serious threat" to world's public health (WHO, 2015).

Antibiotic resistance in bacteria evolves when it can escape the effects of antibiotics by various mechanisms. Some of these are: A. neutralizing antibiotics; B. pumping antibiotics outside of the cell; C. modifying antibiotic's binding sites thus inhibiting the medicines' attachment to microbes.

1.4 Mechanisms of antibiotic resistance

The mechanisms of antibiotic-resistance can be classified into two (02) broad groups that are A. Natural and; B. acquired-resistance. Natural resistance can either be innate (frequently expressed in the organisms) or mediated (genes usually present in the bacteria are only activated to resistant levels after antibiotic treatment) (Reygaert, 2018). On the other hand, acquired resistance can emerge from mutations in the bacteria's chromosomal DNA (Culyba *et al.*, 2015) or from the bacteria receiving genetic material through translation, conjugation, or transposition (Lerminiaux & Cameron, 2019). Drug targ*et al*teration, drug inactivation, drug efflux, and drug uptake limitation are the four categories into which AMR processes can be categorized (Kapoor *et al.*, 2018).

Gram-negative bacteria acquire all these mechanisms for resistance while Gram-positive bacteria acquire only two mechanisms i.e., modification of drug target and drug inactivation because they lack lipopolysaccharide outer membrane so are less likely to utilize the drug-limitingmechanism. They also lack the capacity for some drug efflux processes (Mahon *et al.*, 2018).

1.4.1 Destroying the drug

Penicillin and cephalosporins are two of the most often prescribed beta-lactam antibiotics (Page, 2012). The main structural element of this pharmacological class is a four-sided -lactam loop, which is shared by all members. The activity of -lactamases dismantles the main mechanism of -lactam resistance, the -lactam loop. It is impossible for -lactam ring formation to bind to penicillin-binding proteins (PBP) because -lactamases hydrolyze it (Bush and Bradford, 2016).

1.4.2 Drug target modification

The modification of the drug's target is a typical method by which bacteria develop antibiotic resistance (Reygaert, 2018). Changes in the number and/or organization of PBPs are one of the mechanisms of resistance to -lactam medications. The amount of medication that can bind to the

target is affected by changes in the number of PBPs (Bush and Bradford, 2016). If there is a structural alteration, as the appearance of the mecA gene in S. aureus (Foster, 2017), drug binding will be reduced or deleted. Another example is the family of genes known as erythromycin ribosome methylase (ERM), which methylates 16S rRNA and modifies the drug-binding site to prevent the binding of macrolides, streptogramins, and lincosamines (Peterson & Kaur, 2018). For instance, fluoroquinolones are drugs that prevent the formation of nucleic acids. Changes in DNA gyrase or topoisomerase IV led to resistance to these therapies. Drug connections to gyrase and topoisomerase become more difficult or impossible because of these alterations (Nainu *et al.*, 2021).

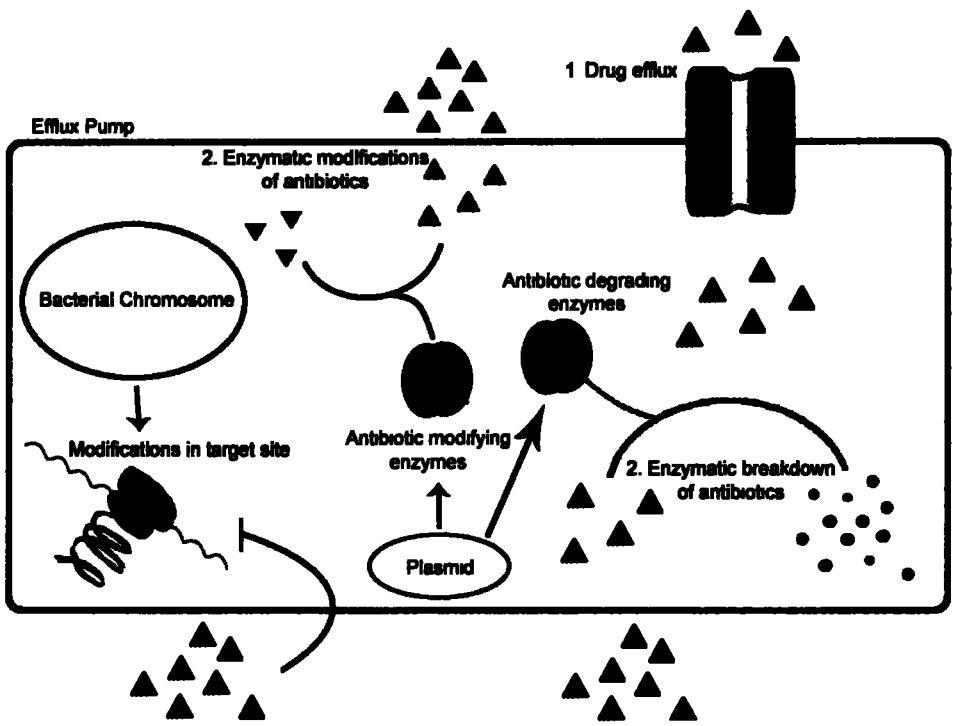

Figure 1.2 Different mechanisms of antibiotic resistance e.g. drug efflux through efflux pump, enzymatic breakdown of the antibiotics, enzymatic modifications of the antibiotic and changes in the target sites (Adapted from Aslam et al., 2018).

1.4.3 Limiting the Drug Uptake

Some sort of molecules are inhibited to pass through the membrane of Gram-negative bacteria due to the structural and functional characteristics of lipopolysaccharide layer present in the outer

membrane (Blair *et al.*, 2014). Gram-negative bacterial strains allow the entry of hydrophilic molecules to pass through the porin channels. The drug uptake can be limited in two main ways by changes in the porin channels either by decreasing the porin number present or by changes in the selectivity ofporin channels by mutation (Kumar & Schweizer, 2005). *Enterobacteriaceae* family members develop resistance either due to the reduction in porin number or completely stoppingthe entire production of specific porins. These bacterial strains develop resistance against carbapenems by this mechanism (Cornaglia *et al.*, 1996). Some other bacterial strains developed resistance against certain antibiotics such as,β-lactam, tetracycline, cephalosporins and imipenem due to changes occurred in porin channels by mutation (Reygaert, 2018).

1.4.4 Modified drug targets

Bacterial cell developed resistance against specific antibiotics by modifying their drug targets. Gram-positive bacterial strains alter the number and structure of PBPs. They are associated with the composition of peptidoglycan layer present in the cell wall. The quantity of drug that is able to bind to its specific target is either increased or decreased by the alteration in PBPs number. Structural change in PBPs resulted in either decreased drug binding ability or completely inhibited itsbinding to the target (Beceiro *et al.*, 2013). Gram-negative bacterial strains wereresistant to vancomycin and daptomycin antibiotics. The former acts by impeding the synthesis of cell wall and the latter acts by depolarization of cell membrane. In case of vancomycin resistance, van genes collectively change the peptidoglycan precursors' structure resulting in the decreased ability of antibiotic to bind. While calcium ions is required for the binding of daptomycin. Its resistance occurred by gene mutation i.e., mprFgene was mutated and changed the surface charge of cell membrane from negative to positive, thus preventing the calcium ions to bind and also daptomycin (Cox & Wright, 2013).

The resistance developed against antibiotics that binds to the ribosomal subunits was due to the occurrence of mutation. Certain genes such as erm genes are involved in interfering with the binding ability of antibiotics to ribosomes. This includes the classes of antibiotics such as aminoglycosides, streptogramins, oxazolidinones and macrolides etc (Roberts, 2004). Mutated structures of topoisomerase and DNA gyrase resulted in resistance against antibiotics such as

fluoroquinolones that targets the synthesis of nucleic acid (Redgrave *et al.*, 2014).

1.4.5 Inactivation of drugs

Inactivation mechanism of drugs was carried out in bacterial strains either by degrading the drug or by transferring a chemical group to it. β-lactamases are the group of enzymes which degrade the β-lactam class of antibiotics. Similarly, tetX gene is responsible for the inactivation of tetracycline. Transferring a chemical group leads to inactivation of drugs. These groups include phosphoryl, adenyl and acetyl. Aminoglycosides were inactivated by the transfer of all three groups to it. While fluoroquinolones, streptogramins and chloramphenicol were inactivated by acetylation (Blair *et al.*, 2015).

1.4.6 Activation of efflux pumps

Bacteria encode genes for the efflux pumps. Under some external stimuli, they areoverexpressed. These pumps are used to eliminate the toxic waste from the bacterial cell. Also, these efflux pumps transport a variety of substances across the cell (Blair *et al.*, 2014).

There are different kinds of efflux pumps in bacterial cell such as ATP-binding cassette family (ABC family), multidrug and toxic compound extrusion family (MATE family), small multidrug resistance family (SMR family), major facilitator superfamily (MFS family) and lastly the resistance-nodulation-cell division family (RND family). These all pumps have certain functions in the bacterial cell. RND transporter family is found in Gram-negative bacteria. They act in coordination with the periplasmic membrane fusion protein(MFP) and an outer membrane porin protein (OMP) to transfer the components across thewhole cell. *E. coli* possesses AcrAB-TolC pump of the RND transporter family. They induce resistance against penicillin, macrolides, tetracycline, fluoroquinolones,and chloramphenicol. The pump protein AcrB is composed of two binding pockets, allowing the substrates of various sizes and properties to bind (Poole, 2007).

1.5 Resistant Gram-negative bacteria

Resistant Gram-negative bacteria which include *Pseudomonas aeruginosa, Enterobacteriaceae, Neisseria gonorrhoeae, Shigella* spp., *Haemophilus influenza, Helicobacter pylori, Acinetobacter baumannii, Salmonella* spp. and *Campylobacter* are the real threat and contributing factors in

sinking health and economy status around the globe. In 2017, the World Health Organization (WHO) released a list of bacteria requiring novel antibiotics on emergency basis to stop the emergence of their resistant variants. WHO grouped these bacteria according to their resistance severity and named them as critical, high, and medium (Figure 1.3).

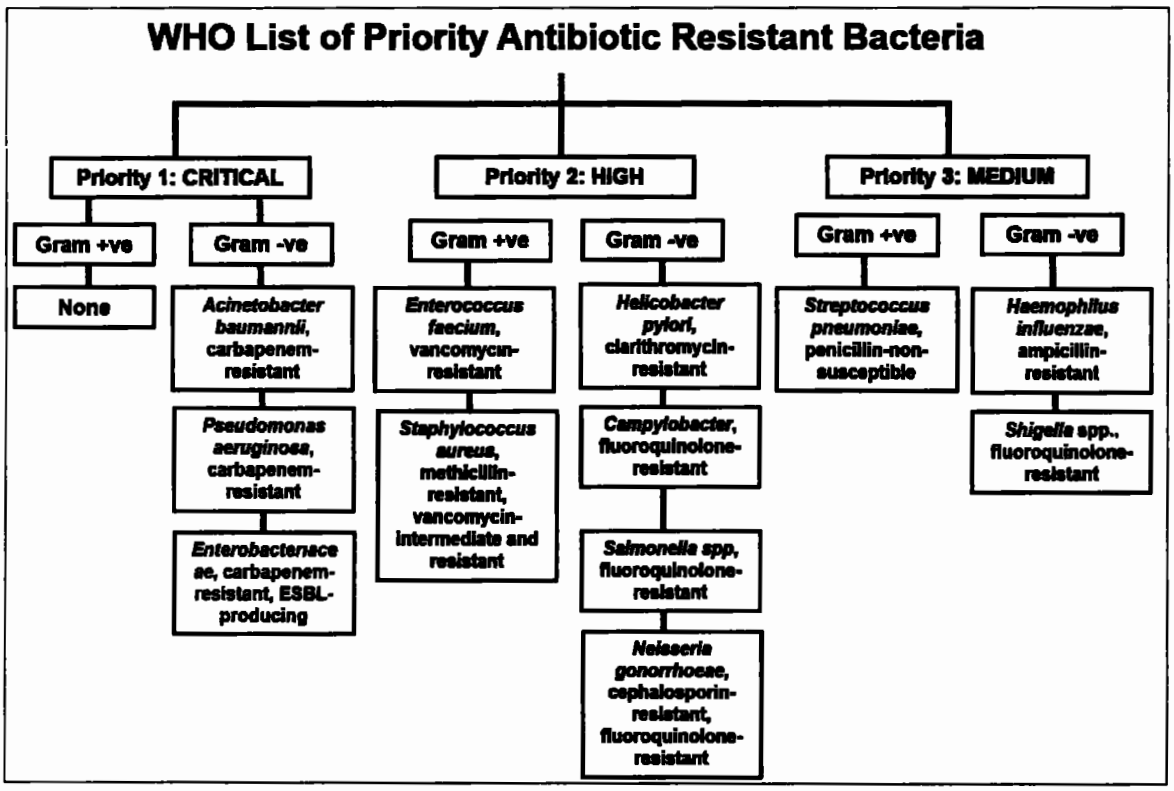

Figure 1.3 WHO list of priority/significant pathogens; These are grouped under three (03) classes based on their antibiotic-resistance i.e. Critical, high or medium. (WHO, 2017)

P. aeruginosa is an aerobic, Gram-negative rod that doesn't form spores and can infect both immunocompetent as well as immunocompromised hosts with a range of diseases (Kerr & Snelling, 2009). It is a very difficult organism to cure in modern medicine due to its propensity to infect immunocompromised hosts, high flexible bacterial resistance, and even a wide spectrum of dynamic defenses. (Mulcahy *et al.*, 2014).

In the environment, *P. aeruginosa* is commonly observed, especially in freshwater. Urban towns have hot tubs, jacuzzies, and swimming pools as reservoirs in which this bacterium is observed. Numerous community-acquired illnesses, including folliculitis, puncture wounds that result in pneumonia, otitis

externa, and many others, can be brought on by it. It is frequently an opportunistic pathogen as well as a significant contributor towards nosocomial infections like pneumonia linked to ventilators, catheter related UTIs, and others. Infections triggered by *P. aeruginosa* are widespread in people with immunocompromised states including cystic fibrosis, burn, uncontrolled diabetes mellitus, bronchiectasis, AIDS, organ transplant, and ICU admissions (Kerr & Snelling, 2009; Mulcahy *et al.*, 2014).

E. coli, commonly known as *E. coli*, is a Gram-negative bacterium. It is present in the gut as a part of gut microbiota in warm-blooded animals and humans. Most *E. coli* strains are not pathogenic, but some causes severe infection to the central nervous system, gastrointestinal tract (GI Tract) and renal system (Alharbi *et al.*, 2019). *E. coli* is classified into two major groups i.e., commensal and pathogenic strains (Alwash & Al-Rafyai, 2019). Commensal bacterial strains are harmless and live in a symbiotic relationship with the host and rarely induce infections (Ramos *et al.*, 2020).

E. coli does not have any known virulence factor (Alwash & Al-Rafyai, 2019). Pathogenic *E. coli is* subdivided into two classes i.e., intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC). Intestinal pathogenic *E. coli* (InPEC) induces diarrheal infections and is classified in six known pathotypes such as, Enterotoxigenic *E. coli* (ETEC), Diffusely Adherent *E. coli* (DAEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC) and Enteropathogenic *E. coli* (EPEC). The classification of these enteropathotypes of *E. coli* depends on their virulence, mechanism of infection, syndromes and symptoms, tissue tropism and association with enterocytes. Recently, studies classified Adherent-Invasive *E. coli* (AIEC) as a new pathotype of intestinal pathogenic *E. coli* (InPEC) which is categorized as a disease related *E. coli* associated to Crohn's disease. Whereas strains of extraintestinal pathogenic *E. coli* (ExPEC) are related to Uropathogenic *E. coli* (UPEC) which causes urinary tract infections (UTIs) and neonatal meningitis (NMEC) (Rojas- Lopez *et al.*, 2018). All these pathotypes cause foodborne illness and their major source of infections are contaminated poultry meat, sprouts, vegetables, fruits, drinking water, dairy products and beverages (Rojas-Lopez *et al.*, 2018).

1.6 Bacterial outer membrane

The outer membrane of bacteria is an intermediator between the external environment and the host cell. It is found in peptidoglycan layer and cytoplasmic membrane (Hejair *et al.*, 2017). These OMPs are present on the outer surface of bacteria. When these pathogens enter in the host cell, it quickly triggers the immune response and recognized these pathogens as a foreign molecule (Wang *et al.*, 2017). Bacterial outer membrane (OM) acts as a barrier in the transportation of toxic substances like antibiotics and bile acids. Hence, the Gram-negative bacteria do not allow chemicals having molecular weight higher than 600 Dalton (600 Da) to enter inside the cell. So, antibiotics like daptomycin and vancomycin whose molecular weight is higher than 1400 Dalton (1400 Da) cannot penetrate the bacteria. This characteristic causes the hurdle in the invention of new antibiotics that target the Gram-negative bacteria (Choi & Lee, 2019).

Outer membrane porins are transmembrane proteins which form pores and structurally comprised of β-barrels. It plays a pivotal role in the transportation of hydrophilic substances through the opening channels which are filled with water. Gram-negative bacteria possess these porins which can be categorized according to their activities as specific and non-specific porins. According to their structural functionality, they are grouped as monomeric, dimeric, and trimeric porins (Pagès *et al.*, 2008). Porin plays a key role to maintain the integrity of envelop in Gram-negative bacteria such as non-specific porin i.e., outer membrane protein A (OmpA) is a protein which is associated with peptidoglycan which forms a non-covalent bond with flexible domains of periplasmic and allow the passively transport of various microscale chemicals (Choi & Lee, 2019).

In Gram-negative bacteria, the passive transportation of antibiotics through these outer membrane porin channels results in the development of resistance such as, fluoroquinolones and β- lactam class of antibiotics easily cross the outer membrane with the help of non-specific porin i.e., outer membrane protein F (OmpF). Hence, the mutants of OmpF develop resistance against various β-lactam class of antibiotics in Gram-negative bacteria like *E. coli, Klebsiella pneumoniae, P. aeruginosa, Enterobacter aerogenes and Serratia marcescens* (Ziervogel & Roux, 2013).

1.6.1 Outer Membrane Protein (OMP) structure

Gram-negative bacteria's outer membrane is made up of lipopolysaccharides. It is comprised of three fragments i.e., 1) lipid A, a hydrophobic membrane which forms the outer cover of outer membrane, 2) Core-OS, a non-repetitive, phosphorylated oligosaccharide core, 3) O-antigen, a hydrophilic coating surface made up of repeated sugar units (Erridge *et al.*, 2002). Lipid A is extremely conserved while O-antigen is really flexible. The core is classified into inner and outer region. The inner region is formulated by 2-keto-3-deoxy-D-manno-octulosonic acid and some residues of L-glycero-D-manno-heptose. The outer region is formulated by various residues of sugar associated with each other. These regions of core help to stabilize the bacterial outer membrane (Caroff & Karibian, 2003). The lipopolysaccharide plays a major role in the permeability and integrity of the outer membrane. Macromolecules having charge on them are not allowed to enter the membrane. However, inadequate diffusion of hydrophobic molecules is permitted (Nikaido, 2003).

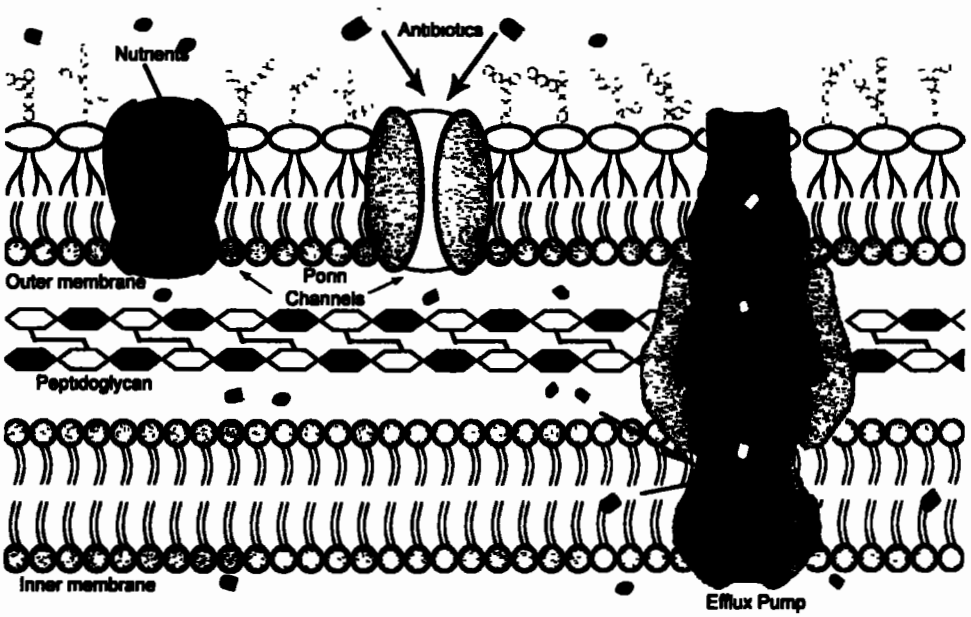

Figure 1.4 Structural representation of outer membrane of Gram-negative bacteria (Adapted from Prajapati et al., 2021)

The outer membrane protein (OMP) is composed of lipopolysaccharides, phospholipids, and proteins. OMPs are structurally composed of β-barrel sheets, usually ranging from 8-26 strands

(Rollauer *et al.*, 2015). They help in the transportation of hydrophillic compounds. According to their function, they are present in mono, di and trimeric forms (Pagès *et al.*, 2008). There are some major porins present in bacterial cell includes OmpA, OmpC, OmpF, OmpD etc. These porins proteins plays a vital role in the transportation of nutrients, salts, ions, and antibiotics etc (Isibasi *et al.*, 1988).

They only allow the molecules having the size of upto 600Da to cross the barrier (Cowan *et al.*, 1995). Mutations or complete loss of porin OmpF and OmpC leads to the resistance against β-lactam class of antibiotics, fluoroquinolones, chloramphenicols and many others in *E. coli* (Kim *et al.*, 2020).

1.7 Problem statement

Although research has been carried out to isolate and characterize bacteria from hospital patients, data on their susceptibility to antibiotics are rare and local data on molecular resistance patterns is not available. Profiling of antibiotic resistant bacteria relative to nosocomial infections in Pakistani Population has never been recorded in available online databases. The data will be significantly beneficial for devising healthcare policies in relevant areas of Pakistan. Moreover, the surveillance study will help to minimize antibiotic resistance in healthcare settings by providing the basis for establishing guidelines against excessive antibiotic use.

1.8 Significance of research

Multidrug resistance is a soaring problem of the modern human life. As the list of multidrug resistant (MDR) bacteria is increasing at an exponential rate, research on finding a way to tackle the problem is of high priority. But before even looking for the solution we need to realize the seriousness of the issue in Pakistan. We need to have an honest, precise picture of the multidrug resistance countrywide. The study will give us a not just a detailed picture of multidrug resistance across Pakistan but will also highlight major mutations in the bacteria leading to the multidrug resistance. This will assist in better and efficient prescription of antibiotics in the future.

1.9 Objectives

Through this study we aimed to compile a comprehensive pattern of antibiotic resistance in different bacterial strains and subsequently filing of collected results to develop demographically specific online database for Pakistan.

i. Isolation, Biochemical and genetic identification of bacteria isolated from healthcare facilities in different parts of Pakistan.

ii. Antimicrobial susceptibility profiling of the *E. coli* and *P. aeruginosa* isolates and profiling strains resistant against commonly used antibiotics.

iii. Identifying genetic diversity among the antibiotic resistant bacterial isolates by molecular characterization of major porins

iv. To get a national level picture of antibiotic resistance in Pakistan

Chapter 2
Literature Review

2 Literature review

Antibacterial resistance is an ever-increasing problem of the current century and contributes to the list of most serious threats to public health globally (WHO, 2017). The number of resistant bacterial strains, degree of resistance in each microorganism and affected geographic areas are mounting with each passing day (Pfeifer *et al.*, 2010). Also, number of organisms showing ABR, especially resistance to multiple antibiotics, are increasing continuously (Noor & Munna, 2015). Thus, disease-causing agents which were previously considered to be susceptible to antibacterials are showing resistance to these therapies (Levy & Marshall, 2004) Increase in morbidity and mortality has been reported by Kapil (2005) and Ventola (2015) caused by resistant microorganisms as it increases the risk of not-so-fit therapy (Kapil, 2005; Ventola, 2015). This resistance may impede treatment or lead to delayed treatment resulting in complications or even death (Fair & Tor, 2014; Prestinaci *et al.*, 2015). Also, a patient may need more care because of the resistance, and may need alternative, more expensive antibiotics, having more serious side-effects. The patient may also require more detailed treatments to be given in the hospitals (Ventola, 2015; Friedman *et al.*, 2016).

The MDR organisms result in a more risky and costly therapy which can also result in being unsuccessful sometimes. In the developing countries, most antibiotics are available over the counter which can be distributed without prescription; Patients and masses are therefore required to be educated and it is a crucial need of the time (Moyes *et al.*,2009). In the developing world, individuals may risk their lives to multidrug-resistance (MDR) diseases as available medications will fail (Levy & Marshall, 2004). For instance, Noor & Munna (2015) reported MDR enteric disease agents which caused danger and threat to public health in developing countries. Over the world, MDR has been stated in *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enterobacter cloacae* and *P. aeruginosa* (Mshana *et al.*, 2013).

Historically, clinicians have been using their past clinical experience to treat infections successfully (i.e., empirical therapy) (Karlowsky *et al.*, 2004; Wong *et al.*, 2017). However, the practice is becoming difficult as resistance has been detected against the majority of the antibiotic agents in clinical medicine currently being used in humans and animals. This situation has made

the selection of a more suitable antibiotic agent a more difficult mission for the clinicians. In this situation, the *in-vitro* antimicrobial susceptibility testing data has become a great source of dependency for the clinicians. It also shows the significance of analytical practices in clinical setups (Walker, 2007).

2.1 Antibiotic-resistant bacterial infections

Antibiotic resistant infections are widely spread throughout the world (Golkar *et al.*, 2014). A national US survey of infectious-disease specialists which was steered by the Emerging Infections Network and reported that more than sixty percent of members had experienced a resistant and untreatable bacterial infection within the last 12 months (Spellberg & Gilbert, 2014). Many health establishments describe this swift rise of resistant microbes as a crisis having disastrous results (Viswanathan, 2014). The Center for Disease Control & Prevention (CDCP) of United States has stated that the humankind is now in the post-antibiotic period. The World Health Organization (WHO) cautioned in 2014 that the antibiotic resistance is in emergency condition (Michael *et al.*, 2014). MDR bacteria are acknowledged as hazards to most of the developed countries (Golkar *et al.*, 2014).

As reported by Davies & Davies (2010) that antimicrobial resistance puts a serious global threat of growing concerns. It is not just to humans but to animal & environment systems as well. Van Beckel *et al.*, (2015) has reported that the global utilization of antibiotics in domestic animals cycling antibiotics use throughout the world. It will have economic and public health effects in the coming years. Their study also shows that antibiotics are commonly used in chicken, cattle and pigs and it is expected that in 2030 the use will rise to 67 percent in major parts of the globe (van Beckel *et al.*, 2015).

Among Gram-positive bacterial strains the biggest risk can arise from resistant *Enterococcus* species and *S. aureus* (CDCP, 2018; Rossolini *et al.*, 2014). An international epidemic may arise as a result. More Americans die from multi-resistant *S. aureus* each year than from murder, emphysema, Parkinson's disease, HIV/AIDS, or any other single cause. Enterococci that are resistant to vancomycin and other expanding bacteria are becoming resistant to the majority of prescribed medicines. It is usual for common respiratory pathogens like *S. pneumoniae* and *M. tuberculosis* to develop drug resistance (Rossolini *et al.*, 2014).

Gram-negative pathogens are especially troublesome as they have acquired resistance to almost all antibacterial medications available, resulting pre-antibiotic era circumstances (Golkar *et al.*, 2014; CDCP, 2018; Rossolini *et al.*, 2014). The development of MDR Gram-negative bacteria has significantly affected the antibiotic potential. Agents of highest concern are the Gram-negative contagions found in medical settings. Acinetobacter species, *P. aeruginosa*, and Enterobacteriaceae (mostly *K. pneumoniae*) are primarily responsible for these. MDR Gram-negative bacteria, such as beta-lactamase-producing *E. coli* and *N. gonorrhoeae*, are also spreading rapidly among people (Rossolini *et al.*, 2014).

Many studies have also put light on the discovery of antibiotics and Antibiotic Resistance Genes in vast environmental settings. High-throughput quantitative PCR (HT-qPCR) with Illumina sequencing has been used to provide a comprehensive insight into the profile of a large variety of ARGs in vast environmental models (Christgen *et al.*, 2015; Garner *et al.*, 2016; Jia *et al.*, 2017; Lau *et al.*, 2017). Su *et al.*, (2017) collected samples from 116 urban sewerage spots and applied methods for characterization of topographical and seasonal dispersals of ARGs and its multitudes. 381 ARGs were identified and extensively publicized in all trials. It was reported by this study that human actions were the main contributors to antibacterial resistance and its dispersal.

The effectiveness of an antimicrobial agent is deteriorated due to the likelihood of resistance or tolerance acquired from the very first use of this compound. This is universally accurate for all antimicrobials that are used to treat bacterial, fungal, viral, and other diseases. Many biochemical & biological systems can speed up this resistance development. It should be avoided to overplay the complexity of all those mechanisms which are linked to the rise and circulation of the resistance. Furthermore, lack of base data on these subjects is a basic need, which resulted in a lack of significant accomplishments to achieve the development of resistance. Different associations have called it a serious global public health issue worldwide. Many reports are compiled, numerous recommendations and proposals are presented, but little progress has been achieved so far. The increase in antibiotic resistance is an insistent concern unfortunately, (Roca *et al.*, 2015).

The second major concern that is observed is that in several countries, antibacterials are un-regulated and are readily available by the counter without any prescription. This results in antibacterials that are easily accessible, cheap and plentiful, which enhances abuse of

antibiotics. The capability to buy online and lack of regulations for purchase of such medicines have also made them available and accessible in countries where antibiotics *are* strictly regulated (Michael *et al.*, 2014).

The third major concern is prescription of the antibiotics; in the U.S. for example, the increasing number of prescribed antibiotics indicates that a lot of work needs to be done to reduce the use of these medicines (Gross, 2013). Even in some states of US, the number of prescribed doses of antibiotics in a year surpass their population, resulting in more than one prescribed treatment per individual per year (CDCP, 2018; Gross, 2013).

Statistics on ABR among local bacteria help in defining the top conceivable treatment for different patients (Ganesh *et al.*, 2013; Johnson, 2015). However, it must not be neglected that the percentage of resistant bacteria varies from one part of world to another (Karam *et al.*, 2016), and in many medical facilities, local data on resistance patterns is lacking (Abejew *et al.*, 2014). The available data can be used at multiple sites including understanding AMR trends, devising community health plan, detecting priority areas for interventions, finding out treatment choices and keeping record of the result of these interventions to decrease or stop resistance (WHO, 2000). Also, there is no adequate statistics on antibiotic resistance profile of antibiotics in developing countries like Pakistan. Therefore, the present study will also cover the screening of the antibiotic resistant profile of antibiotic medications that are used in the handling of infectious microbes in Pakistani hospitals.

In the countries like Pakistan, low dose and inappropriate dose regime is very common because the prescriptions are not appropriate, and the patient compliance is not considered on a serious note. Also, there is no any organized national surveillance program to monitor antibiotic resistance. The emerging mechanisms of resistance in antibiotic resistant bacteria require constant monitoring for accurate and timely detection of novel antimicrobial resistance. The antibacterial surveillance studies have a major part in minimizing antibiotic resistance in health facilities by establishing prescribing regimes at local levels and thus providing the foundation for the establishment of strategies to the healthcare systems for appropriate antibiotic use.

Therefore, this study is planned to discover and expose the resistance pattern in the bacterial pathogens found in Pakistani population.

2.2 *E. coli*

One of the most extensively researched model organisms in the fields of molecular biology and microbiology is *E. coli* (Blount, 2015; Idalia & Bernardo, 2017). One of the earliest commensal bacteria to colonize the gut after birth is the well-known bacterium *E. coli*. However, this bacterium can result in serious systemic infections in immunocompromised patients or in healthy people whose physical, anatomical, and physiological barriers have been weakened (Kaper *et al.*, 2004; Leimbach *et al.*, 2013). Additionally, because of genetic diversity, some strains of *E. coli* vary from their commensal counterparts and encode particular virulence features that enable them to infect a variety of animals with illness (Kaper *et al.*, 2004)

One of the most genetically adaptable microbes is *E. coli*, which can colonize and survive in both primary (associated with hosts such as birds, animals, and humans) habitats and secondary (without hosts) habitats. Due to the great degree of genetic flexibility in this bacterial species, it has a remarkable potential to adapt, which has resulted in the creation of pathogenic strains from commensal populations (Baumgart *et al.*, 2021; Vandecraen *et al.*, 2017, Dobrindt & Hacker, 2001; Darmon & Leach, 2014). Genomes of pathogenic *E. coli* strains are often bigger because these bacteria need more virulence factors (VF) and other adaptive traits. Most of the time, virulence genes are found on transposable genetic elements such plasmids, transposons, bacteriophages, insertion sequences, and pathogenicity islands (Torres & Kaper, 2002; Sabaté *et al.*, 2006); As a result, they may also be swapped horizontally, which may enable creative bacterial rearrangements. On the other hand, commensal bacteria can potentially turn pathogenic through gene loss. For instance, the absence of *E. coli*-specific genes, such as cadA and flagellar genes, caused Shigella to become pathogenic (Maurelli *et al.*, 1998; Lan *et al.*, 2002). Certain examples of VF encoded by phages in *E. coli* strains include Shiga toxins, enterohemolysin, cytolethal distending toxins, superoxide dismutase, and certain OMPs (Schmidt, 2001; Beutin *et al.*, 1990; Boyd and Brüssow, 2002). The spread of acquired virulence genes with unique functions beyond their clonal lineage as well as the emergence of new harmful strains are both outcomes of the horizontal transfer between strains, which promotes variety and adaptability. Through horizontal gene transfer, EHEC gained the stx genes (transfer by phages), OI (O-island), and LEE (locus of enterocyte effacement) (Javadi *et al.*, 2017). EPEC emerged after the acquisition of the LEE island and *espC* serine protease gene (Kaper *et al.*, 1999). Likewise, there are many identified PAIs in

different *E. coli* pathotypes which were required via horizontal gene transfer and can contribute to fitness and the colonization of different niches (Javadi *et al.*, 2017; Messerer *et al.*, 2017).

A better knowledge of *E. coli* VFs, the mechanisms causing species diversification, and the monitoring of foodborne illness outbreaks has been made possible by the growing genomic sequence data. In 2011, raw fenugreek sprout eating in Germany was linked to a foodborne epidemic of diarrhoea caused by Shiga-producing *E. coli* (EHEC-STEC-VTEC) O104:H4 (King *et al.*, 2012). Surprisingly, this strain proved more virulent than the majority of *E. coli* that produce Shiga toxin. DNA analysis revealed that the strain harboured plasmid-borne VF, which is often prevalent in EAEC and promotes aggregative adhesion to intestinal epithelial cells, as well as toxin-encoding phage comparable to the 933W phage identified in EHEC (Beutin & Martin, 2012).

2.2.1 Antibiotic resistance in *E. coli*

According to studies by Marshall *et al.*, (2009) and Tadesse *et al.*, (2012), *E. coli* can have a high level of resistance to several of the antibiotics that people have been using since the 1930s. Uropathogenic *E. coli* is reported to cause most of the clinically important UTIs globally with a considerably high resistance rate to amoxicillin, tetracycline, and trimethoprim/sulfamethoxazole (Kot, 2019).

The ability of *E. coli* strains to horizontally transfer genes and their high rate of gene acquisition may contribute to this. Although the causes of the rise in antibiotic resistance may be complex, it is generally accepted that human activities and increased antibiotic usage for human health, animal health, and food production are the primary culprits (Chokshi *et al.*, 2019; Ma *et al.*, 2021). An example of a very virulent pathogen linked to bloodstream and urinary infections is the widely reported multidrug-resistant *E. coli* ST131, which has aided in the propagation of the CTX-M-15 gene (Johnson *et al.*, 2010, Naseer *et al.*, 2009). In general, the most common kinds of antibiotics that *E. coli* bacteria are resistant to include β -lactams, quinolones, aminoglycosides, sulfonamides, and fosfomycin. Environmental pollution and animal and human gut colonization in poor nations are dominated by strains of *E. coli* that produce ampC (Chokshi *et al.*, 2019; Hasan, 2020). We are limited to the last resource antibiotic classes, such as polymyxins and carbapenems, as Extended Spectrum β-lactamase (ESBL) and AmpC-producing *E. coli* are more frequently reported as the cause of severe infections (Mikhayel *et al.*, 2021; Iseppi *et al.*, 2020). Additionally, 134 instances of *E. coli* infections expressing the OXA-244 variation of OXA-48 were discovered from clinical

samples in Germany (Pfeifer *et al.*, 2012), and Oxa-48-carrying *E. coli* strains have also been found in Europe (Nordmann, 2014). Additional research revealed that 119 *E. coli* strains recovered from additional European nations contained the same mutation (Bakthavatchalam *et al.*, 2016; Dautzenberg *et al.*, 2014, Glupczynski *et al.*, 2012). Similar to this, New Delhi metallo-β-lactamase (NDM-1) and closely related enzymes are a category of zinc-requiring metallo-β-lactamases capable of hydrolysing a wide variety of β-lactams, including all penicillins, cephalosporins, and carbapenems (Williamson *et al.*, 2012). Additionally, NDM-1 and more than 20 NDM variants have proliferated and are linked to illnesses in several regions of the world (Farhat and Khan, 2020). Resistant bacteria have emerged as a global concern to public health as a result of an increase in international travel and trade. Similar to humans, antibiotics are frequently used in food animals for a variety of purposes, including growth stimulation and continuing mass preventive medicine. Third- and fourth-generation cephalosporins (Ceftiofur and Cefquinome) and fluoroquinolones (Enrofloxacin) are often used in food animals in many poor nations (Angulo *et al.*, 2009; Collignon *et al.*, 2016; Naveen, 2018). Because there are more extended-spectrum β - lactamase-producing and fluoroquinolone-resistant *E. coli* in poor nations due to a lack of regulation, funding, controls, and surveillance, the issue is considerably worse there (Founou *et al.*, 2017; Hasan, 2020). Global efforts are therefore required to assure the development of alternative strategies to combat the aforementioned antibacterial resistance of particularly pathogenic strains. Antibiotic-resistant bacteria are now responsible for 700,000 annual fatalities, and by 2050, that figure will rise to 10 million, surpassing the 8.2 million annual deaths currently attributable to cancer (Ventola, 2017; Dadonaite *et al.*, 2022). The creation of new treatment strategies will remain crucial. Vaccination is one alternative sustainable preventative measure that can aid in reducing the spread of antibiotic-resistant *E. coli*. In the sections that follow, we will provide a summary of the vaccination programs used to stop illnesses brought on by various *E. coli* pathotypes.

A study conducted in the USA showed highest resistance against AMP (97.8 %), then against trimethoprim-sulfamethoxazole (TMP-SMX) (92.8 %), and least against CIP (38.8 %) (Karlowsky *et al.*, 2002). Higher rates of ampicillin resistance (55 %) were found in *E. coli* isolates according to a different UK investigation (Bean *et al.*, 2008). In India, the resistance rates were found as TMP-SMX (83.3 %), CN (48.8 %), and CIP (46 %) (Manikandan *et al.*, 2011). Leski *et al.*, in

2016 showed high CN and CIP resistance with 72.9 % and 47.1 %, respectively (Leski *et al.*, 2016). Alanazi *et al.*, (2018) found high CIP (72.7 %) and AMP (42.85 %) resistance in their study.

A study by Sultana *et al.*, (2023) showed that *E. coli* showed greater AMP, AK, cefazolin (Z), CIP, and nalidixic acid (NA) resistance than the others. In all tested Gram-negatives, a 2014 research of UTI patients in Iran revealed the most prevalent resistance against cefixime (CEF), cephalothin, and cotrimoxazole (SXT) (Khosravi *et al.*, 2014)

In a study conducted in hospital of Basrah, Iraq, the *E. coli* isolates were found to be highly resistant for ceftriaxone (CRO), ampicillin (AMP), levofloxacin (LEV), cefotaxime (CTX), aztreonam (ATM), ceftazidime (CAZ), gentamicin (CN), piperacillin (PRL), and TMP-SMX. A substantially lower level of resistance to imipenem, cefepime, and ciprofloxacin was seen in the *E. coli* isolates. The results confirmed that clinical isolates of *E. coli* in UTI patients had significant levels of drug resistance (Jail & Al Atbee, 2022)

In a study conducted in Institute of Child Health, Lahore, Pakistan, Uropathogenic *E. coli* strains were isolated from pediatric patients and subjected to antibiotic susceptibility assays for 21 common antimicrobial drugs. Results revealed that more than 90 % of the uropathogenic strains were not susceptible to cefuroxime (CXM), cefixime (CEF), and CTX. More than 80 % of uropathogenic strains were also found to be resistant to co-amoxiclav (AC), ceftazidime (CAZ), ceftriaxone (CRO), CIP, NA, norfloxacin (NOR), pipemidic acid, and co-trimoxazole (SXT). However, *E. coli* strains were susceptible to colistinsulphate (85 %), polymyxin (BPB) (85 %), chloramphenicol (C) (84 %), nitrofurantoin (nF) (79 %), fosfomycin (FOS) (76 %), and meropenem (MEM) (71 %) (Iqbal *et al.*, 2021).

The population of Islamabad who presented with UTIs at the tertiary care facilities was the subject of retrospective research. According to the findings, *E. coli*'s resistance to Amoxicillin-Clavulanic Acid (47 %), TMP-SMX (27 %), Fluoroquinolones (24 %), and Cephalosporins (38 %), has increased. The use of nF and FOS increased significantly. High rates of resistance to cephalosporins (62 %), fluoroquinolones (76 %), TMP-SMX (73 %), and amoxicillin (AML) (53 %) were recorded. However, FOS (92 %), aminoglycosides (90 %) and nF (80 %) exhibited noticeably high levels of sensitivity (Bangash *et al.*, 2022)

In a research conducted in 2020 by Haq *et al.*, it was discovered that the isolates were least sensitive to NA, which was then followed by AMP, SXT, CTX, CIP, ATM, AML, CN, nF, and IPM. Among

the resistant isolates, the iucC gene was the most prevalent. It was discovered that 86 % of the samples collected were MDR. They discovered a significant relationship (P=0.03) between the genes fimH and resistance to ATM, and iucC with resistance to AMP and AML (Haq *et al.*, 2020)

According to a study by Mubashir *et al.*, (2021), *E. coli* has a high level of resistance to CTX (74.9 %), CAZ (74.6 %), cefoxitin (FOX) (70.1 %), and augmentin (63.2 %). *E. coli* showed lower resistance rates to IMP (7.4 %), FOS (8.9 %), AK (13.2 %), and nF (25.9 %). In their study, Sohail *et al.*, *E. coli* resistance to CTX (72 %), CAZ (71 %) and amoxicillin (84 %) was reported to be roughly comparable to that in our investigation. Similar to this, Sohail *et al.*, (2015) found reduced *E. coli* resistance to IMP (3 %), FOS (10 %). In contrast, considerable *E. coli* AK resistance (91 %) was noted in a research by Muhammad *et al.*, in 2020. According to research carried out by Bashir *et al.*, in Pakistan in 2008, *E. coli* showed resistance to the following antibiotics: AMP (92 %), SXT (80 %), CIP (62 %), CN (47 %), nF (20 %), and AK (4 %).

The discrepancies in bacterial resistance rates between studies can be related to a variety of variables, including the research population and regional disparities. The patient's indiscriminate use of antibiotics without seeking medical advice is one of the factors contributing to the emergence of bacterial resistance to antibiotics. This causes mutant strains to arise, which improves their capacity to block the entry of medicines. Numerous research have revealed that the majority of urinary tract pathogens have changed over time and throughout numerous nations (Gupta *et al.*, 2011; Livermore *et al.*, 2007; Lomazzi *et al.*, 2019; Magliano *et al.*, 2012).

2.3 *P. aeruginosa*

P. aeruginosa is a Gram-negative, aerobic rod-shaped bacterium that is a member of the -proteobacteria and of the bacterial family Pseudomonadaceae. One of the 12 subtypes in the group includes *P. aeruginosa* (Todar, 2008). The pathogen is an organism that lives freely in many planktonic environments. This bacterium, which may also build biofilm, is in-charge of 10–20 % of hospital infections (Fazeli *et al.*, 2012).

One of the most clinically and epidemiologically significant microorganisms is *P. aeruginosa.* According to Kolleff (2013), it is the primary cause of opportunistic infections in immunocompromised individuals as well as the leading cause of nosocomial infections among non-fermenting Gram-negative bacilli. A MDR opportunistic pathogen, *P. aeruginosa* can infect

immunocompromised people with COPD, cystic fibrosis, cancer, injuries, burns, sepsis, and ventilator-associated pneumonia (VAP), including those with COVID-19 (Rossi E, *et al.,* 2021, Jurado-Martin *et al.,* 2021.; Cendra & Torrents, 2021*).* In biofilm states, *P. aeruginosa* can endure hypoxic conditions as well as other hostile environments (Sinha, M., *et al.,* 2021; Tang, P., *et al.,* 2021). Treatments for *P. aeruginosa* infections are also very challenging because of the bacteria's fast mutations and adaptation to develop antibiotic resistance. Additionally, due to its propensity to flourish on moist surfaces, *P. aeruginosa* is one of the top organisms responsible for hospital acquired infections and is frequently detected in medical equipment (ventilation) (Jangra, 2022)

Due of its considerable metabolic flexibility, *P. aeruginosa* may flourish in a wide variety of habitats (Silby *et al.,* 2011). Additionally, it is a very effective opportunistic pathogen that may cause both acute and chronic infections (Winstanley *et al.,* 2016). It is well acknowledged that *P. aeruginosa* is a pervasive opportunistic pathogen prevalent in the environment, despite the fact that transmission mechanisms are still difficult to determine.

It is nonetheless subject to ongoing selection pressure in hospital settings (Zarychanski, 2009) and is one of the primary etiological causes of nosocomial infections in Brazil (Boucher and Crey, 2008; Tavares, 2000).

The *P. aeruginosa* has developed a number of defence mechanisms to withstand antimicrobial effects. The most significant -lactam resistance mechanism involves the synthesis of β-lactamase enzymes; however, resistance can also result from overexpression of β-lactam efflux systems, changes in membrane permeability, and the synthesis of PBPs with low β-lactam affinities. All of these pathways may coexist in *P. aeruginosa* or may exist separately (Antonio *et al.,* 2019).

According to the US CDCP's estimates, *P. aeruginosa* infections caused 32,600 hospital admissions and 2700 fatalities in the USA in 2019 (CDC, 2019). According to Cassini *et al.,* (2019), from 2007 to 2015, there were on average 72,000 infections with antibiotic-resistant *P. aeruginosa* and 4155 fatalities per million individuals in Europe. The number of these infections increased by around three times over this time.

2.3.1 *P. aeruginosa* antibiotic resistance bacterial infections

P. aeruginosa reportedly demonstrates resistance to a variety of antibiotics, including aminoglycosides, quinolones, and β-lactams (Hancock and Speert, 2000). Intrinsic, acquired, and

adaptive resistance are the three primary types of defence used by *P. aeruginosa* against antibiotic attack. *P. aeruginosa's* intrinsic resistance consists of several different mechanisms, including low outer membrane permeability, the evolution of efflux pumps that expel medicines from cells, and the production of enzymes that render antibiotics inactive. Resistance in *P. aeruginosa* might arise through mutations or via the horizontal transfer of resistance genes (Breidenstein *et al.*, 2011). *P. aeruginosa* creates a biofilm in the lungs of infected people that serves as a diffusion barrier to shield the bacteria from drug exposure (Drenkard, 2003).

According to epidemiological research, bacterial infections caused by resistant to antibiotics claimed the lives of up to 700,000 people annually. According to Botelho *et al.*, (2019), 12.9 % of *P. aeruginosa* isolated from European samples have combination resistance. *P. aeruginosa*-related hospital infections continue to result in antibiotic resistance, which is a significant healthcare issue (Haque *et al.*, 2018). *E. coli* and *P. aeruginosa* are the most frequent causes of hospital-acquired infections in Spain, according to the 2016 EPINE study, with *P. aeruginosa* accounting for 10.5 % of clinically isolated bacterial infections (Lopez-Calleja *et al.*, 2011).

P. aeruginosa, the fourth most prevalent pathogen in hospitals in Europe, caused over 9 % of infections, according to the 2011-2012 Health Care Acquired Infections (HCAIs) report. *P. aeruginosa* is also responsible for 7.1 % of HCAI in the US (McCarthy and Paterson, 2017). Additionally, the 2016 European Center for Disease Prevention and Control (ECDC) epidemiological research revealed that *P. aeruginosa* is a common cause of pneumonia flare-ups, urinary tract infections, and bloodstream infections in ICUs and hospitals (Botelho, *et al.*, 2019; Haque *et al.*, 2018; Feng *et al.*, 2017; Xin, *et al.*, 2018). Additionally, data from the China Antimicrobial Surveillance Network (CHINET) revealed 301,917 clinically isolated pathogenic strains, and it was discovered that *P. aeruginosa* ranked fourth among nosocomial infections, accounting for 7.96 %, behind *E. coli*, *K. pneumoniae*, and *S. aureus* which shows much *P. aeruginosa* poses a serious hazard to human health on a worldwide scale, not just locally (CHINET, 2022)

Mubashir *et al.*, (2021) reported that FOX and nF were shown to be completely ineffective against Pseudomonas species. AK and FOS were both 100 % and 80 % sensitive, however there was high resistance to augmentin (60 %), IMP (60 %), CTX (40 %) and CAZ (40 %) (Mubashir *et al.*, 2021). The effectiveness of all other antibiotics was not examined in the study by Sohail *et al.*, (2015),

but they found a lesser resistance to amikacin. According to research done in Khyber Pakhtun Khuwa, 8.3 % of patients showed resistance to AK, 25 % to augmentin, 66.7 % to CTX, 75.0 % to CAZ, 58.3 % to FOS, 41.7 % to IMP, and 100 % to Nf (Muhammad *et al.*, 2020).

The goal of another study conducted in Iran was to investigate the pattern of antibiotic resistance of *P. aeruginosa* isolated from 172 instances of superficial infections reported to Iranian hospitals' emergency departments. The greatest rates of resistance to AMP (93 %), CN (89.5 %), CIP (82.5 %), and AK (77.3 %) were seen in aeruginosa strains. MEM (2.3 %), IMP (2.9 %), polymyxin B (21.5 %), and SXT (31.9 %) were the medications with the highest efficacy rates (Ahmadi *et al.*, 2016)

According to a study from China, *P. aeruginosa* was extremely resistant to conventional antibiotics (57.7 %–100 %) but was vulnerable to polymyxin B. For this investigation, blood samples were taken from 162 badly burnt patients with bloodstream infections who were admitted to burn ICU between January 2011 and December 2014 (including 120 patients with extremely severe burns) (Gong *et al.*, 2016).

P. aeruginosa infections in a University Hospital, Malatya, Turkey were investigated in 80 inpatients. Resistance to the antibiotics tested was in the 12 % to 88 % range; amikacin was the most effective and CRO was the least effective antibiotic (Yetkin *et al.*, 2006)

In total, 52.2 % (n = 60/115) of *P. aeruginosa* isolates from nosocomial infections in southwest Iran were carbapenem resistant; of these, 95.0 %, 55.0 %, and 5.0 % were multidrug-resistant, severely drug-resistant, and pandrug-resistant, respectively (Heidari *et al.*, 2022)

2.4 OmpF porin of *E. coli*

E. coli has the two most important porin proteins named OmpC and OmpF, which play a vital role in transportation of small molecules, toxic salts, antibiotics, and nutrients. OmpF porin is an important membrane protein of *E. coli*, as it has some immunogenic properties in it. It plays a pivotal function in the pathogenesis of bacteria which includes serum resistance, invasion, and adherence (Hejair *et al.*, 2017). OmpF comprises of a barrel formed by 16 antiparallel β-strands placed in membrane and exhibits the surface antigen's eight domains on the N-terminal of extracellular domain (Wang *et al.*, 2017).

Studies showed that OmpF is the major porin protein of *E. coli* because of its immunogenic properties and provides 87.5 % immunity against the virulent strain of *E. coli* i.e. *E. coli* PCN033 (Liu *et al.*, 2012). Studies revealed that the complete loss of OmpF or mutation in active residues of OmpF porin of *E. coli* resulted in alteration of antibiotic susceptibility or resistance will be developed against antibiotics (Ziervogel & Roux, 2013). Many studies revealed that due to depletion of porin proteins i.e., OmpF and OmpC, resulted in the instability and losses the configuration of outer membrane of *E. coli* which ultimately reduces the permeability of membrane triggering the resistance against antibiotics (Kim *et al.*, 2020)

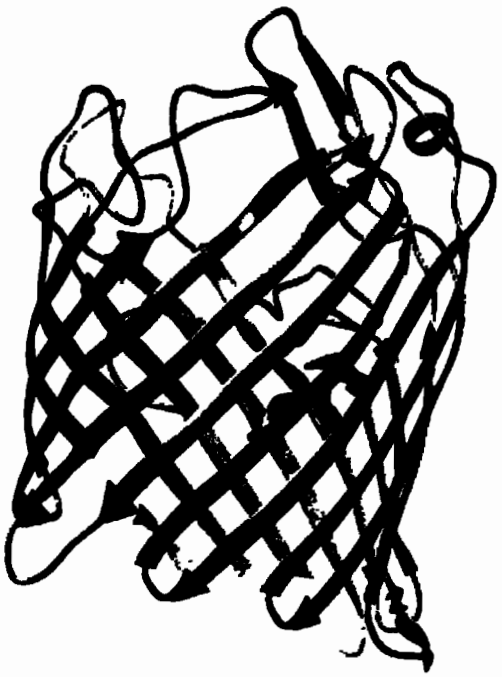

Figure 2.1 Structure of OmpF

Outer membrane protein F (OmpF) is a matrix protein of *E. coli* (Cowan *et al.*, 1995). It is functional in the trimeric form. Each monomer is comprised of 16 antiparallel β-barrel sheets. The 3-D structure of OmpF porin was resolved at 2.4Å through X-ray diffraction (Lou *et al.*, 1996). The molecular weight of monomeric OmpF is 37kDa. The mature part of protein is composed of 340 amino acids that form channels for the diffusion of small hydrophilic substances. Periplasmic side contains eight small β-hairpins while extracellular side comprises of eight large arbitrary loops i.e., L1 to L8 that connects the β-sheets. L2 is associated with the interaction between monomers

(Cowan *et al.*, 1995). L3 is the longest loop with 33 amino acid residues and limits its accessibility by folding itself internally in the porin tightening the pore size (Kefala *et al.*, 2010).

Some studies revealed the immunogenic properties of OmpF porin against *E. coli* PCN033 virulent strain (Liu *et al.*, 2012). Studies revealed the occurrence of resistance in *E. coli* due to complete depletion or mutation in the active sites of OmpF, leads to structural instability and reduced permeability of outer membrane, triggers the antibiotic resistance (Kim *et al.*, 2020).

Various studies showed that resistance mechanism of OmpF gene is regulated by the micF gene. It is a stress response gene which regulates the expression of OmpF gene. A 93 nucleotide non-translated antisense RNA is encoded by micF gene. This antisense RNA binds to OmpF mRNA and controls the expression of OmpF by degrading the message and inhibiting the translation. If the expression of micF increases, the expression of OmpF gene decreases, which ultimately induces resistance in *E. coli* (Delihas & Forst, 2001).

2.5 OmpW of *E. coli*

OmpW is a common Gram-negative bacteria outer membrane protein with an 8-stranded β -barrel structure and a long and narrow hydrophobic channel (Figure 2.2). Although OmpW has been found in several different bacterial species lately (Albrecht *et al.*, 2006; Hong *et al.*, 2006), its biological role is still mostly unknown. OmpW, however, may have a role in defending bacteria against a variety of environmental stresses, including osmosis (Xu *et al.*, 2005), oxidation (Gil *et al.*, 2007), temperature, and the lack of nutrients and oxygen (Nandi *et al.*, 2005). This has been widely shown in earlier research. A link between the presence of OmpW in cells and bacterial resistance to drugs including ampicillin, tetracycline, and CRO has also been shown by proteomic techniques (Goel and Jiang, 2010). That's the main reason that some antimicrobials are less effective at preventing bacterial growth because of OmpW's function in the transfer of minute hydrophobic compounds (Hong *et al.*, 2006).

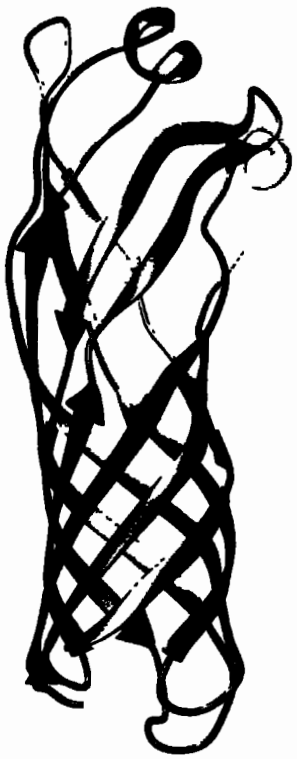

Figure 2.2 Structure of OmpW

OmpW has been found to be increased in NA-resistant *E. coli* strains (Li *et al.*, 2008). OmpW is downregulated in kanamycin-resistant *E. coli* K-12 strains, colistin/carbapenem-resistant *A. baumannii* mutants, and ceftriaxone-resistant *S. typhimurium* strains, however, which is consistent with the idea that porins limit the entry of β-lactams into cells (Hong *et al.*, 2006; Zhnag *et al.*, 2020). OmpW in *E. coli* has been linked by proteomic study to bacterial resistance to medications including ampicillin, tetracycline, and CRO (Wu *et al.*, 2013). Additionally, *E. coli* defends itself against enrofloxacin by decreasing OmpW expression and limiting the drug's transport and intracellular concentration (Piras *et al.*, 2015). Thus, it was shown that *A. baumannii's* OmpW expression was noticeably decreased when subjected to tobramycin stress (Kashyap *et al.*, 2022). OmpW expression changed in *A. baumannii* isolates in response to CIP and IMP, respectively (Gurpinar *et al.*, 2022).

The OmpW gene was knocked out, which also showed that numerous bacteria have antimicrobial resistance to OmpW. The resistance of *E. coli* mutants missing the OmpW to colicin S4, chlortetracycline, neomycin, and AMP suggests that OmpW is the receptor or a component of the receptor for these medications (Pisl *et al.*, 1999; Lin *et al.*, 2010; Wu *et al.*, 2012). OmpW of S.

typhimurium is 2.5 times more sensitive to methyl viologen than the wild type (Gil *et al.*, 2007). Penicillin, kanamycin, and polymyxin B bacterial sensitivity are all impacted by *Actinobacillus pleuropneumoniae's* deletion of OmpW (Chen *et al.*, 2022). In the meanwhile, the *E. coli* OmpW is mechanistically linked to EmrE and participates in the substrate efflux process mediated by ethidium multidrug resistance gene E (emrE) (Pushpker *et al.*, 2022).

A transcriptional SoxS factor associated with oxidative stress was also shown to be adversely regulating OmpW in *E. coli* (Zhang *et al.*, 2020). A phosphotransferase from EnvZ's His243 to OmpR's Asp55 increases the cellular amount of phosphorylated OmpR (OmpR-P) when certain environmental signals are recognized by EnvZ, indicating that EnvZ and OmpR are in an active state. While OmpR directly inhibits OmpW in Salmonella enteritidis, this condition alters the OMP composition and causes differential expression of OmpW and other OMP genes, boosting resistance to β-lactams (Ko, 2022).

2.6 OmpC of *E. coli*

OmpC is made up of negatively charged, 16-stranded beta barrels that help form the porin's eyelet and support its size-exclusion and permeability features (Delcour, 2009). According to research by Chang *et al.*, (2018) and Zhang *et al.*, (2018), OmpC increases the entrance and resistance of antibiotics such β-lactams as well as the transport of hydrophilic molecules with low molecular weight through the outer membrane. OmpC's mutation changes OM permeability and impairs structural integrity (Nikaido, 2003).

According to Lou *et al.*, (2011) OmpC is involved in the transmission of antibiotics. In clinical isolates of Enterobacter aerogenes and Enterobacter cloacae, the deletion or reduction of OmpC is associated with a modest increase in imipenem MIC (Lavigne *et al.*, 2012; Babouee *et al.*, 2016). All carbapenem non-susceptible (CP-NS) *E. coli* isolates had reduced OmpC transcription and protein expression, and one isolate's carbapenem susceptibility was recovered by cloning the OmpC gene (Liu *et al.*, 2008). Clinical isolates of multidrug-resistant *E. coli* exhibit increased CTX resistance due to OmpC mutations (Lou *et al.*, 2011). The OmpC deletion mutant of *E. coli*, however, was susceptible to carbapenems, cefepime, carbapenems, fourth-generation cephalosporins, imipenem, vancomycin, and puromycin (Choi and Lee 2019, Liu *et al.*, 2012, Hakkinen *et al.*, 2020). It was also resistant to streptomycin, fusidic acid, and nitrofurantoin.

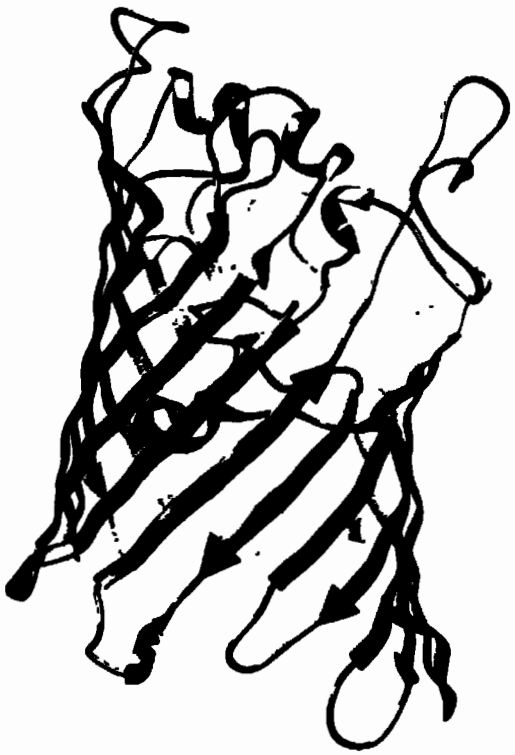

Figure 2.3 Structure of OmpC

Mutations in OmpC were one of the main causes of increased MICs for certain antibiotics. According to mutation prediction, amino acid changes like D192G in the OmpC of carbapenem-resistant *E. coli* are the main cause of carbapenem resistance (Tian *et al.*, 2020). The OmpC protein in *E. coli* also has a duplication of eight amino acids in a nearby location. This duplication may be used to build salt bridges with the negatively charged residues lining the other side of the barrel wall and alter the pore's electrostatic field based on certain R66, L67, Y64, F69, M65, and L67 positions (Hakkinen *et al.*, 2020). The disruption of tertiary or quaternary structure, the prevention of phage attachment and sterically limiting molecule movement, the change of the hydrophobic nature and charge of porins by limiting their interaction with compounds, such as antibiotics, to promote transport, and the disruption of phage attachment are additional factors that can be used to demonstrate how the identified insertion impacted OmpC function (Hakkinen *et al.*, 2020). According to studies using quantitative real-time reverse transcription PCR (RT-qPCR) in avian pathogenic *E. coli*, EnvZ, the histidine kinase (HK) of OmpR/EnvZ, may have an impact on the expression of OmpC and other stress response and biofilm-related genes. Cells can react fast and effectively to various growth situations thanks to small regulatory RNA (sRNA)-dependent

modulation of gene expression (Jorgensen *et al.*, 2020). In the meanwhile, it was shown that the 109-nucleotide MicC sRNA inhibits OmpC production in *E. coli* by directly base-pairing to an OmpC mRNA 5′ untranslated region, which is dependent on the Hfq RNA chaperone for activity (Chen *et al.*, 2004).

2.7 OprD of *P. aeruginosa*

P. aeruginosa is one of the most prevalent triggers of nosocomial infections and is frequently linked to lung infections in cystic fibrosis patients (Lyczak *et al.*, 2000). Due in great part to the poor permeability of its outer membrane, it exhibits a startlingly high inherent resistance to conventional antibiotics (Hancock, 1998; Breidenstein *et al.*, 2011). Drug efflux pumps and -lactamase are two examples of more effective secondary resistance mechanisms that might be introduced as a result of the decreased antibiotic inflow. *P. aeruginosa*, unlike Enterobacteria, uses a variety of substrate-specific channels for nutrient absorption rather than expressing universal porins like OmpF (Chevalier *et al.*, 2017). Although these channels have smaller pores than porins, substrate binding occurs with higher affinities because of their distinctive binding sites, which is helpful in the conditions where these bacteria are located when there is a lack of nutrients. According to (Eren *et al.*, 2012), proteins from the outer membrane carboxylate channels (Occ) family, which is further subdivided into the OccD and OccK subfamilies, are responsible for importing the bulk of small molecules. While the latter favours cyclic molecules, the former carries basic amino acids. OprD (also known as OccD1), the prototype member of the OccD subfamily, is of tremendous pharmacological importance since it not only mediates the absorption of basic amino acids but also permits the passage of carbapenem β-lactam antibiotics (Trias and Nikaido, 1990, Shen *et al.*, 2015). Recent whole cell-based assays have demonstrated the significance of comprehending the molecular processes underlying substrate recognition and penetration by showing that even small alterations to the chemical makeup of new carbapenem analogues can have substantial impacts on OprD-mediated uptake (Iyer *et al.*, 2017).

By using X-ray crystallography, the structure of *P. aeruginosa* OprD has been determined (Biswas *et al.*, 2007; Eren *et al.*, 2012; Eren *et al.*, 2013). It is an 18-stranded monomeric protein having numerous large exterior loops, two of which, L3 and L7, fold into the barrel to create a tight constriction. The substrate permeation route has an asymmetric charge distribution due to the

presence of a line of positive charges (also known as a "basic ladder") produced by five arginine and one lysine residues on one side of the pore and a largely negatively charged side on the other.

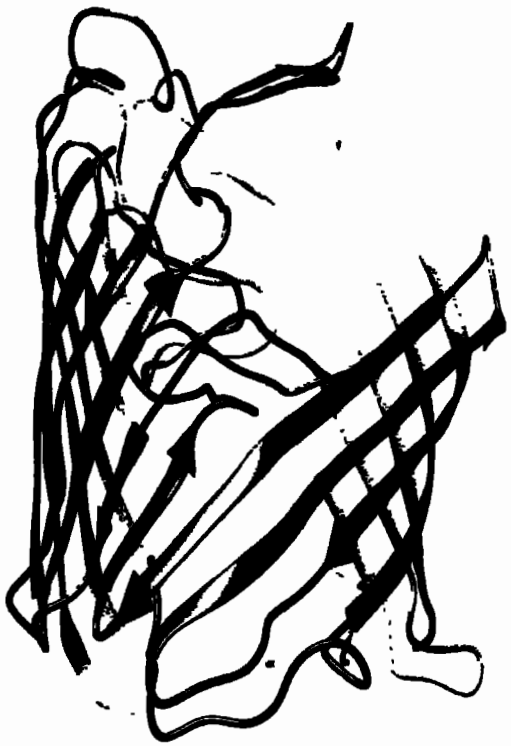

Figure 2.4 Structure of P. aeruginosa OprD

Due to its role in the entrance of carbapenem antibiotics, particularly IMP and MEM, OprD represents the second main porin protein in the outer membrane of *P. aeruginosa* and is one of the most researched (Trias and Nikaido, 1990; Quinn *et al.*, 1991; Yoneyama *et al.*, 1995). Analysis of imipenem-resistant mutants that failed to produce any measurable amounts of the porin as seen by western blot analysis suggested that OprD functions as the gate via which IMP enters the *P. aeruginosa* cell (Yoneyama and Nakae 1993; Wolter *et al.*,2004).

In 2016, Kos *et al.*, discovered that an in-frame deletion within the OprD (occD1) gene resulted in the expression of a smaller protein, while the ability to transport arginine was maintained, leading to carbapenem resistance. However, this could be because a second porin (OpdP/OccD3) is involved in the uptake of arginine. Despite the fact that the porin can bind both MEM and IMP, mutants lacking OprD were solely resistant to IMP while remaining susceptible to MEM, indicating the existence of a different uptake pathway for this particular drug (Perez *et al.*, 1996).

At all three-time intervals examined, OprD was the protein exhibiting the largest over-representation in OMVs from biofilm samples in comparison to the bacterial outer membrane (Park *et al.,* 2015). Since OprD (OccD1) was not notably overrepresented in planktonic OMVs, this overrepresentation was unique to the biofilm lifestyle. According to a theory put up by Park *et al.,* (2015), *P. aeruginosa* may be able to decrease the amounts of porin in its outer membrane by packing OprD (OccD1) into biofilm OMVs. This would decrease the bacteria's susceptibility to carbapenem. By adsorbing these antimicrobial peptides in *E. coli*, OMVs have been demonstrated to shield bacteria against polymyxin B and colistin (Manning and Kuehn 2011). Similar theories include the idea that high OprD levels in OMVs cause the outer membrane's OprD to become depleted, allowing carbapenem to accumulate inside OMVs and reducing the amount of free carbapenem in bacterial cells.

2.8 OpdK of *P. aeruginosa*

The pathogenic bacterium, *P. aeruginosa* has the protein OpdK in its outer membrane. The monomeric, 18-stranded -barrel with a kidney-shaped pore and a constriction with a diameter of 8 Å was discovered in the protein's most recent crystal structure (Figure 2.5).

Figure 2.5 Overall Structure of OpdK.

The OprD outer membrane (OM) protein family is used by the Gram-negative bacteria *P. aeruginosa* for the absorption of several tiny, hydrophilic substances essential for cell development and function. OpdK, a member of the OprD family, may be necessary for the assimilation of vanillate and other aromatic acids (Hancock and Brinkman, 2002; Hancock and Tamber, 2004).

Although, the OprD protein's overall structure and that of OpdK are extremely similar (Biswas *et al.*, 2008; Biswas *et al.*, 2007), there is a notable difference between these two channels in the conformation of the pore-restricting loop L3. The side chain to side chain distance reveals that this loop's conformation in OpdK results in a constriction that is significantly broader than that in OprD (the narrowest diameter is 8.0 Å in OpdK against 5.5 Å in OprD).

Chapter 3

Material and Methodology

3 Material and methodology

3.1 Samples collection

A total of 51 isolates of *P. aeruginosa* and *E. coli* were collected from well-known health care bodies of all four provinces of Pakistan as clinical isolates. These isolates were collected in sterile nutrient agar plates and transferred to the laboratory of SA-Centre for Interdisciplinary Research of Basic Sciences (SA-CIRBS), of International Islamic University Islamabad, Pakistan. All samples were sub-cultured and stored at 4 ^{0}C.

The samples were initially labelled according to the location of isolation (Table 3.1). These samples were renamed later to the simplest numbering format for easier handling. Sampling was conducted from July 2021 to March 2022.

Table 3.1 ***Number of E. coli and P. aeruginosa isolates collected from different provinces of Pakistan***

Province	No of *E. coli* isolates	No of *P. aeruginosa* isolates
Balochistan	3	4
Khyber Pakhtunkhwa (KPK)	6	5
Punjab/ICT	10	10
Sindh	7	6
Total	**26**	**25**

3.2 Preparation of media

Three types of media were used to perform this research:

1. Nutrient agar
2. Muller Hinton agar
3. Nutrient Broth

3.2.1 Nutrient agar

Nutrient agar media prepared in 1000 ml of media bottle. 6.5 g powder of nutrient broth and 5 g powder of agar were added in media bottle. Final volume of media bottle was made 1000 ml by adding distilled water with the help of graduated cylinder.

The media bottle lid shouldn't be tightly closed. The medium was autoclaved for 20 minutes at a pressure of 15 psi and a temperature of 120 °C. To assure sterility, the autoclaved medium was added to petri plates that had already been sterilized. The medium was allowed to cool to room temperature and harden. *P. aeruginosa* and *E. coli* were cultured in the medium.

3.2.2 Muller Hinton agar (MHA)

Muller Hinton agar was used as growth media for antibiotic susceptibility testing. It was prepared by mixing 21 g of MHA powder in the media bottle of 1000 ml in which 100 ml distilled water was added for dissolution purposes and then it was filled with water upto 1000 ml but in some cases, it was noticed that the dissolution of media was not so good even by vigorous shaking, so to overcome this problem magnetic stirrer was used. Before the autoclaving lid of the media bottle was not sealed tightly and it was autoclaved for 20 minutes at 120^0 C. Media was cooled down and in various no of petri dishes, it was poured, solidified and stored in the sterile place.

3.2.3 Nutrient broth media

Nutrient broth medium (brand) was prepared in conical flask containing 1000 ml of distilled water. The flask was tightly closed with aluminum foil and shaken thoroughly to dissolve the components of the media. The broth was autoclaved on 120 °C for 20 minutes at 15 psi pressure. The broth was poured into sterilized test tubes under sterilized environment in laminar flow hood.

3.2.4 Culturing of clinical isolates

A sterile inoculation loop was used to transfer the bacterial inoculum from clinical petri plates to nutritional agar plates. For the purpose of isolating pure cultures, the standard streaking approach was used. The culture was kept in an incubator at 37 °C for 18-24 hours.

For DNA extraction process, already prepared nutrient broth media was used. From this media,5 ml of broth was put in each 15 ml Test tube. With the help of a sterile toothpick, some bacteria cells were picked from the culture and put in the test tube. Another clean toothpick was put in

another test tube as a negative control. These test tubes were placed in a shaking incubator. Bacterial cells were incubated in a shaking incubator overnight at 37 °C for 120rpm. An optimum condition such as aerobic environments, sterile conditions, and 37 °C temperature is necessary for maximum growth of bacterial cells. After 18-24 hours of incubation, the culture was used for DNA extraction process.

3.3 Preparation of McFarland standard

The McFarland standard is used to visually compare the turbidity of the bacterial density to the turbidity of the appropriate standard. For this 85 ml of 1 % sulfuric acid was added to a 100 ml conical flask. Using a micropipette, 0.5 ml (500 l) of 1.175 % barium chloride was gradually added to 1 % sulfuric acid. The conical flask's capacity was raised to 100 ml by adding an additional 1 % sulfuric acid. After being agitated with a magnetic stirrer for three to five minutes, the solution appeared uniform and clump-free. The homogeneous solution was added and then put into sterile screw-cap tubes.

At a wavelength of 625 nm, the optical density of the mixture was determined using a spectrophotometer (Hach, Model: DR/2400). 0.087 was reported as the optical density value, which is within the 0.08 to 0.10 range permitted by the 0.5 McFarland standard. The created standard was stored in the dark and at room temperature.

3.4 Antibiotic Susceptibility test

Antimicrobial susceptibility is a tool for evaluating an antibiotic's effectiveness and its capacity to prevent bacterial growth. To determine if the bacteria is susceptible or resistant to different antibiotics, the Kirby Bauer disc diffusion method of antibiotic susceptibility was used on Muller Hinton agar.

3.4.1 Methodology

With a sterile inoculation loop, a few bacterial colonies were selected from a Nutrient Agar plate. In sterile vials filled with normal saline, the colonies were suspended. Every saline vial was vortexed to combine it. In each vial, a uniform bacterial suspension was produced. Using a black and white card as a background, the turbidity of the suspension in each vial was adjusted to 0.5 McFarland standards. Within 15 minutes, the prepared suspension was employed.

The bacterial suspension tube was dipped into using a sterilized cotton swab. The cotton swab was rotated and softly pressed against the test tube's side above the fluid level to remove any surplus fluid.

By streaking the swab across the whole surface of the MHA plate three times, the surface was infected. The plate was turned about 60 degrees every time to make sure that the inoculum was distributed evenly across the MHA surface.

The agar media's surface was left to dry for 2-3 minutes. Antibiotic discs were extracted from the cartridge using sterile forceps. All of the discs were positioned on one of the designated spots when the MHA plate's lid was partially removed. To establish perfect contact of the discs with the MHA agar surface, the forceps were used to gently push the discs. To reduce the exposure of the agar media to airborne pollutants, the lid was put back on after each disc was placed. All plate lids were closed once all discs had been positioned in their designated positions. All of these MHA plates with discs were incubated for 22–24 hours at 37 °C in an inverted posture. Each plate's incubation result was recorded.

After 22 to 24 hours of incubation, bacterial growth around the antibiotic discs indicated that the bacterial strain was resistant to that antibiotic. The zone of inhibition (ZOI) is the region surrounding the antibiotic discs where no growth had occurred. While gazing at the back of the MHA plate with a ruler, the diameter of the ZOI was measured in millimeters (mm) above the center of the disc. When the zones of neighboring discs intersected, the zone diameter was computed by taking each zone's radius and multiplying it by two. Zone diameter findings were evaluated and contrasted with norms set by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2020). Therefore, isolates of *P. aeruginosa* and *E. coli* that were sensitive, resistant, and intermediate were assessed.

Table 3.2 ***Standard Antibiotics discs (Manufacturer: Oxoid Ltd, UK) used for susceptibility testing against E. coli***

S.No	Antibiotic	Symbol	Concentration
1	Amikacin	AK	30 µg
2	Aztreonam	ATM	30 µg
3	Amoxicillin + clavulanic acid	AMC	30 µg

4	Cefepime	FEP	30 µg
5	Imipenem	IPM	10 µg
6	Meropenem	MEM	10 µg
7	Sulbactam+Cefoperazone	SCF	105 µg
8	Doxycycline	DO	30 µg
9	Ciprofloxacin	CIP	5 µg
10	Levofloxacin	LEV	5 µg
11	Gentamicin	CN	10 µg
12	Ceftriaxone	CRO	30 µg
13	Ampicillin	AMP	10 µg
14	Cephradine	CE	30 µg

Table 3.3 ***Standard Antibiotics discs used for susceptibility testing against P. aeruginosa***

S.No	Antibiotic	Symbol	Concentration
1	Aztreonam	ATM	30 µg
2	Levofloxacin	LEV	5 µg
3	Amikacin	AK	30 µg
4	Doripenem	DOR	10 µg
5	Ticarcillin	TIC	75 µg
6	Pipra-Tazobactam	TZP	110 µg
7	Meropenem	MEM	10 µg
8	Cefepime	FEP	30 µg
9	Colistin	CN	10 µg
10	Imipenem	IPM	10 µg
11	Ceftazidime	CAZ	10 µg
12	Norfloxacin	NOR	30 µg
13	Ciprofloxacin	CIP	5 µg

3.5 DNA extraction

DNA was extracted using the ZymoBIOMICS™ DNA Microprep Kit by following the protocol which was given in the kit.

3.6 Confirmation of genomic DNA

The genomic DNA extracted from clinical isolates was confirmed by gel electrophoresis.

3.7 Gel electrophoresis

2 grams of agarose was dissolved in 100 ml of 1x Tris-Acetate EDTA (TAE) buffer and heated for two to three minutes in a microwave to make 2 % agarose gel. The horizontal gel tank (Major science, Model: ME10-7-10) was filled with a buffer solution containing dissolved agarose, and the mixture was left to harden for 30 minutes. For 60 minutes, the gel was operated at 100 volts. The gel was stained in 0.5 µg/ml ethidium bromide for 10 minutes and DNA bands were confirmed after the gel was observed under UV light in the gel documentation system (SYNGENE, Serial Number: SYDR/ 2138).

3.7.1 Preparation of Tris Acetate Buffer

Reagents which were used in the preparation of 1X Tris-acetate buffer are glacial acetic acid (1.2 ml/L), EDTA salt dihydrate (0.372 g/L), Tris base (4.84 g/L). First of all these basic components were completely dissolved in 300 ml of water and then the final volume was made up to 1000 ml. Sometimes EDTA was not homogeneously dissolved, so magnetic stirrer was used in such a case.

3.7.2 Preparation of agarose gel

2 g of agarose was mixed in 20 ml TAE buffer and final volume was made up to 100 ml for preparing 2 % agarose. In the conical flask the mixture of both gel and TAE was mixed. After mixing it was kept in the microwave oven for 2-3 minutes in such a way that its lid was not closed tightly. To overcome the evaporation and alteration in the concentration of agarose, this solution was prevented from overheating. At the end it was cooled down to 45 °C.

Well comb was fixed carefully as its reserved position in the casting tray and the pouring of agarose gel was done consciously. For the prevention of formation of bubbles, gel was poured very slowly into the casting tray. When gel was solidified, the casting tray was fixed in the gel box, comb was

removed, and tris acetate buffer was poured into the gel box so that the gel was completely covered with buffer.

3.7.3 Loading sample and running of agarose gel

4 µl genomic DNA was mixed with 1µl 6x loading dye loaded into agarose wells. The middle well was loaded with DNA ladder of specific size very carefully. The gel was run at 100 volts for 1 hour. As we know that DNA has negatively charge because of the presence of phosphate group and was travel toward positive electrode (red is positive and black is negative). From the gel box, solidified gel was removed very carefully and was placed in a mixture of 0.5 µg/ml ethidium bromide and TAE buffer, for 10 minutes for staining purposes. As the staining was completed the gel was removed from this box and was placed in the UV tray for visualizing the band of extracted DNA relative to the ladder DNA by using software known as image lab software.

Gel was visualized under UV light in gel documentation system BIO-RAD Gel Doc™ EZ Imager) and DNA bands were confirmed and visualized using Image Lab software.

3.8 Polymerase Chain Reaction technique (PCR)

Table 3.4 and 3.5 shows primers sequences used for detection of different OMPs. To design these primers SnapGene software was used. Any type of DNA primer must be highly specific to some basic parameters. Complementarity of PCR primer to the extracted DNA is very important. The importance of the size of the primer also an important role in primer designing. For the amplification of genomic DNA, primer with a simple fragment must be used. The size of the primer must be <30 base pairs so that it must not be very small or too long. Amplification will be inaccurate and non-specific if the length of the primer is too long.

The following properties should be possessed for obtaining better amplification products.

1. It has a length in the range of 18-24 bps

2. 40-60 % GC-content

3. The melting temperature difference of the PCR primer must be 5 °C.

Table 3.4 ***Primers sequence used for detection of different OMPs of E. coli***

Gene	Primer Sequence 5' → 3'	Tm (°C)	Product Size (bp)
OmpC	F: 5'-ATGAAAAAGTTAACAGTGGCGGCTTTG-3' R: 5'-TTAAAAACGATATCCTGCTGAGAACATAAACACC -3'	67.2 66.3	1128
OmpF	F: 5'-ATGATGAAGCGCAATATTCTGGCAGT-3' R: 5'-TTAGAACTGGTAAACGATACCCACAGCA-3'	61.3 63.0	1089
OmpW	F: 5'-ATGAAAAAGTTAACAGTGGCGGCTTTG-3' R: 5'-TTAAAAACGATATCCTGCTGAGAACATAAACACC -3'	61.5 61.3	639

Table 3.5 ***Primers sequence used for detection of different OMPs of P. aeruginosa***

Gene	Primer Sequence 5' → 3'	Tm (°C)	Product Size (bp)
OprD	F: ATGAAAGTGATGAAGTGGAGCGC R: TTACAGGATCGACAGCGGATAGTC	61.4 62.3	1332
OpdK	F: 5'-ATGAAAATCCGTCGTAAAACCGCA-3' R: 5'-TCACCACGACAACGGATAACTCAC-3'	60.8 63.4	1255

3.8.1 PCR reagents

PCR reaction mixture of 50 µl was prepared (Table 3.6) which contained: 10 µl Master Mix (SOLIS BIODYNE), 18 µl PCR grade water (**BIO BASIC WW 1002**), 10 µl of each primer (1µM) and 2 µl genomic DNA.

Table 3.6 ***PCR reagents with final quantity and concentration***

Components	Final concentration	Final volume
Matrix	1 X	10 µl
Reverse primer	1 µM	10 µl
Forward primer	1 µM	10 µl
DNA template	50-100 ng/µl	2 µl
PCR grade water	-	18 µl
Total volume (PCR)		**50 µl**

Table 3.7 ***Thermo Cycler conditions for PCR***

S.No	Steps	Temperature	Duration	Cycles
1	Initial denaturation	95 °C	3 minutes	1
2	Denaturation	95 °C	30 seconds	30
3	Primer annealing	63 °C	30 seconds	30
4	Elongation	62 °C	2 minutes	30
5	Final elongation	72 °C	7 minutes	1
6	-	10 °C	Infinite	1

3.8.2 Protocol

The reaction components were prepared as mentioned in table 3.5. A solution of all the reagents were prepared in the PCR tube which included master mix, primers, PCR grade water and DNA template. Final volume of all the solution of reagents were made upto 60 µl and for better mixing PCR tubes were tapped with the help of index finger. For the purpose of settling down all the reagents, PCR tubes were spun around for a short interval of time. In the separate tube negative control was also prepared which was composed of all the reagents except DNA templates. Amplification was done by using thermocycler. Table 3.6 represents PCR conditions for these genes. PCR products were analysed using agarose gel (2 %) and 100 bp DNA ladder was used as size marker.

3.8.3 Validation of Polymerase Chain Reaction

To validate PCR reaction, electrophoresis was performed. 5 µl of the each of final reactions were loaded on the 2 % agarose gel wells with a reference DNA ladder of appropriate concentration and size. The gel was run for 1 hour and was visualized under Gel Doc System. A band on the gel confirms successful amplification of the gene.

3.9 Purification of DNA

DNA samples were purified after PCR reactions. DNA purification kit (SolarBio Cat#D1300) was used to extract DNA. The purified samples were sent to Korea (Macrogen) for Sanger sequencing.

3.10 Insilico studies of OMPs of *P. aeruginosa* and *E. coli*

3.10.1 Expert Protein Analysis System (ExPASy)

Using the Expert Protein Analysis System (ExPASy) program, the gene sequence was converted into the protein sequence. Finding the code region was the major goal of using this program.

3.10.2 BLASTN

Basic Local Alignment Search Tool (BLAST) is an online sequence similarity search program that can be used to compare a user's query to a database of sequences (Altschul *et al.*, 1990). BLAST has different tools, but BLASTN is their most widespread tool used for nucleotide homology search. It compares new nucleotide sequences with sequences that have already been reported and stored in their database.

The sequences of porins of clinical isolates of *P. aeruginosa* and *E. coli* in FASTA format were compared with the database sequences of using nucleotide BLAST. The nucleotide BLAST was utilized to examine the identity or likeness between our sequences and other available sequences in the database. The topmost result was selected for each sample and a summarized table was formulated.

3.10.3 Clustal Omega

Clustal Omega, a bioinformatics technique, was used to examine the multiple sequence alignment of protein and nucleotide. It offered several sequence alignments of various, physiologically significant sequences. Clustal Omega was also utilized in this work to establish the amino acids in terms of whether their locations have changed. These generated amino acid sequences were later used in formation of 3D structures using Phyre2 platform.

3.10.4 Phyre2

Phyre2, an online platform, was used to predict the 3D structure of the desired proteins. The FASTA format of amino acid sequence was pasted in the available space and the result was generated and emailed after some time.

3.10.5 UCSF Chimera

It was downloaded and used to highlight the mutated regions of amino acids sequences on 3d protein structures as compared to wild type protein sequences.

Chapter 4

Results

4 Results

4.1 Antimicrobial susceptibility assay

13 frequently used antimicrobials were utilized to evaluate all isolates for antimicrobial drug resistance utilizing disc diffusion techniques. Zone inhibition sizes were assessed, and the CLSI guideline, 2020 was used to interpret the data. Figures 4.1 and 4.2 shows antibiotic susceptibility of a few isolates of *E. coli* and *P. aeruginosa*, respectively, with disc diffusion method. Antibiotic sensitivity data for induvial discs is given in table 4.1 and 4.2.

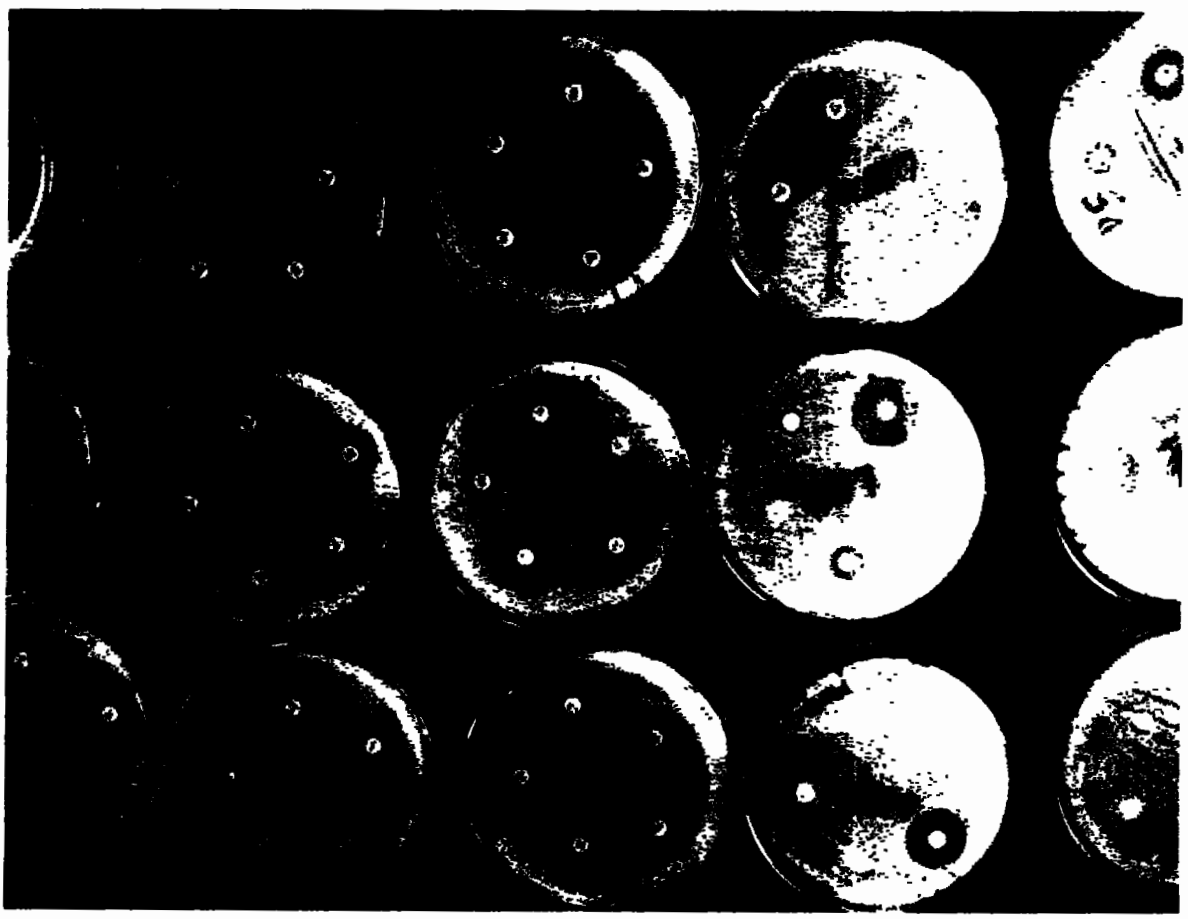

Figure 4.1 Antibiotic susceptibility tests against some clinical isolate of E. coli

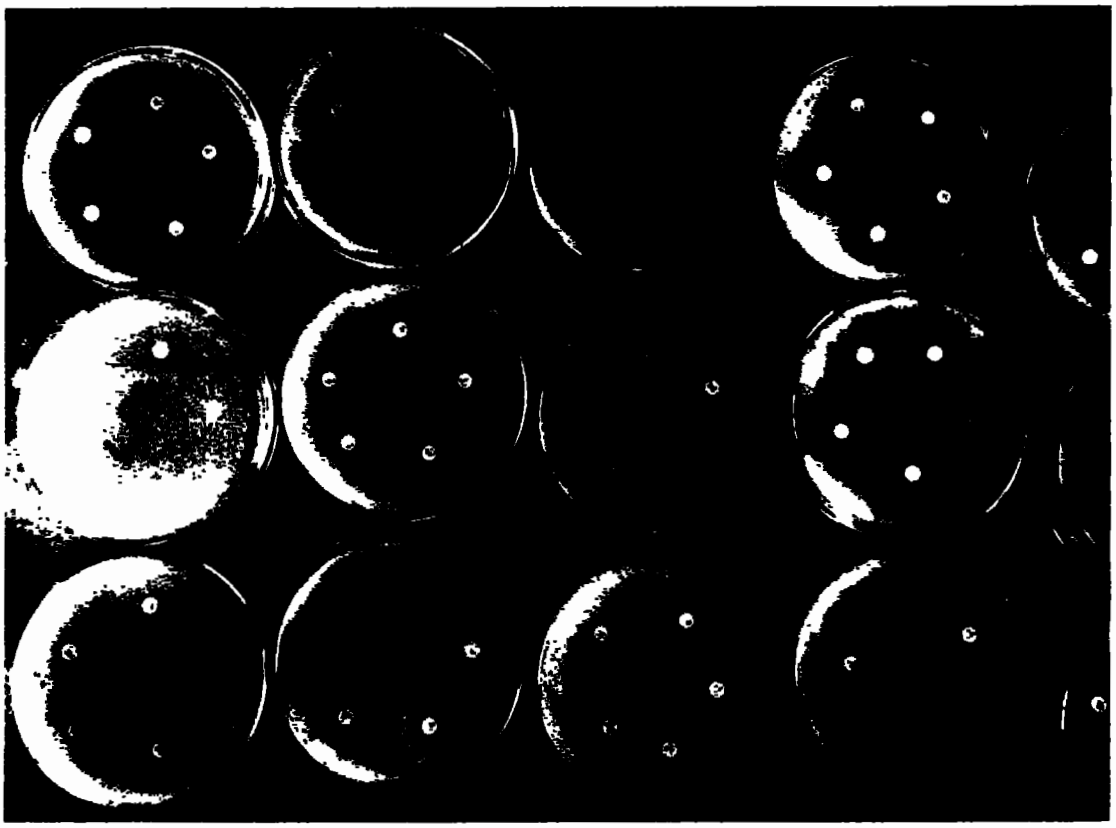

Figure 4.2 Antibiotic susceptibility tests against clinical isolate of P. aeruginosa.

Table 4.1 Antibiotic susceptibility of clinical isolates of E. coli against various antibiotics

Sample No	AK 30	ATM 30	AMC 30	FEP 30	IPM 10	MEM 10	SCF 105	DO 30	CIP 5	LEV 5	CN 10	CRO 30	AMP 10	CE 30
1	R	R	R	R	R	R	R	I	R	R	R	R	R	R
2	S	R	R	R	I	S	S	R	S	S	S	R	R	R
3	I	R	S	R	S	S	R	R	S	R	R	R	R	R
4	S	R	I	R	S	S	I	R	R	I	S	R	R	R
5	S	R	I	I	S	S	S	R	R	R	R	R	R	R
6	S	R	I	R	S	S	S	R	R	R	I	R	R	R
7	R	R	R	R	I	S	R	R	R	R	R	R	R	R
8	S	S	S	I	S	S	S	S	R	R	S	R	R	R
9	S	R	R	R	I	S	S	R	R	R	R	R	R	R
10	S	R	R	R	S	S	R	S	R	I	R	R	R	R
11	S	R	R	R	S	S	I	S	R	I	R	R	R	R
12	S	R	R	R	S	S	I	R	I	S	S	R	R	R
13	S	R	I	R	S	S	S	S	R	R	R	R	R	R
14	R	R	R	R	I	I	R	R	R	R	R	R	R	R
15	S	R	I	R	S	S	I	S	R	R	R	R	R	R

16	S	R	I	R	S	S	S	I	I	R	S	R	R	S
17	R	R	R	R	S	S	R	R	R	R	R	R	R	R
18	I	R	R	R	S	I	R	R	R	R	R	R	R	R
19	I	R	S	R	S	S	S	I	R	R	S	R	R	R
20	S	R	R	R	S	R	S	S	R	R	R	R	R	R
21	R	R	R	R	R	I	R	S	R	R	R	R	R	R
22	S	R	R	R	S	S	R	S	R	R	R	R	R	R
23	S	R	S	R	S	S	I	R	R	R	S	R	R	R
24	S	S	S	S	S	S	S	S	R	I	S	S	S	R
25	S	R	S	R	S	S	I	R	R	I	S	R	R	R
26	S	R	I	R	S	S	I	R	R	I	S	R	R	R

Table 4.2 Antibiotic susceptibility of clinical isolates of P. aeruginosa against various antibiotics.

Sample No	ATM 30	LEV 5	AK 30	DOR 10	TIC 75	TZP 110	MEM 10	FEP 30	CN 10	IPM 10	CAZ 30	NOR 10	CIP 5
1	S	S	S	S	S	S	S	S	S	S	S	R	S
2	R	S	S	S	R	I	S	S	S	S	R	R	R
3	I	R	S	S	R	S	S	R	S	S	S	R	R
4	R	R	S	S	R	R	S	I	S	S	R	R	R
5	R	S	I	S	R	S	S	S	S	S	S	S	R
6	R	R	R	R	R	R	R	R	R	R	R	R	R
7	R	R	R	R	R	R	R	R	I	R	R	R	R
8	R	R	R	R	R	R	R	R	R	R	R	R	R
9	I	R	R	R	R	R	R	R	R	R	S	R	R
10	I	R	R	S	R	S	R	S	R	R	R	R	R
11	I	R	R	R	R	S	R	I	R	R	R	R	R
12	R	S	R	R	R	S	R	I	S	R	I	S	R
13	I	R	R	R	R	I	R	R	R	R	R	R	R
14	S	S	S	S	S	S	S	S	S	S	S	S	S
15	S	S	S	S	S	S	S	S	S	S	S	S	S
16	R	R	I	R	R	R	R	I	R	R	S	R	R
17	R	R	R	R	R	R	R	R	R	R	R	R	R
18	R	R	S	S	I	I	S	I	R	S	R	R	R
19	R	S	S	S	I	S	S	R	S	S	R	I	R
20	R	S	S	S	R	S	S	R	S	S	R	I	R
21	R	S	S	S	I	I	S	R	R	S	R	R	R
22	S	S	S	S	S	S	S	S	S	S	S	S	S
23	S	S	S	S	S	S	S	S	S	S	S	S	S
24	S	S	S	S	S	S	S	S	S	S	S	S	S
25	S	S	S	S	I	S	S	S	S	S	S	S	S

The statistical analysis shows that Clinical Isolate No. 24 of *E. coli* samples is showing resistance against only 2 antibiotics (Figure 4.3). All others are showing resistance against at least 5 antibiotics.

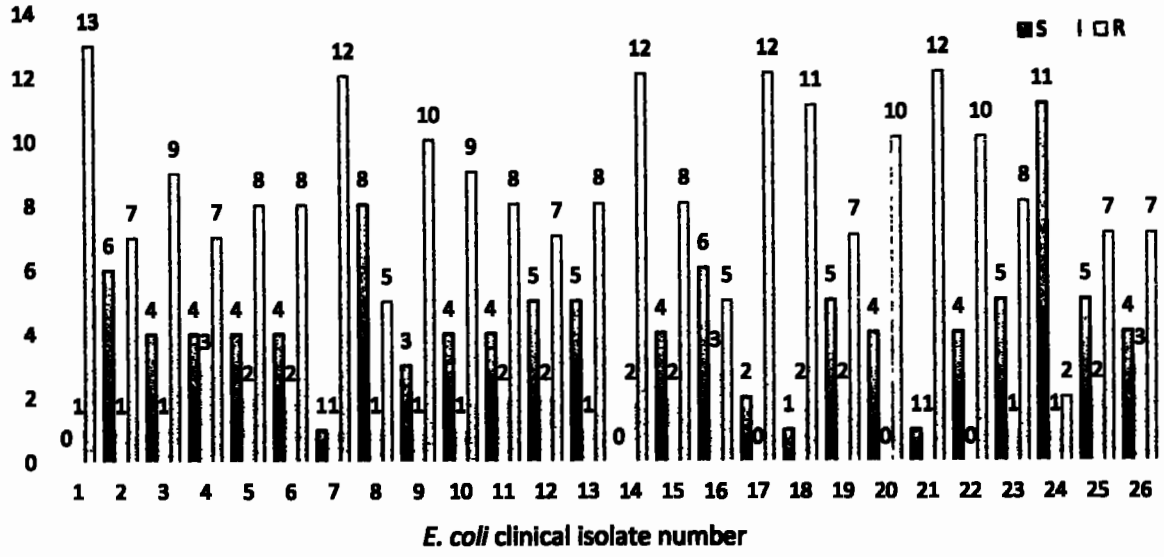

Figure 4.3 Antimicrobial susceptibility pattern for isolates of E. coli against 14 antibiotics.

While in *P. aeruginosa* species, the statistical analysis shows that Clinical Isolates No. 14, 15 and 22-25 are sensitive to all antibiotics as shown in figure 4.4. Sample No. 1 is showing resistance against only 1 antibiotic. All others are showing resistance against at least 3 antibiotics.

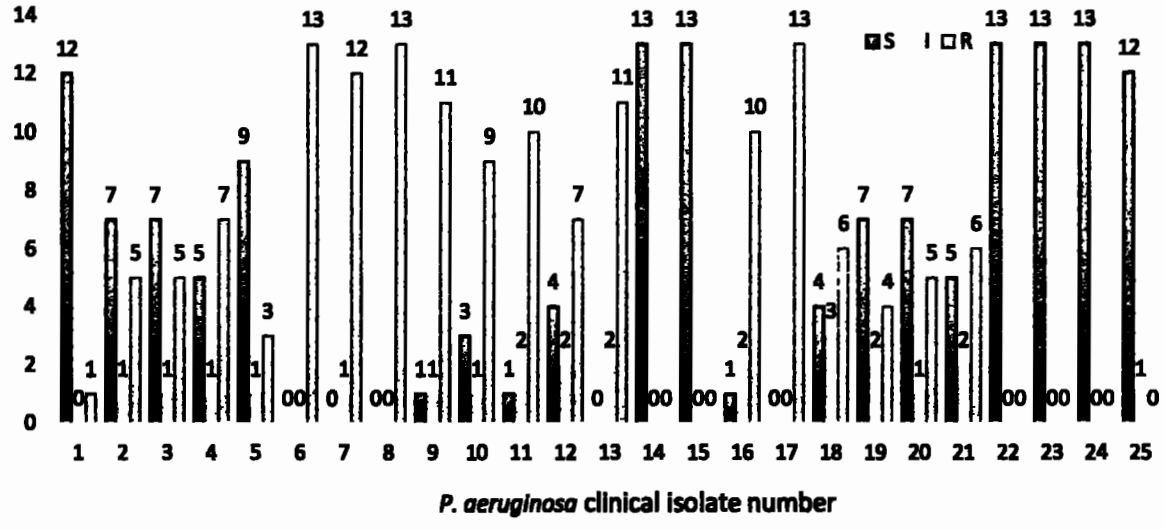

Figure 4.4 Antimicrobial susceptibility pattern for isolates of P. aeruginosa against 13 antibiotics.

The consolidated antibiotics results reveal that MEM 10, IPM 10 and AK30 were the most effective antibiotics against all these *E. coli* strains with 80.76 %, 76, 92 % and 69.23 % efficacy

rate, respectively (Figure 4.5). While ATM 30, FEP 30, CIP 5, CRO 30, AMP 10 and CE 30 had the lowest efficacy against *E. coli* samples.

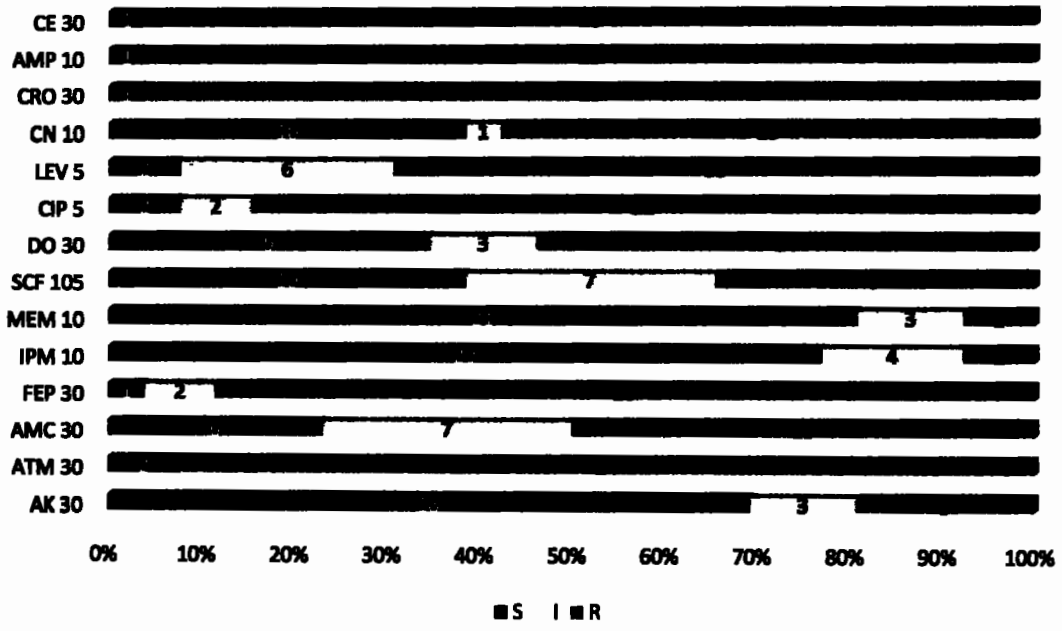

Figure 4.5 Antimicrobial susceptibility pattern for 26 isolates of E. coli against different antibiotics

The efficacy of antibiotics susceptibility results against *P. aeruginosa* showed that DOR 10 (64 %), MEM 10 (60), IPM 10 (60 %), AK30 (56 %), TZP 110 (56), CN 10 (56 %) and LEV 5 (52 %) are the most effective antibiotics (figure 4.6) but no antibiotic one has efficacy above 70 %. CIP 5 (72 %), NOR 10 (60 %), TIC 75 (60 %) and ATM 30 (52 %) were having the highest resistance against *P. aeruginosa* species in our study.

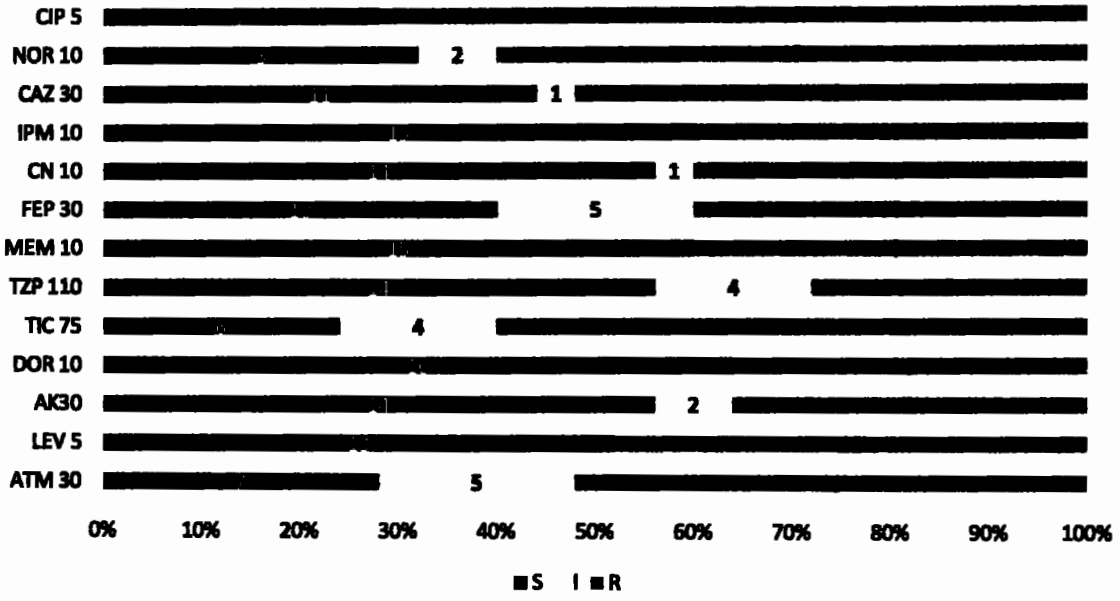

Figure 4.6 ***Antimicrobial susceptibility pattern for 25 isolates of P. aeruginosa against different antibiotics***

4.2 Province wise susceptibility pattern

E. coli from Balochistan province showed the highest percentage (71.43 %) of resistance against 26 antibiotics. KPK and Punjab provinces were in 60s range (66.67 % and 60 %, respectively) while Sindh samples showed least resistance with only 53.06 % (Figure 4.7 & 4.8).

KPK had the highest rate of resistance (69.23) against antibiotics in *P. aeruginosa* species, followed by Balochistan with 63.46 %. Sindh (35.9 %) and Punjab/ICT (34.62 %) have comparatively lower resistance rate (Figure 4.9 & 4.10).

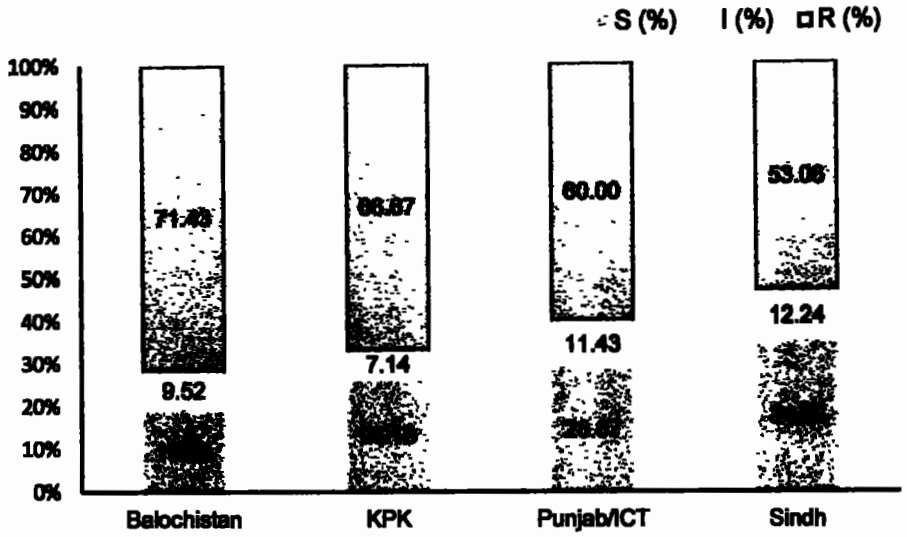

Figure 4.7 *Province wise susceptibility results in E. coli isolates*

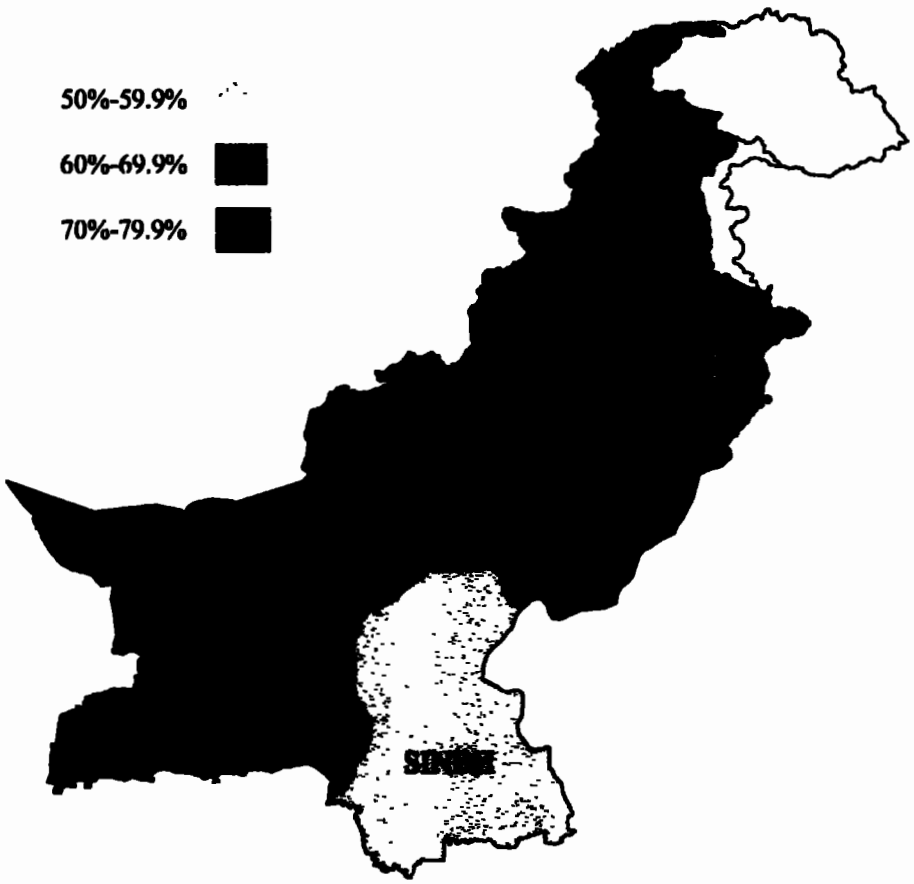

Figure 4.8 *Province wise Antibiotic Resistance Pattern in Pakistan for E. coli resistance. The darker the region, the more the resistance.*

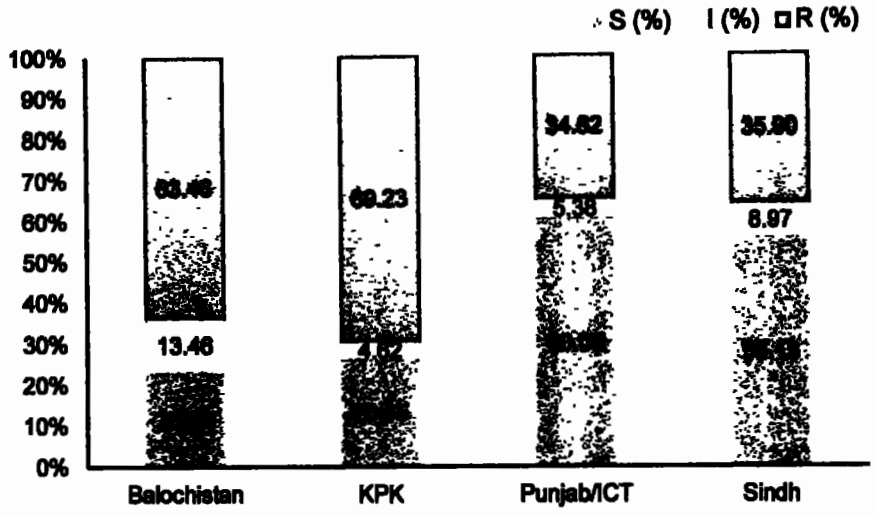

Figure 4.9 Province wise susceptibility results in P. aeruginosa isolates

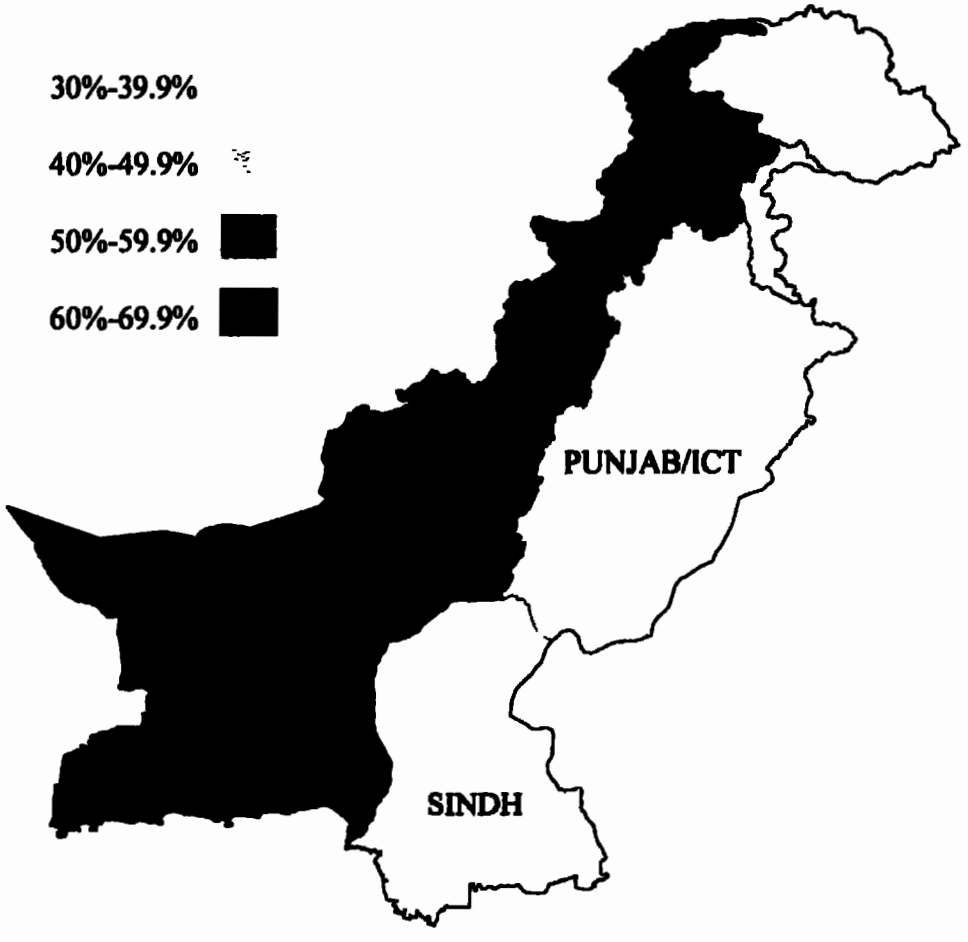

Figure 4.10 Province-wise Antibiotic Resistance Pattern in Pakistan for P. aeruginosa resistance. The darker the region, the more the resistance.

4.3 Multiple Antibiotic Resistance Index (MARI)

A single isolate's MAR index is calculated as follows: MAR index = *a/b*, where *a* is the number of antibiotics to which the isolate was resistant, and *b* is the number of antibiotics to which the isolate was exposed. The isolate's index would be 5/10, or 0.50, if it had been exposed to 10 drugs but only showed resistance to 5. When indexing is used on a sample from which many isolates were obtained, the sample's index would be calculated as a/(b * c), where a represents the total score for antibiotic resistance of all isolates from the sample, b represents the number of antibiotics, and c represents the total number of isolates from the sample. For instance, if a sample of 20 isolates had an overall antibiotic resistance score of 160, the sample's MAR index would be 160/(10 x 20), or 0.8 (Krumperman, 1983). Higher than 0.25 MAR values indicate a high danger of contamination.

Multiple antibiotic resistance (MAR) index values for *P. aeruginosa* and *E. coli* isolates are given in table 4.3. In total, 68 % (n=17) *P. aeruginosa* isolates showed MAR index greater than 0.25 and 32 % (n=08) isolates showed MAR index value less than 0.25. While 96.15 % (n=25) of *E. coli* isolates showed MAR index greater than 0.25 and 3.85 % (n=01) isolates showed MAR index value less than 0.25.

Table 4.3 MAR Index of E. coli (n=26) and P. aeruginosa isolates (n=25)

MAR Index	Number of *E. coli* isolates, (%)	Number of *P. aeruginosa* isolates, (%)
0.00	0, (0)	6, (24)
0.05	0, (0)	1, (4)
0.10	1, (3.8)	0, (0)
0.20	0, (0)	1, (4)
0.30	2, (7.7)	4, (16)
0.40	0, (0)	2, (8)
0.50	12, (46.2)	2, (8)
0.60	2, (7.67)	1, (4)
0.70	4, (15.4)	2, (8)
0.80	4, (15.8)4	2, (8)
0.90	1, (3.9)	1, (4)
1	0, (0)	3, (12)

Aggregate MAR index values were taken for provincial data (Table 4.4) which showed that all the provinces had higher than 0.25 MAR Index value and thus indicating a higher degree of infection.

Table 4.4 ***Province wise MAR Index of E. coli (n=26, antibiotics=14) and P. aeruginosa isolates (n=25, antibiotics=13)***

Province	*E. coli*		*P. aeruginosa*	
	Total no of isolates	MAR Index	Total no of isolates	MAR Index
Balochistan	3	0.71	4	0.63
KPK	6	0.67	5	0.69
Punjab/ICT	10	0.60	10	0.35
Sindh	7	0.53	6	0.36
Total	**26**	**0.61**	**25**	**0.46**

4.4 Detection of OmpC

PCR reaction was used to analyse the outer membrane porin gene i.e., OmpC whose mutation or depletion results in causing resistance in the *E. coli* clinical isolates. The DNA sample was examined and amplified by using the specially designed forward and reverse primers of OmpC gene for all 26 isolates. The PCR product was then run on 1.5 % agarose gel along with 1kb sized DNA ladder for their comparison. The gel was stained and then visualized. Clear bands of OmpC genes having 1128 bp size fragment were seen on the computer screen. Out of 26 isolates, OmpC gene was detected in 96.15 % (n=25) isolates (Figure 4.11A&B).

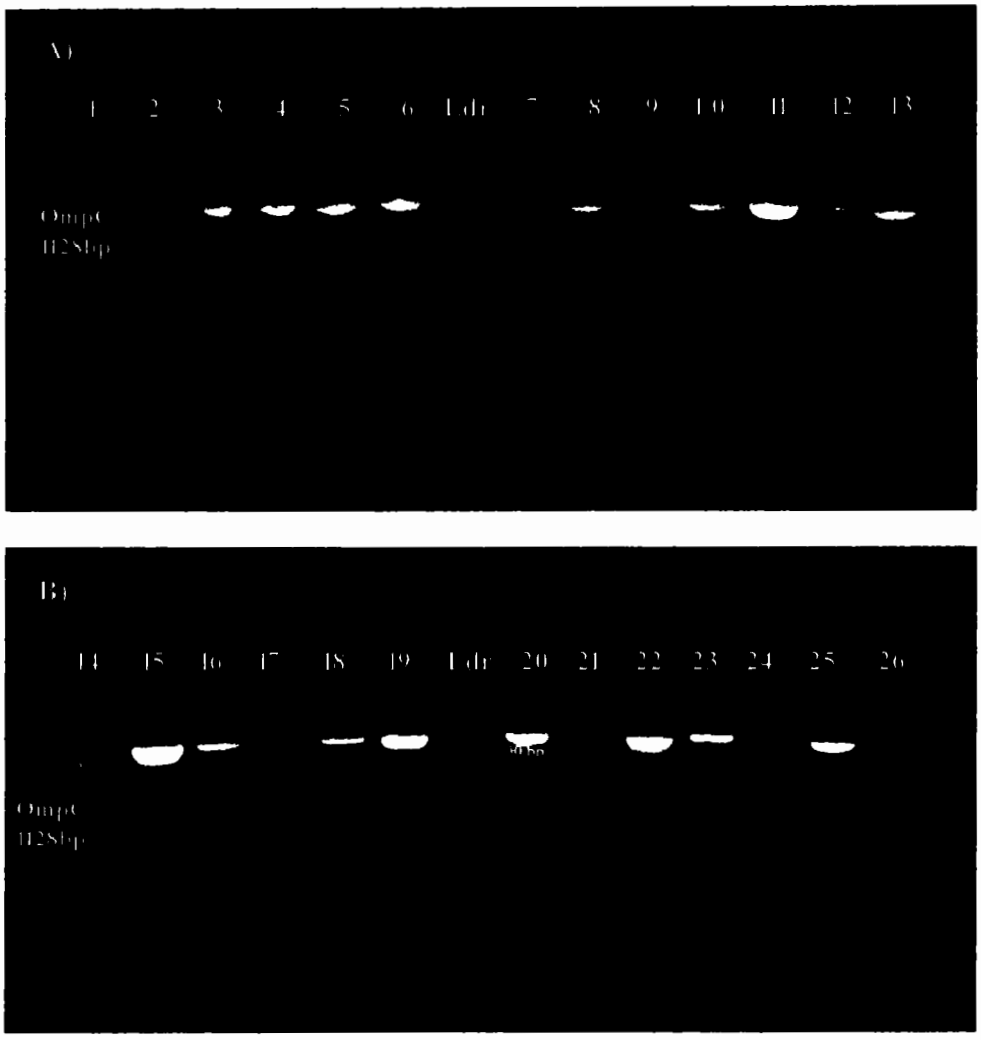

Figure 4.11 ***PCR amplification of OmpC gene. Lanes 2-26 = positive samples of E. coli for OmpC (1128 bp).***

Table 4.5 shows PCR results for OmpC gene detection in different provinces of Pakistan. Gene was knocked out in 10 % isolates of Punjab/ICT.

Table 4.5 ***Province wise PCR results for OmpC gene detection***

Province	Total Isolates	PCR band detected	%age
Balochistan	3	3	100.00
KPK	6	6	100.00
Punjab/ICT	10	9	90.00
Sindh	7	7	100.00

4.4.1 Insilico studies of OmpC

Eight (08) out of total 26 *E. coli* samples were sent for sequencing. The data obtained, in the form of nucleotide sequences, were used further for in-silico studies.

4.4.1.1 Protein translation

ExPASy tool was used for the conversion of nucleotide sequences of OmpC CIs into its one letter amino acid sequence. Six results were shown by the program and one of the best reading frames was selected in which the larger number of amino acids were present. The sequence was from 5`-3` in results (from amino acid to carboxy terminus) indicating the direction in which this protein is translated.

1) OmpC-CI-2

AEVYNKDGNKLDLYGKVDGLHYFSDNKSEDGDQTYMRLGFKGETQVTDQLTGYGQWEYQIQ
GNSAENENNSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNFMQQ
RGNGFATYRNTDFFGLVDGLNFAVQYQGKNGSVDGEGMTNNGRGALRQNGDGVGGSITYDYE
GFGIGAAVSSSKRTDDQNFGLNGYGERYLGNGDRAETYTGGLKYDANNIYLAAQYTQTYNATR
VGSLGWANKAQNFEAVAQYQFDFGLRPSVAYLQSKGKNLGVINGRNYDDEDILKYVDVGATY
YFNKNMSTYVDYKINLLDDNRFTRDAGINTDDIVALGLVYQF

2) OmpC-CI-9

AEVYNKDGNKLDLYGKVDGLHYFSDNKSEDGDQTYVRLGFKGETQVTDQLTGYGQWEYQIQG
NTSEDNKENSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNFMQQ
RGNGYATYRNTDFFGLVDGLNFAVQYQGKNGSVSGEGMTNNGRGALRQNGDGVGGSITYDYE
GFGIGAAVSSSKRTDDQNFGLNGYGERYLGNGDRAETYTGGLKYDANNIYLAAQYTQTYNATR
VGSLGWANKAQNFEAVAQYQFDFGLRPSVAYLQSKGKNLGVVAGRNYDDEDILKYVDVGATY
YFNKNMSTYVDYKINLLDDNQFTRDAGINTDNIVALGLVYQS

3) OmpC-CI-11

AEVYNKDGNKLDLYGKVDGLHYFSDDKSVDGDQTYMRLGFKGETQVTDQLTGYGQWEYQIQ
GNSAENENNSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNFMQQ
RGNGFATYRNTDFFGLVDGLNFAVQYQGKNGSVDGEGMTNNGRGALRQNGDGVGGSITYDYE
GFGIGAAVSSSKRTDAQNGTYVVDNVTHNYIGTGDRAETYTGGLKYDANNIYLAAQYTQTYNA
TRVGSLGWANKAQNFEAVAQYQFDFGLRPSLAYLQSKGKNLGVINGRNYDDEDILKYVDVGAT
YYFNKNMSTYVDYKINLLDDNQFTRDAGINTDNIVALGLVYQF

4) OmpC-CI-16

AEVYNKDGNKLDLYGKVDGLHYFSDNKSEDGDQTYMRLGFKGETQVTDQLTGYGQWEYQIQ
GNSAENENNSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNFMQQ
RGNGFATYRNTDFFGLVDGLNFAVQYQGKNGSVDGEGMTNNGRGALRQNGDGVGGSITYDYE
GFGIGAAVSSSKRTDDQNFGLNGYGERYLGNGDRAETYTGGLKYDANNIYLAAQYTQTYNATR
VGSLGWANKAQNFEAVAQYQFDFGLRPSVAYLQSKGKNLGVINGRNYDDEDILKYVDVGATY
YFNKNMSTYVDYKINLLDDNRFTRDAGITLMTSYSGVCLCSSWT-DVAPSIQSHIKVWNTGLITV
AQAINTDDIVALGLVYQF

5) OmpC-CI-19

AEVYNKDGNKLDLYGKVDGLHYFSDNKDVDGDQTYMRLGFKGETQVTDQLTGYGQWEYQIQ
GNSAENENNSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNFMQQ
RGNGFATYRNTDFFRLVDGLNFAVQHQGKNGNPSGEGFTTGLTNPCPDALPLFAHGFNGSITCDYQ
SFRIRGAICPNHFLTLP-RLFSNLCVSTPI-GYTGVRESHPHTPRSYVRTGTRKNPTSWNFQICGRHTGS
GSCFPFSALVALASDLPRWPATGSAGSINVKFPPFGGHHPKKVWGALLFFWLAKKLWKAPFFP

6) OmpC-CI-22

AEIYNKDGNKLDLYGKVDGLHYFSDNDSKDGDKTYMRLGFKGETQVTDQLTGYGQWEYQIQG
NEPESDNSSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYDSDNFMQQR
GNGFATYRNTDFFGLVDGLDFAVQYQGKNGSAHGEGMTTNGRDDVFEQNGDGVGGSITYNYE
GFGIGAAVSSSKRTWDQNNTGLIGTGDRAETYTGGLKYDANNIYLAAQYTQTYNATRVGSLGW
ANKAQNFEAVAQYQFDFGLRPSLAYLQSKGKNLGRGYDDEDILKYVDVGATYYFNKNMSTYV
DYKINLLDDNRFTRDAGINTDDIVALGLVYQF-

7) OmpC-CI-23

AEVYNKDGNKLDLYGKVDGLHYFSDDKSVDGDQTYMRLGFKGETQVTDQLTGYGQWEYQIQ
GNSAENENNSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNFMQQ
RGNGFATYRNTDFFGLVDGLNFAVQYQGKNGTVCGEGPTHNGRGALPLNRHFFPWSITYDFQR
FRIRERTL-P-RSKPLHHPVVKC-QNRG-NHNCRAESRPRNTSSWTAPYLRP-SKHPTSR-FQT--CHTG
PGVVFVFSAVALDLICPLAVTGRLVSGLSFSLKPRRKYVGTPCR-WSSKNFGKPLPSGTKKNCGR

8) OmpC-CI-25

AEVYNKDGNKLDLYGKVDGLHYFSDDKSVDGDQTYMRLGFKGETQVTDQLTGYGQWEYQIQ
GNSAENENNSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNFMQQ
RGNGFATYRNTDFFGLVDGLNFAVQYQGKNGSVSGEGMTNNGRGALRQNGDGVGGSITYDYE

GFGIGGAISSSKRTDDQNSPLYIGNGDRAETYTGGLKYDANNIYLAAQYTQTYNATRVGSLGWA
NKAQNFEAVAQYQFDFGLRPSVAYLQSKGKNLGTIGTRNYDDEDILKYVDVGATYYFNKNMST
YVDYKINLLDDNQFTRDAGINTDNIVALGLVYHS

4.4.1.2 Comparing Sequences using BLASTN

BLASTN was performed to find the similarity of eight nucleotide sequences of OmpC-CI-2, Om pC-CI-9, OmpC-CI-11, OmpC-CI-16, OmpC-CI-19, OmpC-CI-22, OmpC-CI-23 and OmpC-CI-25. Dozens of results were generated for each query, but we have selected only the top result and added in table 4.6. Six samples had 100 % query coverage with more than 99 % percent identific ation. All the matching results had OmpC genes in their results confirming our result too.

Table 4.6 Summary of Nucleotide alignment by BLASTN for OmpC samples

Isolate	GenBank	Source	Query coverage	E value	Percent Ident	Acc lens	Gene found
2	CP120633.1	*E. coli* O25b H4-ST131	100 %	0.0	100.00 %	5098635	OmpC
9	CP129259.1	*E. coli*	100 %	0.0	99.91 %	4967596	OmpC
11	CP054214.1	*E. coli*	100 %	0.0	99.91 %	5025664	OmpC
16	CP120633.1	*E. coli*	100 %	0.0	100.00 %	5098635	OmpC
19	CP119008.1	*E. coli*	61 %	0.0	95.61 %	5246002	OmpC
22	CP124970.1	*E. coli*	100 %	0.0	100.00 %	4920518	OmpC
23	CP054227.1	*E. coli*	86 %	0.0	96.08 %	4796742	OmpC
25	CP054227.1	*E. coli*	100 %	0.0	99.90 %	4796742	OmpC

4.4.1.3 3D Structures of selected OmpC samples

Phyre2 tool was used for the prediction of protein 3D structure. Figure 4.12A-D represents the 3D structures of OmpC *E. coli* based on d2zfga1 template.

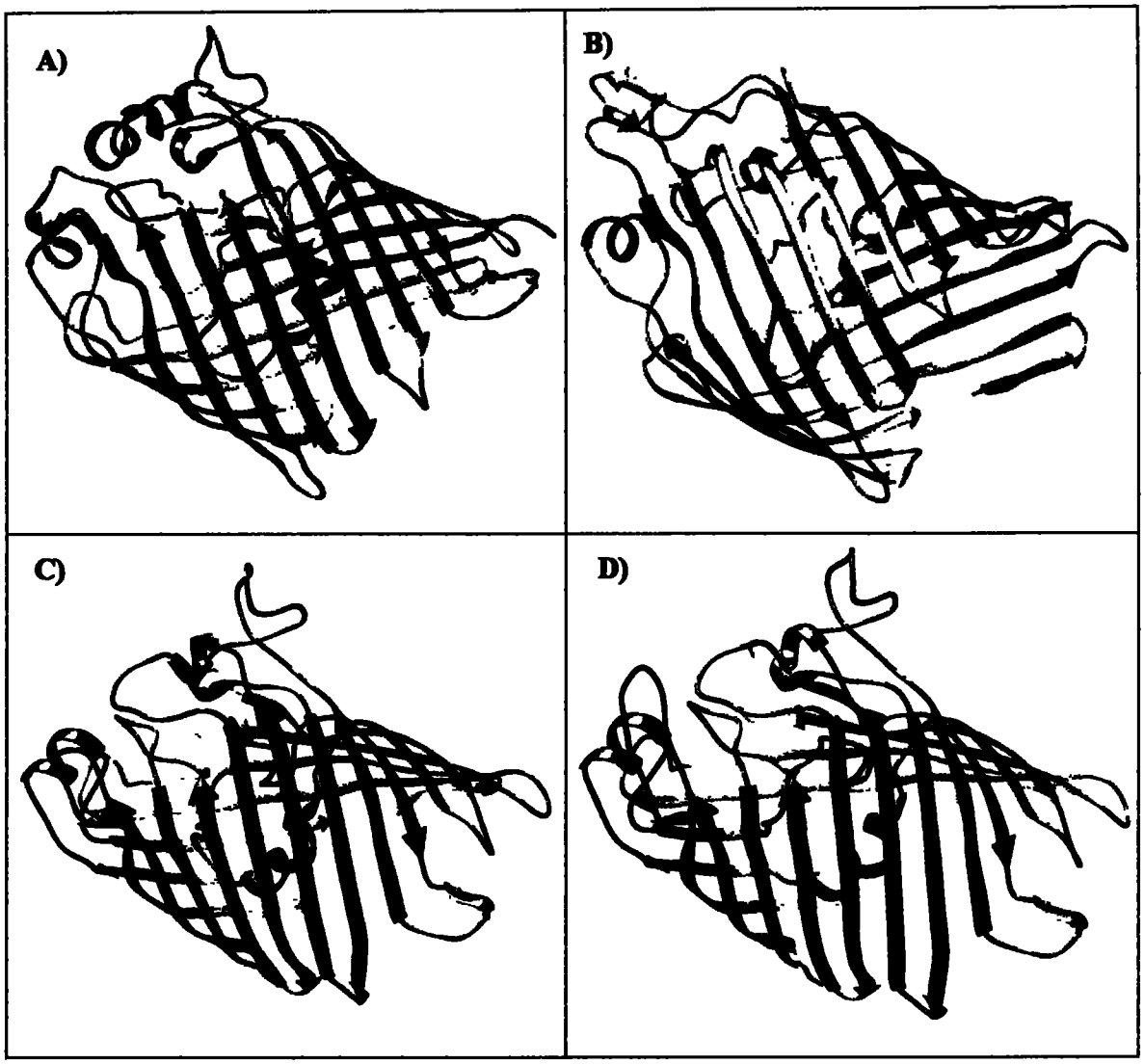

Figure 4.12 *3D structure of OmpC E. coli clinical isolates A) CI-2, B) CI-11, C) CI-22 and D) CI-25*

4.4.1.4 Mutation analysis

The Clustal omega tool was used to compare OmpC nucleotide and protein sequences of both wild type and clinical isolates. As a result, various number of point mutations were observed both in nucleotide sequences and protein sequences.

UCSF Chimera was used to highlight the mutated amino acids on 3D image of the protein. We are presenting here only four elected examples of isolates no 2 (OmpC-CI-2), 11 (OmpC-CI-11), 22 (OmpC-CI-22), and 25 (OmpC-CI-25) as given below from figure 4.13 to figure 4.21.

1) OmpC-CI-2

Figure 4.13 ***Protein alignment results of Clustal omega by comparing OmpC-CI-2 and OmpC-WT.***

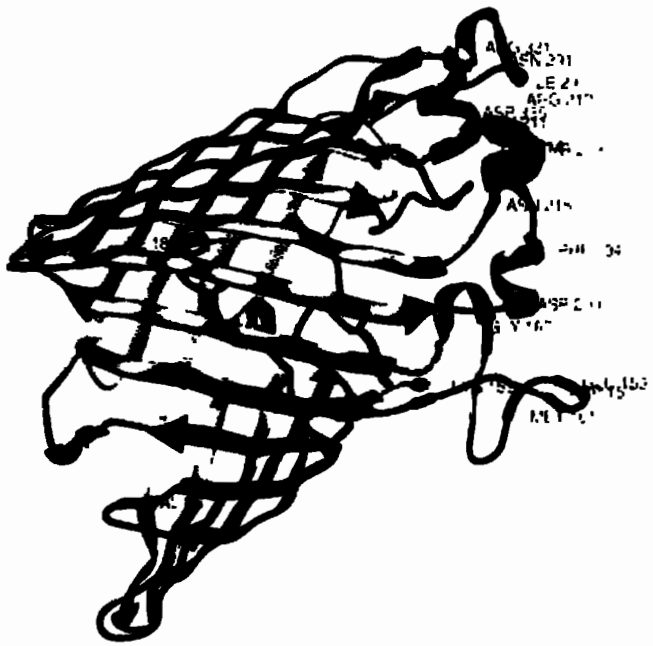

Figure 4.14 ***CI-2 of E. coli having OmpC mutations at different regions as highlighted in the image.***

The details of alterations in amino acids of OmpC of isolate No 2 in comparison to wild type is given in table 4.7.

Table 4.7 Alteration in the amino acids by comparing OmpC-CI-2 and OmpC-WT.

S.No	Change in Amino Acid	S.No	Change in Amino Acid
1	Asp 26 Asn	13	Trp 200 Asp
2	Val 29 Glu	14	Asn 204 PHe
3	Ala 64 Ser	15	Thr 211 Glu
4	Pro 65 Ala	16	Gly 212 Arg
5	Ser 67 Asn	17	Leu 213 Tyr
6	Ile 85 Val	18	Ile 214 leu
7	Gln 152 Lys	19	Thr 216 Asn
8	Ser 157 Asp	20	Val 290 Ile
9	His 159 Glu	21	Ala 291 Asn
10	Ile 161 Met	22	Gln 331 Arg
11	Lys 167 Gly	23	Ala 335 Asp
12	Val 189 Ile		

2) OmpC-CI-11

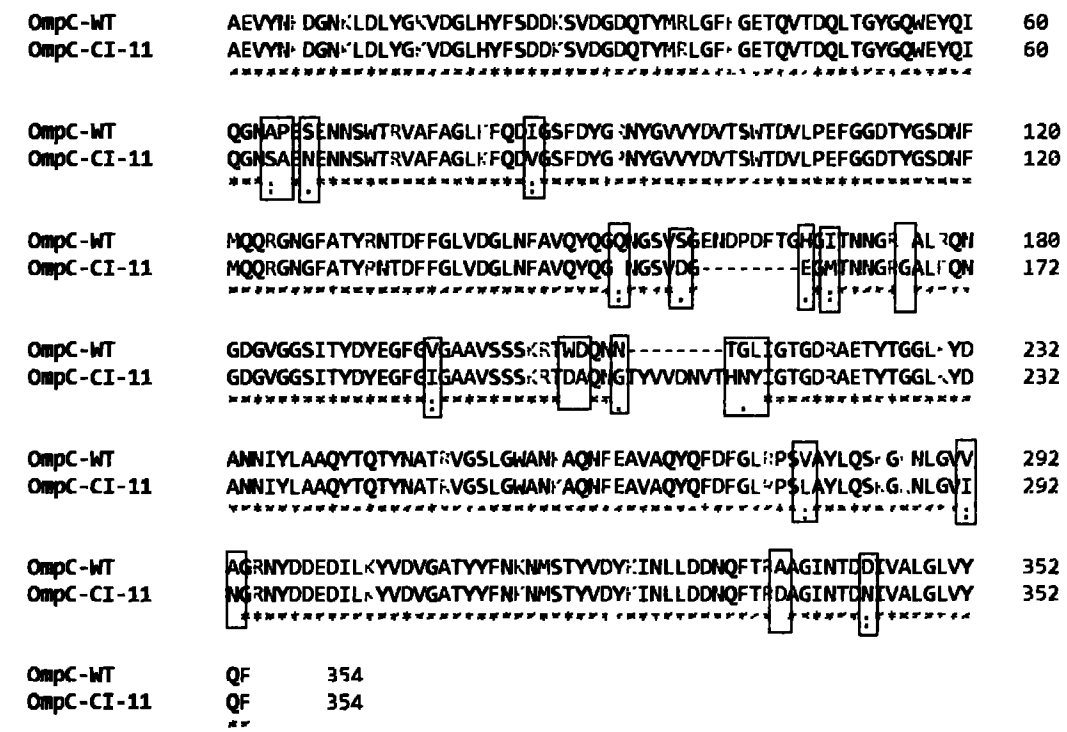

Figure 4.15 Protein alignment results of Clustal omega by comparing OmpC-CI-11 and OmpC-WT.

The details of alterations in amino acids of OmpC of isolate No 11 in comparison to wild type is given in table 4.8.

Table 4.8 Alteration in the amino acids by comparing OmpC-CI-11 and OmpC-WT

S.No	Change in Amino Acid	S.No	Change in Amino Acid
1	Ala 64 Ser	12	Asp 201 Ala
2	Pro 65 Ala	13	Asn 204 Gly
3	Ser 67 Asn	14	Thr 213 His
4	Ile 85 Val	15	Gly 214 Asn
5	Gln 152 Lys	16	Leu 215 Tyr
6	Ser 157 Asp	17	Val 279 Leu
7	His 159 Glu	18	Val 292 Ile
8	Ile 161 Met	19	Ala 293 Asn
9	Lys 167 Gly	20	Ala 337 Asp
10	Val 189 Ile	21	Asp 344 Asn
11	Trp 200 Asp		

Figure 4.16 CI-11 of E. coli having OmpC mutations at different regions as highlighted in the image.

3) OmpC-CI-22

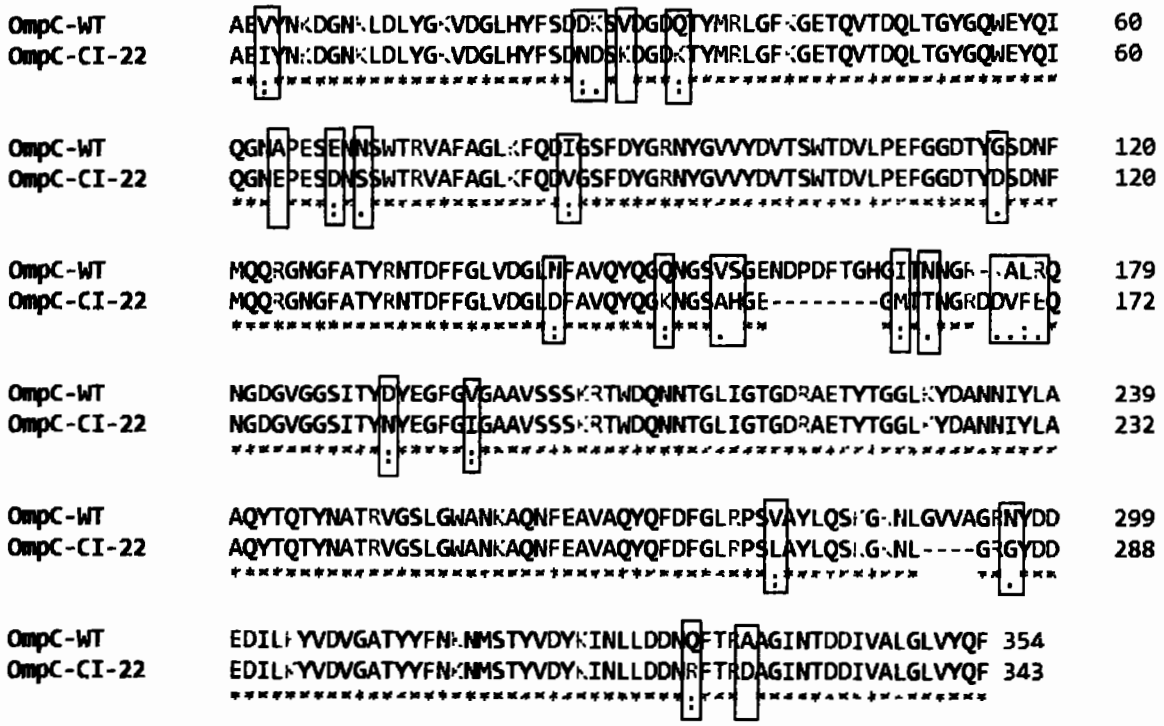

Figure 4.17 ***Protein alignment results of Clustal omega by comparing OmpC-CI-22 and OmpC-WT.***

The details of alterations in amino acids of OmpC of isolate No 22 in comparison to wild type is given in table 4.9.

Table 4.9 ***Alteration in the amino acids by comparing OmpC-CI-22 and OmpC-WT***

S.No	Change in Amino Acid	S.No	Change in Amino Acid
1	Val 3 Ile	14	Ser 157 His
2	Asp 26 Asn	15	Ile 161 Met
3	Lys 27 Asp	16	Asn 163 Thr
4	Val 29 Lys	17	Lys 168 Asp
5	Gln 33 Lys	18	Ala 169 Val
6	Ala 64 Glu	19	Leu 170 Phe
7	Glu 68 Asp	20	Arg 171 Glu
8	Asn 70 Ser	21	Asp 184 Asn
9	Ile i85 Val	22	Val 190 Ile
10	Gly 116 Asp	23	Val 272 Leu
11	Asn 144 Asp	24	Asn 273 Gly
12	Gln 152 Lys	25	Gln 322 Arg
13	Val 156 Ala	26	Ala 326 Asp

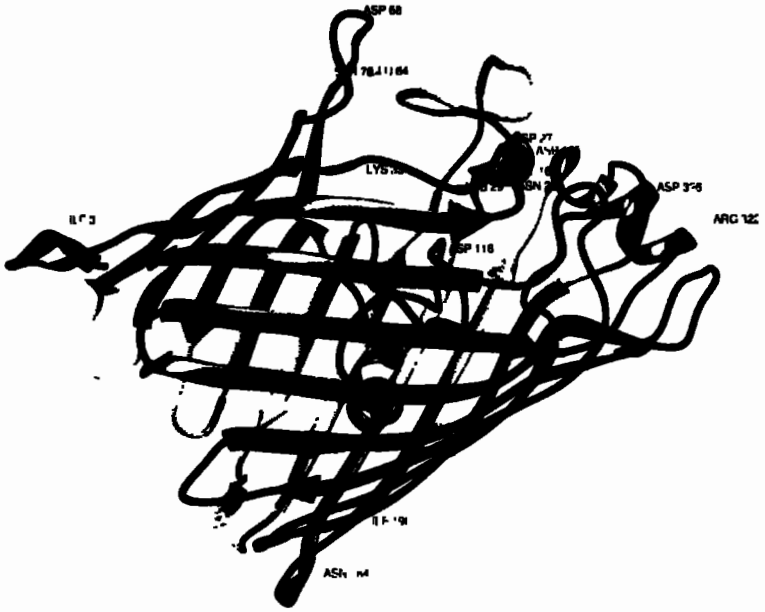

Figure 4.18 ***CI- 22 of E. coli having OmpC mutations at different regions as highlighted in the image.***

4) OmpC-CI-25

```
OmpC-WT      AEVYNKDGNKLDLYGKVDGLHYFSDDKSVDGDQTYMRLGFKGETQVTDQLTGYGQWEYQI  60
OmpC-CI-25   AEVYNKDGNKLDLYGKVDGLHYFSDDKSVDGDQTYMRLGFKGETQVTDQLTGYGQWEYQI  60
             ************************************************************

OmpC-WT      QGNAPESENNSWTRVAFAGLKFQDIGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNF  120
OmpC-CI-25   QGNSAENENNSWTRVAFAGLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYGSDNF  120
             **.:.*.**********.:**********************************

OmpC-WT      MQQRGNGFATYRNTDFFGLVDGLNFAVQYQGQNGSVSGENDPDFTGHGITNNGKPALKQN  180
OmpC-CI-25   MQQRGNGFATYRNTDFFGLVDGLNFAVQYQGKNGSVSG--------EGMTNNGRGALFQN  172
             **********************************.:*****          .*.:*****.*****

OmpC-WT      GDGVGGSITYDYEGFGVGAAVSSKRTWDQMNTGLIGTGDRAETYTGGLKYDANNIYLAA  240
OmpC-CI-25   GDGVGGSITYDYEGFGIGGAISSSKRTDDQMSPLYIGNGDRAETYTGGLKYDANNIYLAA  232
             **********.*.*.*****.**.*.**********************************

OmpC-WT      QYTQTYNATRVGSLGWANKAQNFEAVAQYQFDFGLRPSVAYLQSKGKNLGVVAGKNYDDF  300
OmpC-CI-25   QYTQTYNATRVGSLGWANKAQNFEAVAQYQFDFGLRPSVAYLQSKGKNLGTIGTKNYDDE  292
             ************************************************.:. ******

OmpC-WT      DILKYVDVGATYYFNKNMSTYVDYKINLLDDNQFTKAAGINTDDIVALGLVYQF  354
OmpC-CI-25   DILKYVDVGATYYFNKNMSTYVDYKINLLDDNQFTDAGINTDNIVALGLVYHS  346
             ***********************************.*******.********:
```

Figure 4.19 ***Protein alignment results of Clustal omega by comparing OmpC-CI-25 and OmpC-WT.***

The details of alterations in amino acids of OmpC of isolate No 22 in comparison to wild type is given in table 4.10.

Table 4.10 Alteration in the amino acids by comparing OmpC-CI-25 and OmpC-WT

S.No	Change in Amino Acid	S.No	Change in Amino Acid
1	Ala 64 Ser	14	Thr 205 Pro
2	Pro 65 Ala	15	Gly 206 Leu
3	Ser 67 Asn	16	Leu 207 Tyr
4	Ile 85 Val	17	Thr 210 Asn
5	Gln 152 Lys	18	Val 283 Thr
6	His 159 Glu	19	Val 284 Ile
7	Ile 161 Met	20	Ala 285 Gly
8	Lys 167 Gly	21	Gly 286 Thr
9	Val 189 Ile	22	Ala 329 Asp
10	Ala 191 Gly	23	Asp 336 Asn
11	Val 193 Ile	24	Gln 345 His
12	Trp 200 Asp	25	Phe 346 Ser
13	Asn 204 Ser		

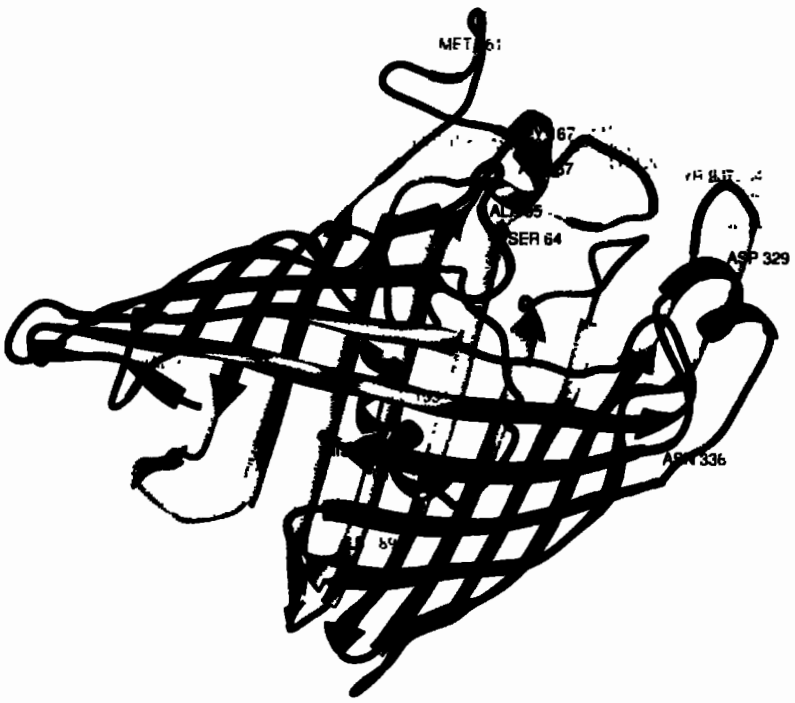

Figure 4.20 CI- 25 of *E. coli* having OmpC mutations at different regions as highlighted in the *image.*

4.5 Detection of OmpF

PCR reaction was used to analyse the outer membrane porin gene i.e., OmpF whose mutation or depletion results in causing resistance in the *E. coli* clinical isolates. The DNA sample was examined and amplified by using the specially designed forward and reverse primers of OmpC gene for all 26 isolates. The PCR product was then run on 1.5 % agarose gel along with 1kb sized DNA ladder for their comparison. The gel was stained and then visualized. Clear bands of OmpF genes having 1089 bp size fragment were seen on the computer screen. Out of 26 isolates, OmpF gene was detected in 92.31 % (n=24) isolates (Figure 4.22 A&B).

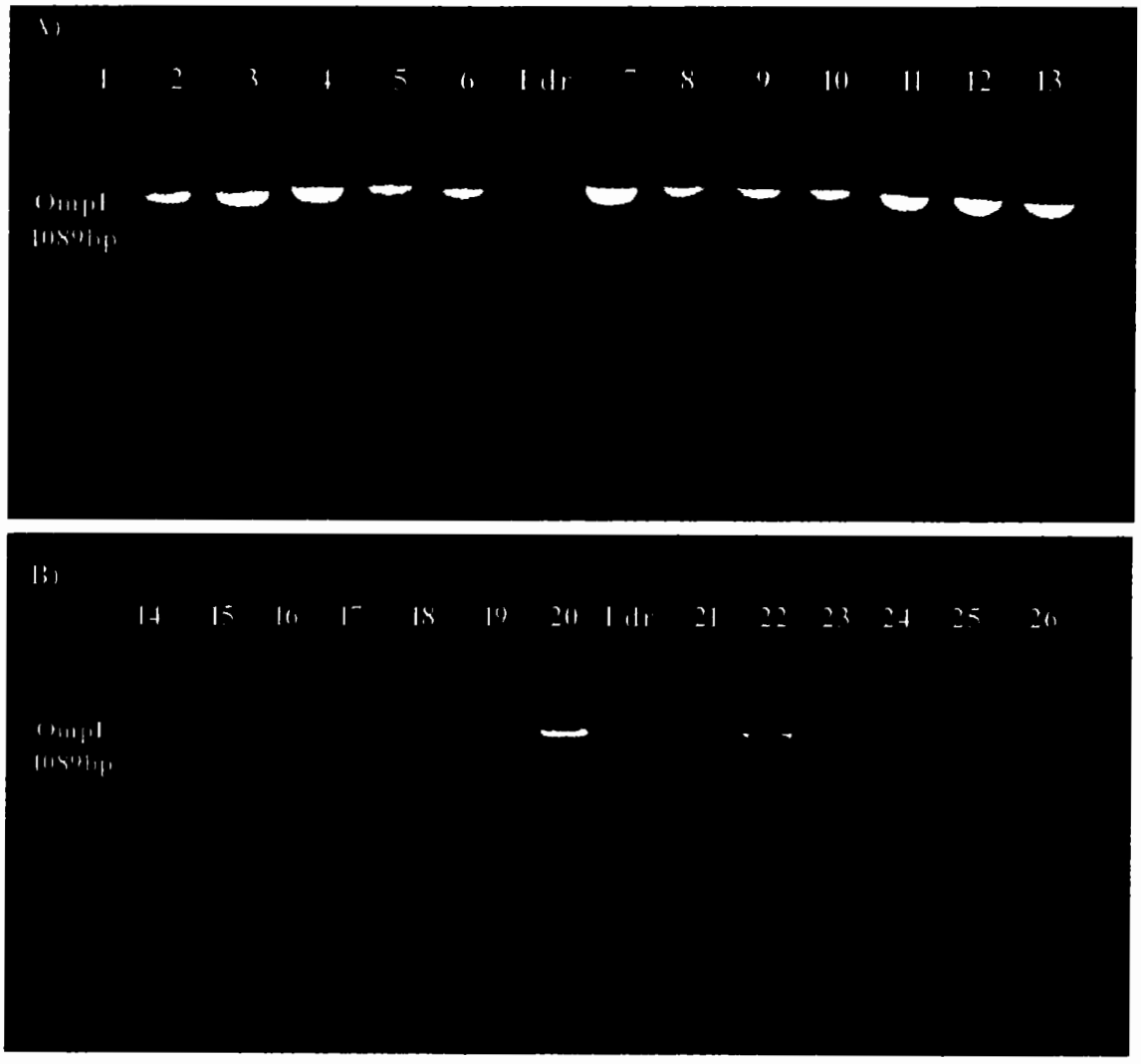

Figure 4.21 (A&B) ***PCR amplification of OmpF gene. Lanes 2-24 and Lane 26 = positive samples of E. coli for OmpF (1089 bp).***

Table 4.11 shows PCR results for OmpF gene detection in different provinces of Pakistan. Gene was knocked out in 10 % of isolates of Punjab/ICT and in 14 % of isolates of Sindh.

Table 4.11 Province wise PCR results for OmpF gene detection

Province	Total Isolates	PCR band detected	%age
Balochistan	3	3	100.0
KPK	6	6	100.0
Punjab/ICT	10	9	90.0
Sindh	7	6	85.71

4.5.1 Insilico studies of OmpF

Nine (09) out of a total of 26 *E. coli* samples were sent for sequencing. The data obtained, in the form of nucleotide sequences, were used further for in-silico studies.

4.5.1.1 Protein Translation

ExPASy tool was used for the conversion of nucleotide sequences of OmpF samples into its one letter amino acid sequence. Six results were shown by the program and one of the best reading frames was selected in which the larger number of amino acids were present. The sequence was from 5`-3` in results (from amino acid to carboxy terminus) indicating the direction in which this protein is translated.

1) **OmpF-CI-2**

VAGTANAAEIYNKDGNKVDLYGKAVGLHYFSKGNGEDSYGGNGDMTYARLGFKGETQINSDL
TGYGQWEYNFQGNNSEGADAQTGNKTRLAFAGLKYADVGSFDYGRNYGVVYDALGYTDMLP
EFGGDTEYSDDFFVGRVGGVATYRNSNFFGLVDGLNFAVLYLGKNERDTARRSNGDGVGGSIS
YKYKSFGIDGAYGAADRTSLPEKSPLGKG-PPEQ-ATGL--D-SEQPPGSELRSTR-PQHLCILHFTM
HYLP-CYHTQRHSPSSLNPHYQY-SFGLSPRLTPKPDNQKEGTPWPGDH-FCINHF-TILLKPSYMLL
KKRTNFFGWPFFDTSIYSAYWFGSSLL

2) **OmpF-CI-9**

IQGYCKRCRNL-QRWQQSRSVR-SCWSALFFQG-R-KQLLWQWQHDLCPSWVLGKT-IQSQLNRE
LANEKKTFEVSCQNGGCSQIDNETRRGFGGLKNSDLGSFYYGRN-GVVHCALFLPLTRG-SSCRM
RRSAAPYARRVGGFAIRS-IKLLCPSLMETTCTVTLLGYQGTEHRSSGHQANERSWSYDRKQRAQ
HDQRISHRCKHTHHQILPPYRCNPVHHKPRRSYGRNRKNHRQGI-DPRMPDAFC--FERQRPSELF
PWKLYSL-L-AHRPY-I-LSPSKLDGHLSCRHCSVTVFPLLLEFNGATDYSTDLRRRIFGKIPRRCGP
GLTKWTDSHVVPYIHKTQ

3) OmpF-CI-11

NLWVPGKDKNTGVAKVYTKWSQCKFDGQSFCVLFFPRVTVAIITVALATRPMPVLVLKGKLKSI
FI-PVMVAGNITSMVTTLRARTLELETKDVWHSPVLNTLTLVLSITAVTTVGFRMHWVTPIYCQN
FELLLHTPMICSMVWKAALLPIVTPTSLNWLMV-ASLFTTWLKKNVTLRSSLTATVLAVSSARNT
KALGCLVLMVPLTVPTCLKILPLVNAMILNSWLPGL-FDSNNIYLVANYSETPYRYSPSLIRFYKP
QLLLHSPLLTYCYLLQYQFEFGSASVHRIKKF-DLNTEKVSVMVIWLKLS-SGSNLLFLQIFSTLWL
TSSAIIFYYNNILDLGFLTSPVACRVSFNHFEKSSW

4) OmpF-CI-13

EGPAAVSSRYCNAAESITKMATK-ICTVKLSVCIISPRVTVNTVTVAMAT-PMPVLVLKGKLKSIPI
-PVMVSVNITSIVTTPDVLSLHVETKHTYGFTGKNYLIAIFVIDFRTQSSSRWCTIFDHG-GKILRED
NTVSGTQARTRPKLRNRSEQPPTPWPIETACSVPAPFFTQGPEKRSSDPSSQTEKGLEFTIGNKRRP
TQPKEKIIACYTVPPPKFGHPIRCKPNASKTTP-VTAVIRKKPTSGYLKPGKARSVLVPSLERLRLS
KLFPWKVIFPLDYYRVRSELDLSCPL-NQNGNRSCTHLHRNVSFRYR-KIMETDPFTVKNYFCCIF
VNISATFFYLF-ERGPTCTCANNIQKTERK

5) OmpF-CI-15

AGPTLSVAGTATLQKSITKMATK-ICTVKLSVCIIFPRVTVTTVTVGLGNRPSARLGCKRETHVVQ
IGPVRAMGNSLEGSCRDGGGSQIGNEPRLAFAGLKYADVGSFDYGRYYRVAHCAAVLHVAEW
MIFVQDETVTMHFFLDRYQSLRTYS--NCQRRRRWTDLRCHARVYPGTEQ-TQINHQTKEVVLTI
GSNTANTTNEQVIAVCVSPPNSGSISV-PNASKTAP-LRNVIERTIVSVFKIRECQTRYVTR-SVQRL
QSCSPGNTLRLGYLRTDRYLF-LPPSTHTEIEHDTVQPYWSFIITLRKLSGEHFTSQIYFGRMLVIIS
ATFSDLLLAEQRQHRDAFYLKKREKGP

6) OmpF-CI-17

AGRCVVAGTANAAEIYNKDGNKVDLYGKAVGLHYFSKDNGVNSYCGNGNKTYARLGFKEQT-
FIPI-PVKAGVNLTSAVRG-GRWCAKLVKVAEGYGCLKTLAWYC-SRSTTVWLLAWVYRATGRT
GVG-TVRAPRHGREGGLHLIRSTARRRGGGAGRSGRFLTQGTGTGKVRPINQTKEVVIYE-ENNA
PPGPTQRNKSSLYA-SPTKILAPLLGVAPMHHKTAPASYGP-SKKPSFSGI-HTANARTRFVNQL-T
VSAFQTLFPLESHNYPLDYIAGTT-LIVFEFPPFNPHKRDIMFLNAGIRGSLVFSILVYYKYRMGDR
TSFLRTETTFGRHFWLYIFPNGCRNPEFVRRKVGPVPAMHV

7) OmpF-CI-18

CTGACTACTGAGAGAGCTCGACGAACTTTCACATGCT

RTLSVSGTADAAEIYNKDGNKVDLYGKAVGLHYFSKDNGVNSYGGNGDKTYARLGFKGETQIN
SDLTGYGQWEYNFQGNNSEGADAQTGNKTRLAFPGLKYADIGSFDYGRNFRVGLRGTSNYDN-
QNNVLANK-DNVLQVKGSPAFLNIKLKKPLGRGRWNGVQWHERGKKVLDKGSSGPTDQSRRK
LGIKKRKTKPPQHNQRKNARPLCDHHQPKFRAPFRSDPSAFKNRPRGFRP-SKRTIVSVF-NPAKC
QTAFLLPVLSVSALQRLFTPGKFIIPYRTINPDQNRNLILWFPPFNTKHGA-IFVGRFRHNCFTTLSIE
IIMQDRLLLLVQELLCRHLLVIRLSGDVLR-PDY-ESSTNFHM

8) OmpF-CI-20

GRLLVAGTANAAEIYNKDGNKVDLYGKAVGLHYFSKDNGVNSYGGNGDKTYARLGFKGETQI
NSDLTGYGQWEYNFQGNNSEGADAQKGNKTRLAFAGLKFADAGSIDYGRNYGVVYDALGYTD
MLPEFGGDTAYSDDFFVGRVGGVATYRNSNFFGLVDGLNFAVQYLGKNERAGIPERSNGDGVG
GSISYEFEGFGIVGAYGAADRTDAQEAEFRGQGKKAEQWATGLKYDANNIYLAANYGETRNAT
PIEGGFANKTQDVLLVAQYQFDFGLRPSIAYTKSKAKDVEGIGDVDLVNYFEVGATYYFNKNMS
TYVDYIINQIDSDNKLGVGSDDTVAVVVLSKKK-KKKKTGMYIRENRRGGIQDGGEVG-GGVQA
GGRSAMRRGIWR

9) OmpF-CI-22

GRLLVAGTANAAEIYNKDGNKVDLYGKAVGLHYFSKDNGVNSYGGNGDKTYARLGFKGETQI
NSDLTGYGQWEYNFQGNNSEGADAQKGNKTRLAFAGLKFADAGSIDYGRNYGVVYDALGYTD
MLPEFGGDTAYSDDFFVGRVGGVATYRNSNFFGLVDGLNFAVQYLGKNERAGIPERSNGDGVG
GSISYEFEGFGIVGAYGAADRTDAQEAEFRGQGKKAEQWATGLKYDANNIYLAANYGETRNAT
PIEGGFANKTQDVLLVAQYQFDFGLRPSIAYTKSKAKDVEGIGDVDLVNYFEVGATYYFNKNMS
TYVDYIINQIDSDNKLGVGSDDTVAVVVLSKKK-KKKKTGMYIRENRRGGIQDGGEVG-GGVQA
GGRSAMRRGIWR

4.5.1.2 Comparing sequences using BLASTN

BLAST was performed to find the similarity of nine nucleotide sequences of OmpF-CI-2, OmpF-CI-9, OmpF-CI-11, OmpF-CI-13, OmpF-CI-15, OmpF-CI-17, OmpF-CI-18, OmpF-CI-20 and O mpF-CI-22. Dozens of results were generated for each query, but we have selected only the top r esult and added in table 4.12. Four samples had more than 80 % query coverage with more than 9 9 % percent identification. All of the matching results had OmpF genes in their results confirmin g our result too.

Table 4.12 Summary of Nucleotide alignment by BLASTN for OmpF samples

Isolate	GenBank	Source	Query coverage	E value	Per Ident	Acc lens	Gene
2	CP048326.1	*E. coli* O25b:H4-ST131	67 %	0.0	95.66 %	4890300	OmpF
9	CP048322.1	*E. coli*	92 %	6e-145	81.96 %	4771203	
11	CP028742.1	*E. coli*	94 %	0.0	80.98 %	5117905	
13	CP104618.1	*E. coli*	21 %	1e-101	95.47 %	4633559	
15	CP125731.1	*E. coli*	94 %	4e-127	79.51 %	4857268	
17	CP065615.1	*E. coli*	20 %	2e-81	92.07 %	5272040	
18	CP128587.1	*E. coli*	30 %	2e-164	98.80 %	5057461	
20	CP054335.1	*E. coli*	86 %	0.0	100.00 %	5196142	
22	CP029687.1	*E. coli*	16 %	4e-94	90.04 %	4696142	

4.5.1.3 Mutation analysis

All the samples run for mutation analysis in Clustal omega online software showed a great number of mutations against wild type sequence. Figure 4.23 and 4.24 show protein sequences of samples No 2 and 9, respectively, against wild type protein sequences. Both samples resulted in more than 50 mutations individually. Same is the result for samples no 11, 13, 15, 17 and 18. Only Samples no 20 and 22 had relatively lower number of mutations.

4.5.1.4 3D Structures of selected OmpF samples

Due to truncated proteins, as seen in amino acid sequences of these samples, it was impossible to make 3D structures of OmpF porin so no further computational work was carried out.

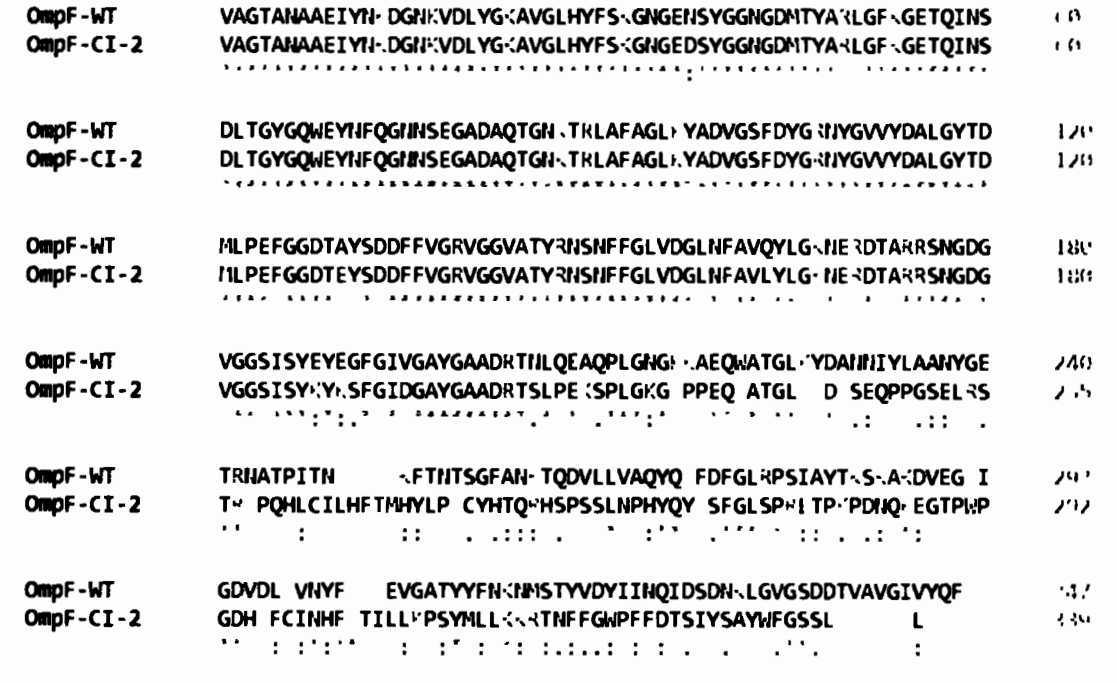

Figure 4.22 ***Protein alignment results of Clustal omega by comparing OmpF-CI-2 and OmpF-WT.***

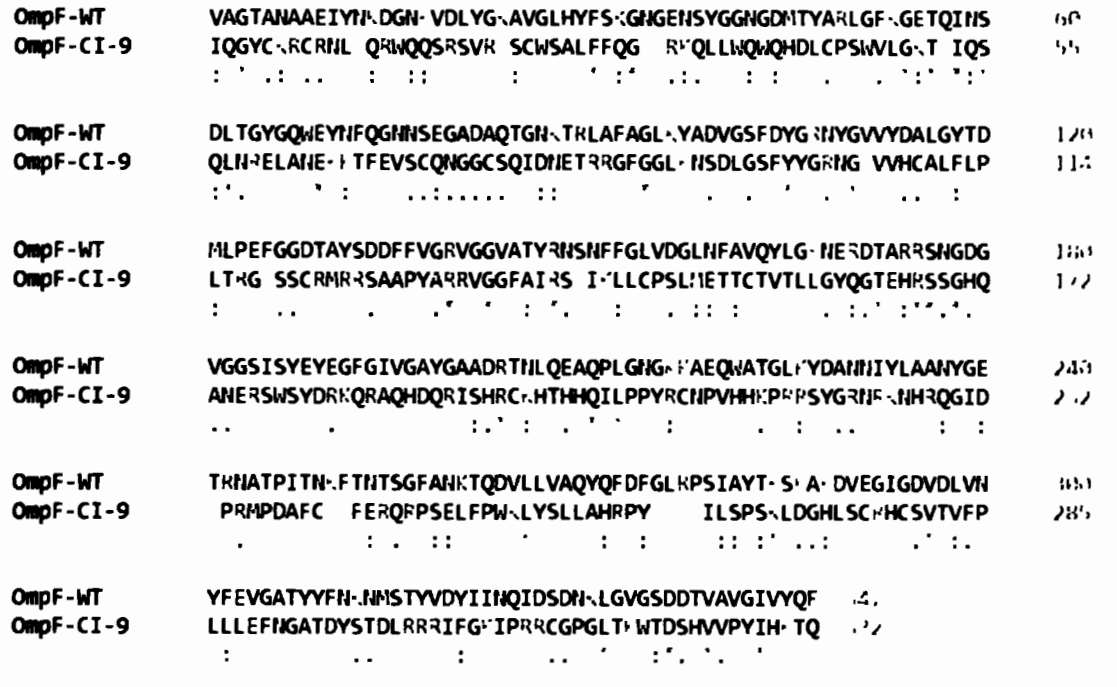

Figure 4.23 ***Protein alignment results of Clustal omega by comparing OmpF-CI-9 and OmpF-WT.***

4.6 Detection of OmpW

PCR reaction was used to analyze the outer membrane porin gene i.e., OmpW whose mutation or depletion results in causing resistance in the *E. coli* clinical isolates. The DNA sample was examined and amplified by using the specially designed forward and reverse primers of OmpW gene for all 26 isolates. The PCR product was then run on 1.5 % agarose gel along with 1kb sized DNA ladder for their comparison. The gel was stained and then visualized. Clear bands of OmpW genes having 639 bp size fragment were seen on the computer screen. Out of 26 isolates, OmpW gene was detected in 73.08 % (n=19) isolates (Figure 4.24A&B).

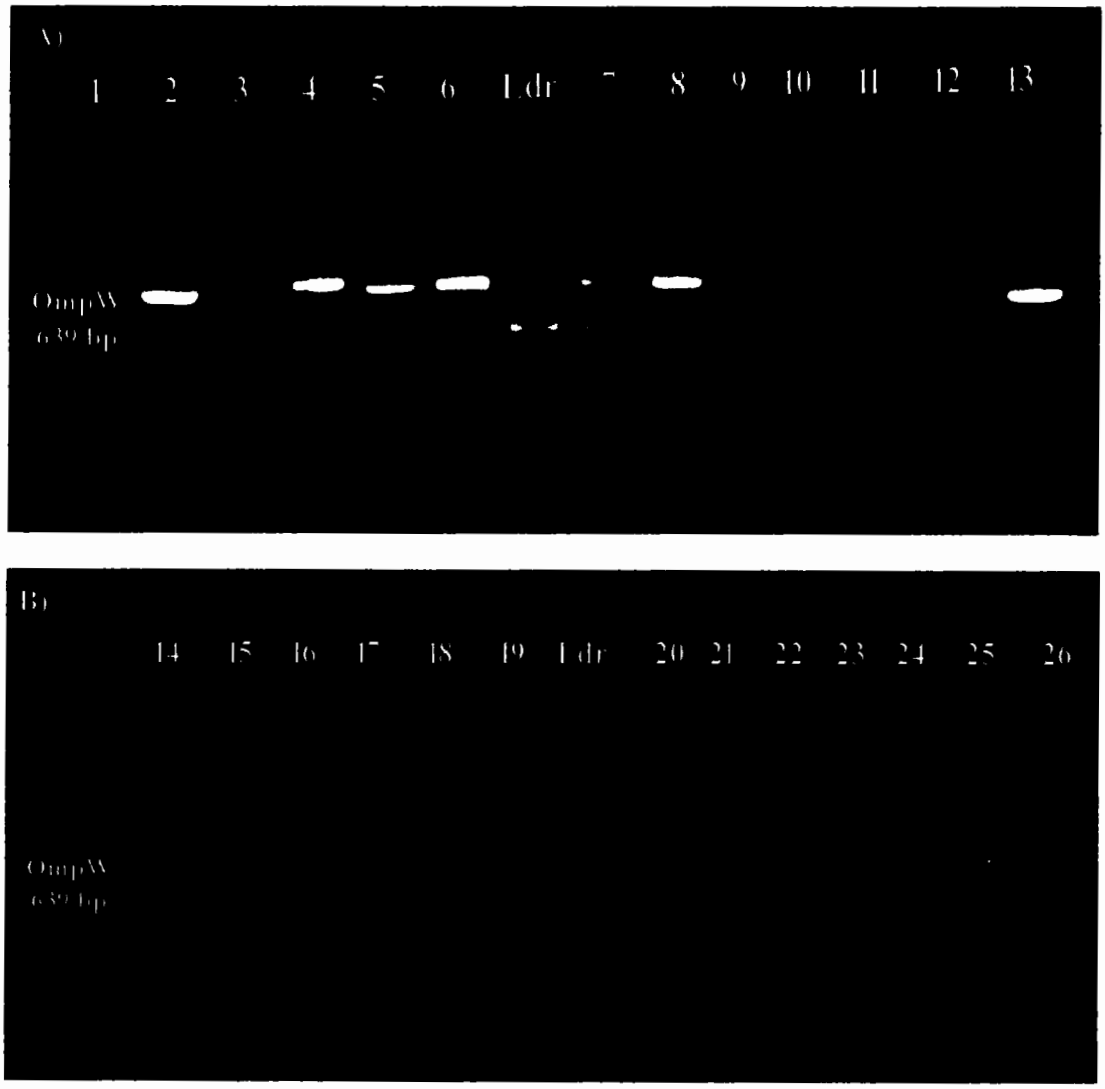

Figure 4.24 (A&B) PCR amplification of OmpW gene: Lanes 2-8, 12-18, 20, 21, 24, 25 = positive samples of E. coli for OmpW (639 bp).

Table 4.13 shows PCR results for OmpW gene detection in different provinces of Pakistan. Gene was knocked out in 33.3 % of isolates of Balochistan, 40 % isolates of KPK, 20 % isolates of Punjab/ICT and in 14 % of isolates of Sindh.

Table 4.13 Province wise PCR results for OmpW gene detection

Province	Total Isolates	PCR band detected	%age
Balochistan	3	2	66.67
KPK	5	3	60
Punjab/ICT	10	8	80
Sindh	7	6	85.71

4.7 Detection of OpdK

PCR reaction was used to analyse the outer membrane porin gene i.e., OpdK whose mutation or depletion results in causing resistance in the *E. coli* clinical isolates. The DNA sample was examined and amplified by using the specially designed forward and reverse primers of OpdK gene for all 25 isolates. The PCR product was then run on 1.5 % agarose gel along with 1kb sized DNA ladder for their comparison. The gel was stained and then visualized. Clear bands of OpdK genes having 1255 bp size fragment were seen on the computer screen. Out of 25 isolates of *P. aeruginosa*, OpdK gene was detected in 805 % (n=20) isolates (Figure 4.26A&B).

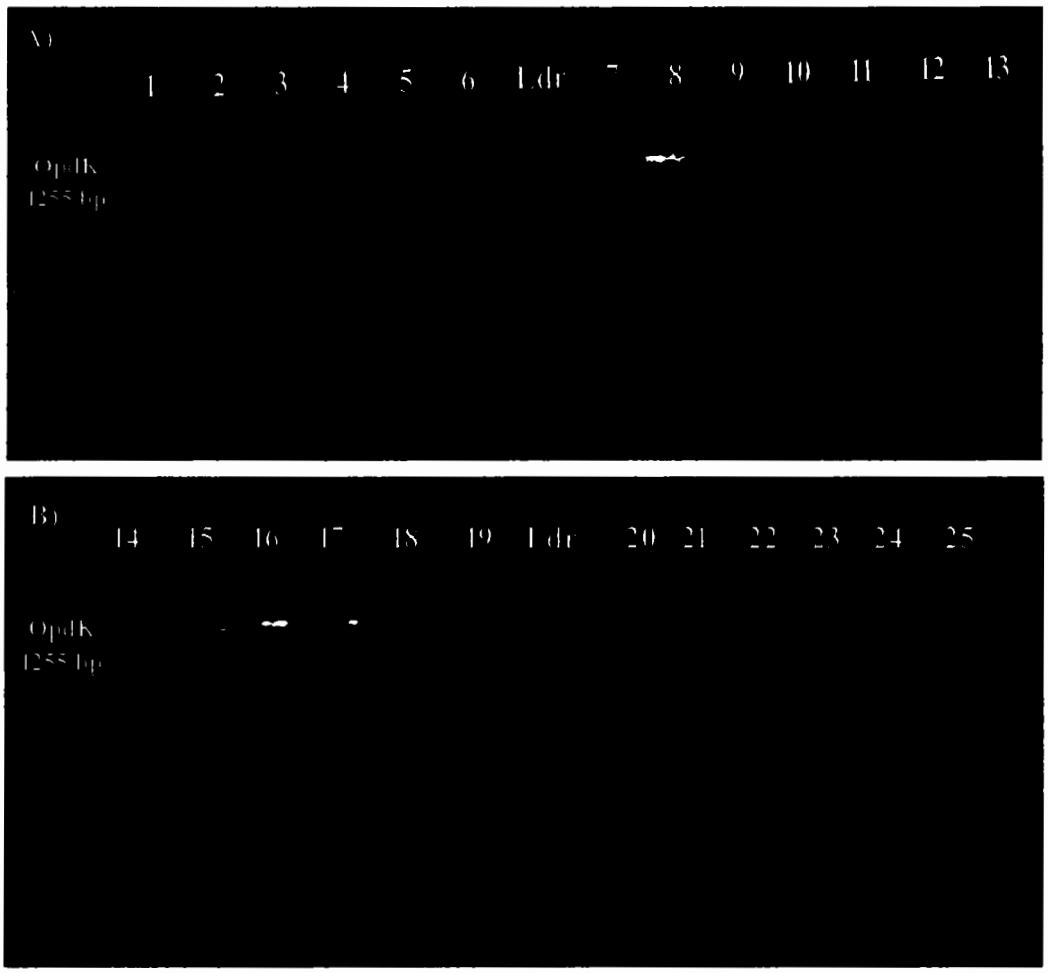

Figure 4.25 ***PCR amplification of OpdK gene: Lanes 1,2, 4-17, 22-25 = positive samples of P. aeruginosa for OpdK (1255 bp).***

Table 4.14 shows PCR results for OpdK gene detection in different provinces of Pakistan. Gene was knocked out in 50 % of isolates of Balochistan and in 40 % of isolates of Punjab/ICT.

Table 4.14 ***Province wise PCR results for OpdK gene detection***

Province	Total Isolates	PCR band detected	%age
Balochistan	4	2	50
KPK	5	5	100
Punjab/ICT	10	6	60
Sindh	6	6	100

4.7.1 Insilico studies of OpdK

Eight (08) out of total 25 *P. aeruginosa* samples were sent for sequencing. The data obtained, in the form of nucleotide sequences, were used further for in-silico studies.

4.7.1.1 Protein translation

ExPASy tool was used for the conversion of nucleotide sequences of OpdK samples into its one letter amino acid sequence. Six results were shown by the program and one of the best reading frames was selected in which the larger number of amino acids were present. The sequence was from 5`-3` in results (from amino acid to carboxy terminus) indicating the direction in which this protein is translated.

1) OpdK-CI-2

GQGKSGVVAGLMLCLSCRASCRGRLPGGCEDRPGPAQLLLQPRFPRPRCRQEPGGRVGAGLHPQ
VQFRLYPGHGRRRPRRHRPVRREAEQRTWHQQFRTSSVAL-RSRGGQLWAPPRGRQTAHFCQPT
EDPKRCSQSFRCRLYHQRPLPPQLFRIRAMFPHMLGPETRCRALQGRVTCADTHPMLARSDAAA
LRTPKSLRASTYARAPK-TTVNFLLSSHCDLESEGALVRTHLPRSIIRASMRAATDSRSGDGNLVE
NNTGGMFPSNSLTDSQPLRALDKSQTDAYRLGQHSSSHPFSLLWHKIATCVRWHKASRPRNKFA
TARRLTTSLAGDGEKDRQKFRLVTNLPCFLPFFTAKETWHGGGFRLPKTKNFGSPPGVWARPDR
KNVLRE-HSRGPPLFLPPPPPKGRGFTGPRQGKLGSF-NFLKFPAKTTQGVKNFVPPSFSPRKSPPLP
PLKFFRAGKKTEGQNLSKKGKPPPQNRGGGGGCYNTRGWW--KKIKK-KIIFFFWGKEPRVVCFY
SCEFFFY-KKEG

2) OpdK-CI-4

DLTLARVEKPFSLLTSPLPMPSSWKMRRPTWSCATTTSTAISATTMPARAWWTSGRRASSSSSVP
AIPRARSPSSSTPSACSA-T-TADVAPSIPIFFLCTMTVARRTTIAASPSPPTYAFRPTNSLSKISSQIFR
Y-RTTSHFFSFTDPRSLNYFPSVNTLRCSSR-SHFIVFYRNRSPSVSPSSSSIDYPLI-PCSRAARVAPF
KLQPPPLHKYLYPYSTLFDRAPAISACSAVISSVHHRCELVTSARVYHDTRPHIMSSL-ASYHLPPL
PFSHSLMIAIPRIVPLISCLSYLLHFPFA-PYRVAAMLLLHCSAVFLTVSDLHSSPHVRACI-INIHLST
SYGHTRTNKCIAQGVFI-FYPLT

3) OpdK-CI-7

GTIPVARS-TLMLACLAAQAAEGGFLEDAKTDLVLRNYYFNRDFLDHHPGKILVYDSSPYLHPPD
QFRPSHAHDLLLLPHLPPALLDVDQLACHHVFPPSPPTPSLYRLPTSFRPPPSPTPLQISPRPMPSPK
YSLKFFSVALSTAPASLIASPFQPDFPPPSPPPYSLLALQLRLPPPIHLPLLPVPTCTSCDL-SQTISTSP
RLPSNPSRLSTATPTCCLIYTPDLSRRSYLAASPPSSAFHTLTAVRPTCTLCCDQPSTTAPSNLLPTA
TRFPPSITSNIPKPLYKHSSSHPSIFPCITFPPFYPPNASARPIAFSAHRHPLTRC-RKKHLNKLTFFPP

SLFLPSSPPKHPRLNVFPYTHNTISFFPSFFMPPNPQNLHE-PTPPPPPPSSTPPPPPIAPR-PGLPHRN
WPNPPNFLNFSPPTPRSKTLFPPSPRKIPPFPLLFSPPQKTKTQIFSPTQPPPPLPAFIVGVLFFFFFFIF
FFFFFIFFFFIFFLFFFFFFFLRPSFLQYSLSFLPISP

4) OpdK-CI-10

QLSC-YMTLPALPRKLPRAASWRMRRPTWSCPTITSTAISATTMPARAWWTSGRRASSSSSVPAIP
RARSASASTPSACSA-S-TADVAPAIPSFFPCTMTVARRTAMGASALPPEEAFLGEGGRSKGSHRK
WLVI-QERPAPS-RSGITLSFPDMDGPATRGGSLDSGSLPNWRPGWARATGEVRALTGGVSLGPG
QSTVQGLPRPTAR-VRVHVGRLAAELA-QRLNAGVGDWTSGETSG-SATLRRHASAS-INR-IALQP
LSICISLRNIDIVRAE-TLHMCSSSDCYRTRRNVRNFMSSSACTASNKPGKGSIQAPFQPMDDLN-L
RGHARPSRTPPPCADTVPEWMQFSRRRCEVPCKNS

5) OpdK-CI-12

PGGDTSASLS-PRKLPRAASWRMRRPTWSCATITSTAISATTMPARAWWTSGRRASSSSSVPAIPR
ARSALASTPSSLFGVNLNSGRGTSNSNLLPFHNDGPPANNYGLLPLAPNLPFWASKFRITKCLPNF
RFLSSKERTPAFVKGSPNFAKVFPPGSPSPDPSGL-SLVRVSSGRYTSVPCCQLLAANVVSHLHML
KGVFAARRGSVISSFQFAELPRLLRRVGASIVGPDYIFGAAFCRHNECAPVSGRAHSDICGDNTLS
DCVVIQSDGQSARSALSLIIPQPPYVL-EALFFLGFQFGPGTKNFYQLGGWHKKVPPDPLVRLCPG
RVPARSAGGGRKTMSAKLIFGVQPVLSFFFKTFPKPKHLAGSVGS-RPKLTISPGPSLGLVSTRKPF
-SLLGGIPPAPPPPPPPPPPTPPPPHPPRGPRGGCCGGDKGEKGVEKT-KIFV-NFWGHQKSPGCFLIG
FSPLLGGEGVPPCGVCPF-PPPPKTQRTEKQTFFIHPPPPPPRRWVGVGFISSKKKKNKKKKKIIKN
NKYYKYKKKIFSLGLGR

6) OpdK-CI-13

PLPCGKNQLSPG-FLPFLANFLF-FTQPTCKLPNYYFNRYFLDHNAGNSLVDEWTHGFIL-FSSSYT
PHSLGILLSSIILFFFSLSSLPITRTTKSHPLLPFFLQTPPIHRASASSPILYSWRSFAQLTTISLSADDL
MTPCFRLLGRYCSGVQYQHLHDSLCIHIPGVDIGSPRFLYTTTDSQMPTPSITNLSPHIRHSTTLLC
LCSTLRVLP-CLAR-

7) OpdK-CI-16

DTRWRCVMLPLPVRAAHAAEGGFLEDAKTDLVLRNYYFNRDFRDHDAGKSLVDEWAQGFILK
FSSGYTPGTVGVGLDAIGLFGVKLNSGRGTSNSELLPLHDDGRAADNYGRVGVAAKLRVSASEL
KIGEMLPDIPLLRYDDGRLLPQTFRGFAVVSRELPGLALQAGRFDAVSLRNSADMQDLSACSAPT
QKSDCFNYAGSYYRFNRDRTLLVL-H-LLDDFCRINSSKLLTTESCTAAPRRQQSECSETSADTTPS
RPETSTAHISREASFRTCISAGITLYRWCCTQGRRPICVRPPV-GPQ-GKNMAKISVLPTSSFFSLPN

KQLPEGLPKTNKFFSCPPGCARPLKAGGGALPPPRLSHPPPPCGPGIAGAPGKMGESSKI-KIAAEH
PRVKIFFPVLPREKNRVP-NFGAREKKEGRFLKGNSPLQIAGGGGGFFNPAGGC

8) OpdK-CI-17

FPCPCLN-SFFPLLPSQAADGVFLYNANTDLILRNYYFNRYFRDHDAGKSLVYECAHGFILKFSSG
YTPGTIRLLLNPISLFRLNLNS-RGTCNSSLLPFRYHIPTANLYRRLPSAAHRLISPGLCHLKILPKM
YLFRSLQTALLRHRFPLSPRFSPPFPAPLRSPYASTPCTSPNSTPHSLPYPTSTLFRPPKLYQFLLSPP
PITPFQPLPHPTPPLSLPPCPPTYRRIFASLLPHPTDPRPLATLRRQPSAILRQPSTNKHPLPSSITSTTP
YLSTAFFFHPSFPLPNLSTLFAAQLLHPPHYFLHPRLLLISCNAKNISTNYLSSHLPFFLQSFPTKPL
AKVFPHPPTPIFSPPPFLFPHPPFLF-LSTSRPPPPPHPPPSPLPPFFRPLPKKISETSKLFKIFPPHPPVS
KPFSPSFPTDIPTPLSPLPSFPPQKKTKSQYFF-KRNLSPPHFPPGYWFSFTKHGCFFLYYIIFKPPLPR
LPFN

4.7.1.2 Comparing Sequences using BLASTN

BLASTN was performed to find the similarity of seven nucleotide sequences of OpdK-CI-2, Opd K-CI-4, OpdK-CI-6, OpdK-CI-6, OpdK-CI-7, OpdK-CI-, 10 OpdK-CI-13, OpdK-CI-16 and Opd K-CI-17. Dozens of results were generated for each query, but we have selected only the top resu lt and added in table 4.15.

Table 4.15 Summary of Nucleotide alignment by BLASTN for OpdK samples

Isolate	GenBank	Source	Query coverage	E value	Per Ident	Acc lens
2	LT673656.1	*P. aeruginosa*	24 %	2e-171	95.37 %	6288645
4	CP047069.1	*P. aeruginosa*	90 %	0.0	96.79 %	6129812
6	CP050331.1	*P. aeruginosa*	29 %	4e-107	88.96 %	6522476
7	CP121766.1	*P. aeruginosa*	11 %	1e-54	89.94 %	7082165
10	CP050331.1	*P. aeruginosa*	29 %	1e-151	97.24 %	6522476
16	CP045002.1	*P. aeruginosa*	49 %	0.0	94.93 %	6716578
17	CP121766.1	*P. aeruginosa*	16 %	2e-72	88.61 %	7082165

4.7.1.3 Mutation analysis

All the samples run for mutation analysis in Clustal omega online software showed a great number of mutations against wild type sequence. Figures 4.26 and 4.27 show protein sequences of samples No 16 and 17 against wild type protein sequences. Both samples resulted in more than 50 mutations individually. Same is the result for samples no 2,4, 7, 10, 12 and 13.

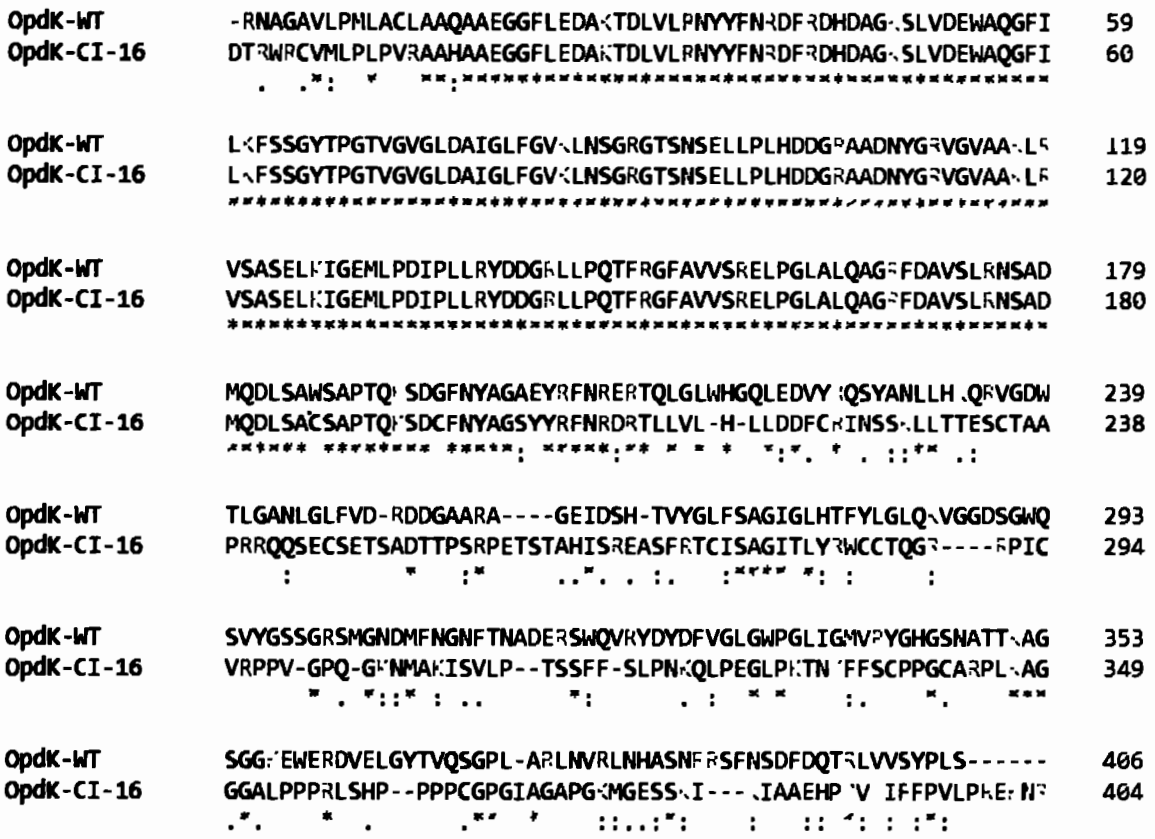

Figure 4.26 ***Protein sequences of sample No 16 against WT sequences***

```
OpdK-WT          -RNAGAVLPMLACLAAQAAEGGFLEDA-TDLVLRNYYFNRDFRDHDAG- SLVDEWAQGFI   59
OpdK-CI-17       FPCPCLN-SFFPLLPSQAADGVFLYNANTDLILRNYYFNRYFRDHDAG SLVYECAHGFI   59
                          ::  * :***:* ** :*:***:********* ********:*** * *:***

OpdK-WT          LKFSSGYTPGTVGVGLDAIGLFGVKLNSGRGTSNSELLPLHDDGFAADNYGRVGVAANLR  119
OpdK-CI-17       LKFSSGYTPGTIRLLLNPISLFRLNLNS-RGTCNSSLLPFRYHIPTANLYRRLPSAAHRL  118
                 **********: : *: *.** ::*** ***.**.***:: .  :*: * *:  **:

OpdK-WT          VSASELKIGEMLPDIPLLRYDDGRLLPQTFRGFAVVSRELPGLALQAGRFDAVSLRNSAD  179
OpdK-CI-17       ISPG-LCHLYILPKMYLFRSLQTALLRHRFPLSPF FSPPFPAPLP--SPYAS--------  167
                 :* . *   ::**.: *:*  :  ** : *     .*  :*.     . : :

OpdK-WT          MQDLSAWSAPTQKSDGFNYAGA-------EYRFNFERTQLG-----------LWHGQLED  221
OpdK-CI-17       ----TPCTSPNSTPHSLPYPTSTLFRPPRLYQFLLSPPPITPFQPLPHPTPPLSLPPCPP  223
                     :  ::*... ..: *  :        *:*  .   :             *

OpdK-WT          VYRQSYANLLHKQRVGDWTLGANLGLFVDRDDGAARAGEIDSHTVYGLFSAGIGLHTFYL  281
OpdK-CI-17       TYRRIFASLLPHPTDPR-----PLATLRRQPSAILRQPSTNAHPLP----SSITSTTPYL  274
                 .**: :*.** :           *. :  : ..  *  . :.* :     :.*   * **

OpdK-WT          GLQKVGGDSGWQSVYGSSGRSMGNDMFNGNFTNADERSWQVRYDYDFVGLGWPGLIGMVA  341
OpdK-CI-17       STAFF-----FHPSFPLP---NLSTLFAAQLLHPPH--Y-------F---LHPRLLLIS-  313
                 .   .     ::  :        . :* .:: :  .  :       *     * *: :

OpdK-WT          YGHGSNATTYAGSGGKFEWERDVELGYTVQSGPLARLNVRLNHA----------------  384
OpdK-CI-17       C-NANNIST-------NY-LSSHLPFFLQSFPT-PLAPVFPHPPTPIFSPPPFLFPHPPF  364
                   :..* :*       ::  . .* : :** *   *   : *

OpdK-WT          ----------------------------------------------SNIRSFNSDFDQTPL  399
OpdK-CI-17       LF-LSTSRPPPPPHPPPSPLPPFFRPLPNKISETSNLFKIFPPHPPVSKPFSPSFPTDIP  423
                   *                                                 : *. .*

OpdK-WT          V-------------------------VSY-PLSW-----------------------  407
OpdK-CI-17       TPLSPLPSFPPQVATYSQYFF--RNLSPPHFPPGYWFSFTHGCFFLYYIIFFPPLPILP  482
                 .                      *       : *  *

OpdK-WT          --      407
OpdK-CI-17       FN      484
```

Figure 4.27 Protein sequences of samples No 17 against WT sequences

4.7.1.4 3D Structures of selected OpdK samples

Due to truncated proteins, as seen in amino acid sequences of these samples, it was impossible to make 3D structures of OpdK porin so no further computational work was carried out.

4.8 Detection of OprD

PCR reaction was used to analyse the outer membrane porin gene i.e., OprD whose mutation or depletion results in causing resistance in the *E. coli* clinical isolates. The DNA sample was examined and amplified by using the specially designed forward and reverse primers of OprD gene

for all 25 isolates. The PCR product was then run on 1.5 % agarose gel along with 1kb sized DNA ladder for their comparison. The gel was stained and then visualized. Clear bands of OprD genes having 1332 bp size fragment were seen on the computer screen. Out of 25 isolates of *P. aeruginosa*, OprD gene was detected in 76.0 % (n=19) isolates (Figure 4.29A&B).

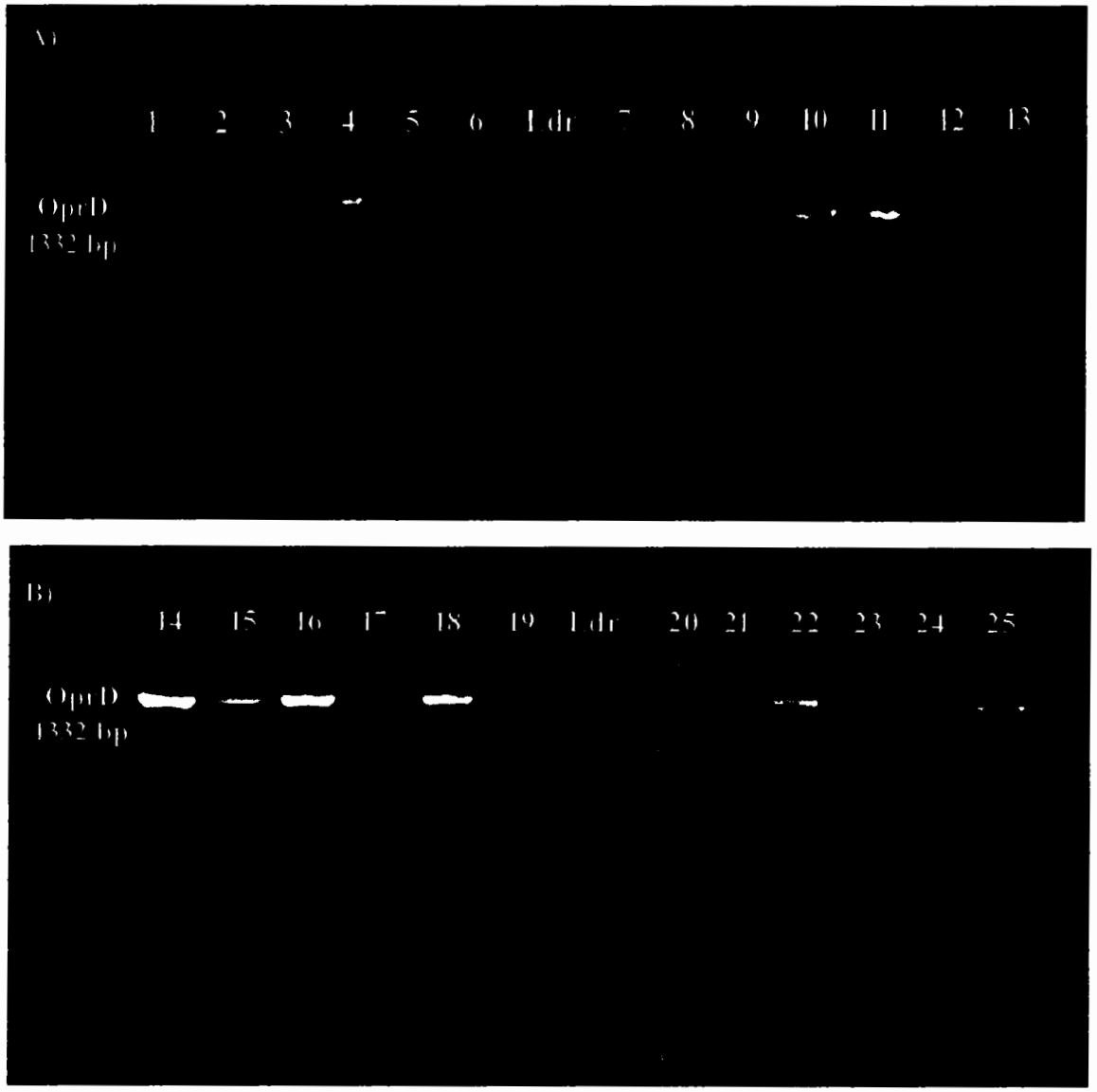

Figure 4.28 (A&B) PCR amplification of OprD gene: Lanes 1, 4-16, 18, 22-25 = positive samples of P. aeruginosa for OprD (1332 bp).

Table 4.16 shows PCR results for OprD gene detection in different provinces of Pakistan. Gene was knocked out in 50 % of isolates of Balochistan and in 30 % of isolates of Punjab/ICT.

Table 4.16 Province wise PCR results for OprD gene detection

Province	Total Isolates	PCR band detected	%age
Balochistan	4	2	50
KPK	5	5	100
Punjab/ICT	10	7	70
Sindh	6	6	100

4.8.1 Insilico studies of OprD

Eight (08) out of total 25 *P. aeruginosa* samples were sent for sequencing. The data obtained, in the form of nucleotide sequences, were used further for in-silico studies.

4.8.1.1 Protein translation

ExPASy tool was used for the conversion of nucleotide sequences of OprD samples into its one letter amino acid sequence. Six results were shown by the program and one of the best reading frames was selected in which the larger number of amino acids were present. The sequence was from 5`-3` in results (from amino acid to carboxy terminus) indicating the direction in which this protein is translated.

1) OprD-CI-4

DAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSGDRVDWTQGFLTTYESGFTQGTVGFGVD
AFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDYSRAGGAVKVRISKTMLKWGEMQPTAPVFA
AGGSRLFPQTATGFQLQSSELEGLDLEAGHFTEGKQGTTTKSRGELYATYAGETAKSADFIGGRY
AITDNLSASLYGAELEDIYRQYYLNSNYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLA
AAYTLDAHTFTLAYQKVHGDQPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARY
DLNLASYGVPGLTFMVRYINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGP
AKDLSFRIRQAWHRANADQAEGDQNEFRLIVDYPL

2) OprD-CI-6

DAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSGDRVDWTQGFLTTYESGFTQGTVGFGVD
AFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDYSRAGGAVKVRISKTMLKWGEMQPTAPVFA
AGGSRLFPQTATGFQLQSSELEGLDLEAGHFTEGKQGTTTKSRGELYATYAGETAKSADFIGGRY
AITDNLSASLYGAELEDIYRQYYLNSNYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLA
AAYTLDAHTFTLAYQKVHGDQPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARY
DLNLASYGVPGLTFMVRYINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGP
AKDLSFRIRQAWHRANADQAEGDQNEFRLIVDYPL

3) OprD-CI-7

DAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSGDRVDWTQGFLTTYESGFTQGTVGFGVD
AFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDYSRAGGAVKVRISKTMLKWGEMQPTAPVFA
AGGSRLFPQTATGFQLQSSELEGLDLEAGHFTEGKQGTTTKSRGELYATYAGETAKSADFIGGRY
AITDNLSASLYGAELEDIYRQYYLNSNYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLA
AAYTLDAHTFTLAYQKVHGDQPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARY
DLNLASYGVPGLTFMVRYINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGP
AKDLSFRIRQAWHRANADQAEGDQNEFRLIVDYPLS

4) OprD-CI-10

DAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSGDRVDWTQGFLTTYESGFTQGTVGFGVD
AFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDYSRAGGAVKVRISKTMLKWGEMQPTAPVFA
AGGSRLFPQTATGFQLQSSELEGLDLEAGHFTEGKQGTTTKSRGELYATYAGETAKSADFIGGRY
AITDNLSASLYGAELEDIYRQYYLNSNYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLA
AAYTLDAHTFTLAYQKVHGDQPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARY
DLNLASYGVPGLTFMVRYINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGP
AKDLSFRIRQAWHRANADQAEGDQNEFRLIVDY

5) OprD-CI-12

DAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSGDRVDWTQGFLTTYESGFTQGTVGFGVD
AFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDYSRAGGAVKVRISKTMLKWGEMQPTAPVFA
AGGSRLFPQTATGFQLQSSELEGLDLEAGHFTEGKQGTTTKSRGELYATYAGETAKSADLQSPIT
SAPPCTVLNSKTSIVSIT-TATTPSHWHPTNRWASISTSTAQTMKARPRPATSATPLGPWRQPTLW
MRTLSPWPTRRSMAISRLIISASARTVPAAAVTRFSSPTPCSTPTSTAPARNPGRPATT-TSPPMAFP
A-LSWSAISMARTSMAPRCLTTTSAIRTTATARTASTTRPTSKPSTWSSPVRPRTCRSASARPGTAP
TPTRPKATRTSSA-SSTIRCR

6) OprD-CI-13

DAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSGDRVDWTQGFLTTYESGFTQGTVGFGVD
AFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDYSRAGGAVKVRISKTMLKWGEMQPTAPVFA
AGGSRLFPQTATGFQLQSSELEGLDLEAGHFTEGKQGTTTKSRGELYATYAGETAKSADLQSPIT
SAPPCTVLNSKTSIVSIT-TATTPSHWHPTNRWASISTSTAQTMKARPRPATSATPLGPWRQPTLW
MRTLSPWPTRRSMAISRLIISASARTVPAAAVTRFSSPTPCSTPTSTAPARNPGRPATT-TSPPMAFP
A-LSWSAISMARTSMAPRCLTTTSAIRTTATARTASTTRPTSKPSTWSSPVRPRTCRSASARPGTAP
TPTRPKATRTSSA-SSTIRCR (3' to 5' frame)

7) **OprD-CI-16**

RQRIVDDQAELVLVAFGLVGVGAVPGLADAERQVLGRTGLDHVLGFQVGLVVLAVLAVAVVLI
ADVVVRHLGAIDVLAIDIADHESQAGNAIGGEVQVVAGLPGFLAGAVEVGVLHGVGEENRVTA
AAGTVLAEADIIKRLIAMDLLVGQGESVRIQSVGCRQGPSGVADVAGLGLAFIVCAVDVEIEAQR
LVGCQWDGVVAVQVILTIDVFEFSTVQGGAEVIGDCVAAPNEIGALGGLACIGCVEFAARLGGG
ALLAFGEVARFEIEPFEFAALQLEAGRGLREQAAAAGGEDRRSRPTSTWSWRCVPSRRHRRGCS
RHGACRRSSPAGCRYRSCPRCRRASDRGSRRHRRRSPRCLG-SRIHRW-GSLGSSRRGPRCRSCRH
G-NSSCGAAGRGCCLR-TPSLRPDR-RMRRPRRTECYLRKP

8) **OprD-CI-18**

DAFVSDQAEAKGFIEDSSLDLLLRNYYFNRDGKSGSGDRVDWTQGFLTTYESGFTQGTVGFGVD
AFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDYSRAGGAVKVRISKTMLKWGEMQPTAPVFA
AGGSRLFPQTATGFQLQSSELEGLDLEAGHFTEGKQGTTTKSRGELYATYAGETAKSADFIGGRY
AITDNLSASLYGAELEDIYRQYYLNSNYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISNTTWSLA
AAYTLDAHTFTLAYQKVHGDQPFDYIGFGENGSGGGGDSIFLANSVQYSDFNGPGEKSWQARY
DLNLASYGVPGLTFMVRYINGKDIDGTKMSDNNVGYKNYGYGEDGKHHETNLEAKYVVQSGP
AKDLSFRIRQAWHRANADQAEGDQNEFRLIVDYP

4.8.1.2 Comparing sequences using BLASTN

BLASTN was performed to find the similarity of eight nucleotide sequences of OprD-CI-4, OprD -CI-6, OprD-CI-7, OprD-CI-10, OprD-CI-12, OprD-CI-13, OprD-CI-16 and OprD-CI-18. Dozen s of results were generated for each query, but we have selected only the top result and added in t able 4.17. All samples had 100 % query coverage with more than 98 % percent identification. All the matching results had OprD genes in their results confirming our result too.

Table 4.17 Summary of Nucleotide alignment by BLASTN for OprD samples.

Isolate	GenBank	Source	Query coverage	E value	Per Ident	Acc lens	Gene
4	CP046402.2	*P. aeruginosa*	100 %	0.0	100.00 %	7056430	OprD
6	CP046402.2	*P. aeruginosa*	100 %	0.0	100.00 %	7056430	OprD
7	CP046402.2	*P. aeruginosa*	100 %	0.0	100.00 %	7056430	OprD
10	CP046402.2	*P. aeruginosa*	100 %	0.0	100.00 %	7056430	OprD
12	CP039293.1	*P. aeruginosa*	100 %	0.0	98.77 %	6879622	OprD
13	CP039293.1	*P. aeruginosa*	100 %	0.0	98.76 %	6879622	OprD

16	CP041772.1	*P. aeruginosa*	100 %	0.0	99.15 %	6514208	OprD
18	CP046402.2	*P. aeruginosa*	100 %	0.0	100.00 %	7056430	OprD

4.8.1.3 3D Structures of selected OprD samples

Phyre2 tool was used for the prediction of protein 3D structure. Figure 4.30A-D represents the 3D structures of OprD *P. aeruginosa* CIs based on c2odjA template.

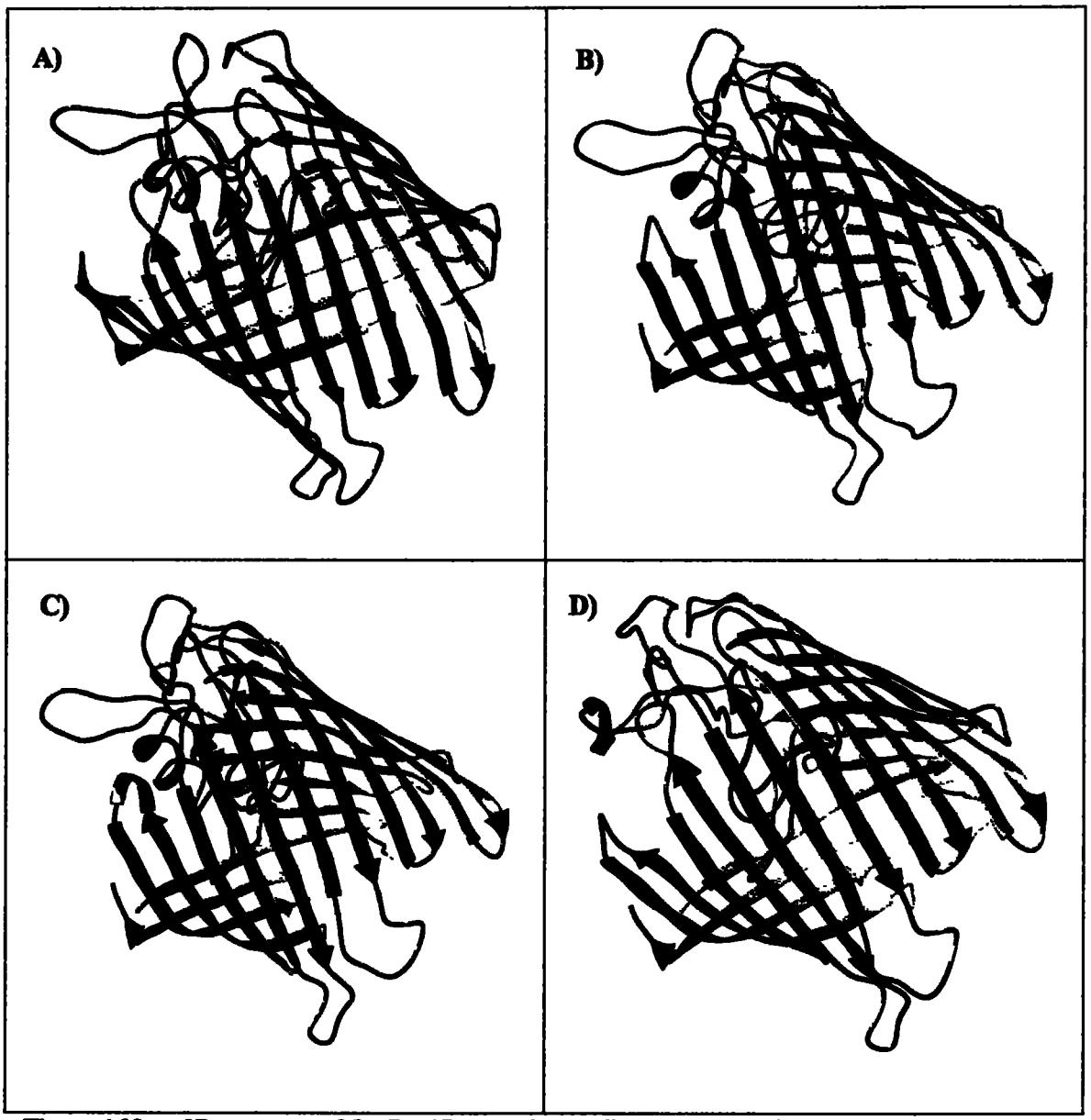

Figure 4.29 3D structure of OprD of P. aeruginosa clinical isolates A) CI-4, B) CI-6, C) CI-7 and D) CI-10

4.8.1.4 Mutation analysis

The Clustal omega tool was used to compare OprD nucleotide and protein sequences of both wild type and clinical isolates. As a result, various number of point mutations were observed both in nucleotide sequences and protein sequences.

UCSF Chimera was used to highlight the mutated amino acids on 3D image of the protein. We are presenting here only four elected examples of isolates no 4 (OprD-CI-4), 6 (OprD-CI-6), 7 (OprD -CI-7), and 10 (OprD -CI-10) as given below from figure 4.30 to figure 4.37.

4.8.1.4.1 OprD-CI-4

Figure 4.30 Protein alignment results of Clustal omega by comparing OprD-CI-4 and OmpC-WT.

The details of alterations in amino acids of OprD of isolate No 4 in comparison to wild type is given in table 4.18.

Table 4.18 *Alteration in the amino acids by comparing OprD-CI-4 and OprD-WT*

S.No	Change in Amino Acid
1	Thr 80 Ser
2	Lys 92 Thr
3	Phe 147 Leu
4	Glu 162 Gln
5	Pro 163 Gly
6	Val 166 Thr
7	Arg 287 Glu
8	Ala 292 Gly
9	Gly 402 Ala

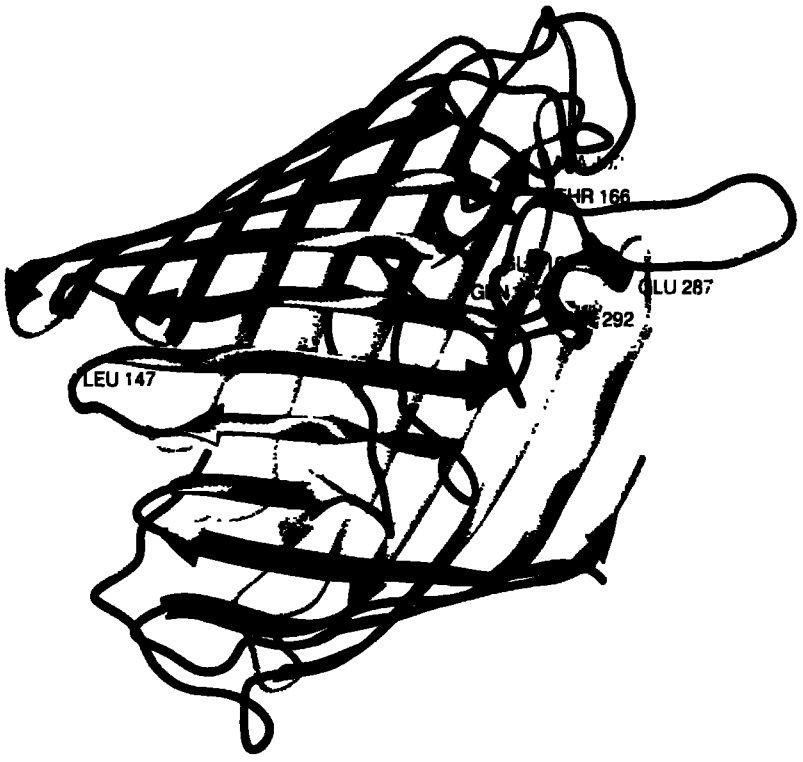

Figure 4.31 **CI-4 of *P. aeruginosa* having OprD mutations at different regions as highlighted in the *image*.**

4.8.1.4.2 OprD-CI-6

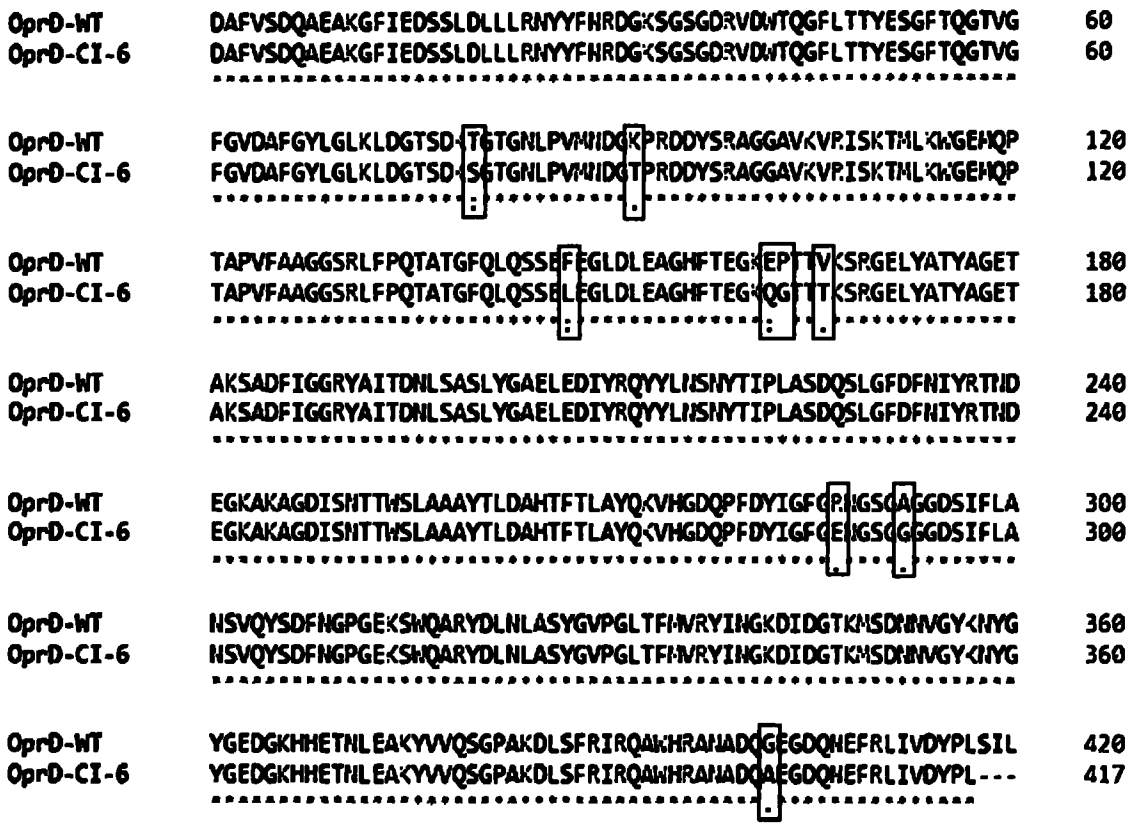

Figure 4.32 Protein alignment results of Clustal omega by comparing OprD-CI-6 and OmpC-WT.

The details of alterations in amino acids of OprD of isolate No 6 in comparison to wild type is given in table 4.19.

Table 4.19 Alteration in the amino acids by comparing OprD-CI-6 and OprD-WT

S.No	Change in Amino Acid
1	Thr 80 Ser
2	Lys 92 Thr
3	Phe 147 Leu
4	Glu 162 Gln
5	Pro 163 Gly
6	Val 166 Thr
7	Arg 287 Glu
8	Ala 292 Gly
9	Gly 402 Ala

Figure 4.33 CI-6 of P. aeruginosa having OprD mutations at different regions as highlighted in the image.

4.8.1.4.3 OprD-CI-7

The details of alterations in amino acids of OprD of isolate No 7 in comparison to wild type is given in table 4.17.

```
OprD-WT      DAFVSDQAEA<GFIEDSSLDLLLRNYYFNRDG<SGSGDRVDWTQGFLTTYESGFTQGTVG  60
OprD-CI-7    DAFVSDQAEA<GFIEDSSLDLLLRNYYFNRDG<SGSGDRVDWTQGFLTTYESGFTQGTVG  60
             ************************************************************

OprD-WT      FGVDAFGYLGLKLDGTSD-TGTGNLPVWNDG<PRDDYSRAGGAVKVRISKTWL<WGEWQP  120
OprD-CI-7    FGVDAFGYLGLKLDGTSD-SGTGNLPVWNDG<PRDDYSRAGGAVKVRISKTWL<WGEWQP  120
             ********************.***********.************************.***

OprD-WT      TAPVFAAGGSRLFPQTATGFQLQSSEFEGLDLEAGHFTEG<EPTTVKSRGELYATYAGET  180
OprD-CI-7    TAPVFAAGGSRLFPQTATGFQLQSSELEGLDLEAGHFTEG<QGTTTKSRGELYATYAGET  180
             ************************.***********.**.*********************

OprD-WT      AKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNSNYTIPLASDQSLGFDFNIYRTND  240
OprD-CI-7    AKSADFIGGRYAITDNLSASLYGAELEDIYRQYYLNSNYTIPLASDQSLGFDFNIYRTND  240
             ************************************************************

OprD-WT      EGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQ<VHGDQPFDYIGFGRNGSGAGGDSIFLA  300
OprD-CI-7    EGKAKAGDISNTTWSLAAAYTLDAHTFTLAYQ<VHGDQPFDYIGFGENGSGGGGDSIFLA  300
             ************************************************.*.*.*********

OprD-WT      HSVQYSDFNGPGE<SHQARYDLNLASYGVPGLTFNVRYING<DIDGTKMSDNNVGY<NYG  360
OprD-CI-7    HSVQYSDFNGPGE<SHQARYDLNLASYGVPGLTFNVRYING<DIDGTKMSDNNVGY<NYG  360
             ************************************************************

OprD-WT      YGEDGKHHETNLEA<YVVQSGPAKDLSFRIRQAWHRANADQGEGDQNEFRLIVDYPLSIL  420
OprD-CI-7    YGEDGKHHETNLEA<YVVQSGPAKDLSFRIRQAWHRANADQAEGDQNEFRLIVDYPLS--  418
             ************************************.*.*************************
```

Figure 4.34 Protein alignment results of Clustal omega by comparing OprD-CI-7 and OmpC-WT.

The details of alterations in amino acids of OprD of isolate No 7 in comparison to wild type is given in table 4.20.

Table 4.20 Alteration in the amino acids by comparing OprD-CI-7 and OprD-WT

S.No	Change in Amino Acid
1	Thr 80 Ser
2	Lys 92 Thr
3	Phe 147 Leu
4	Glu 162 Gln
5	Pro 163 Gly
6	Val 166 Thr
7	Arg 287 Glu
8	Ala 292 Gly
9	Gly 402 Ala

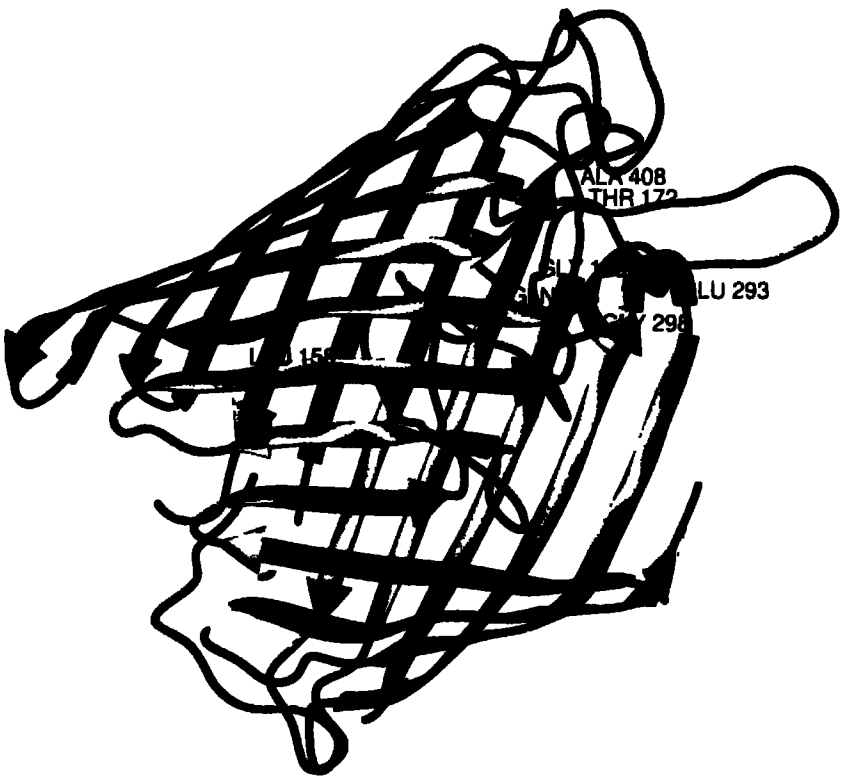

Figure 4.35 CI-7 of P. aeruginosa having OprD mutations at different regions as highlighted in the image.

4.8.1.4.4 OprD-CI-10

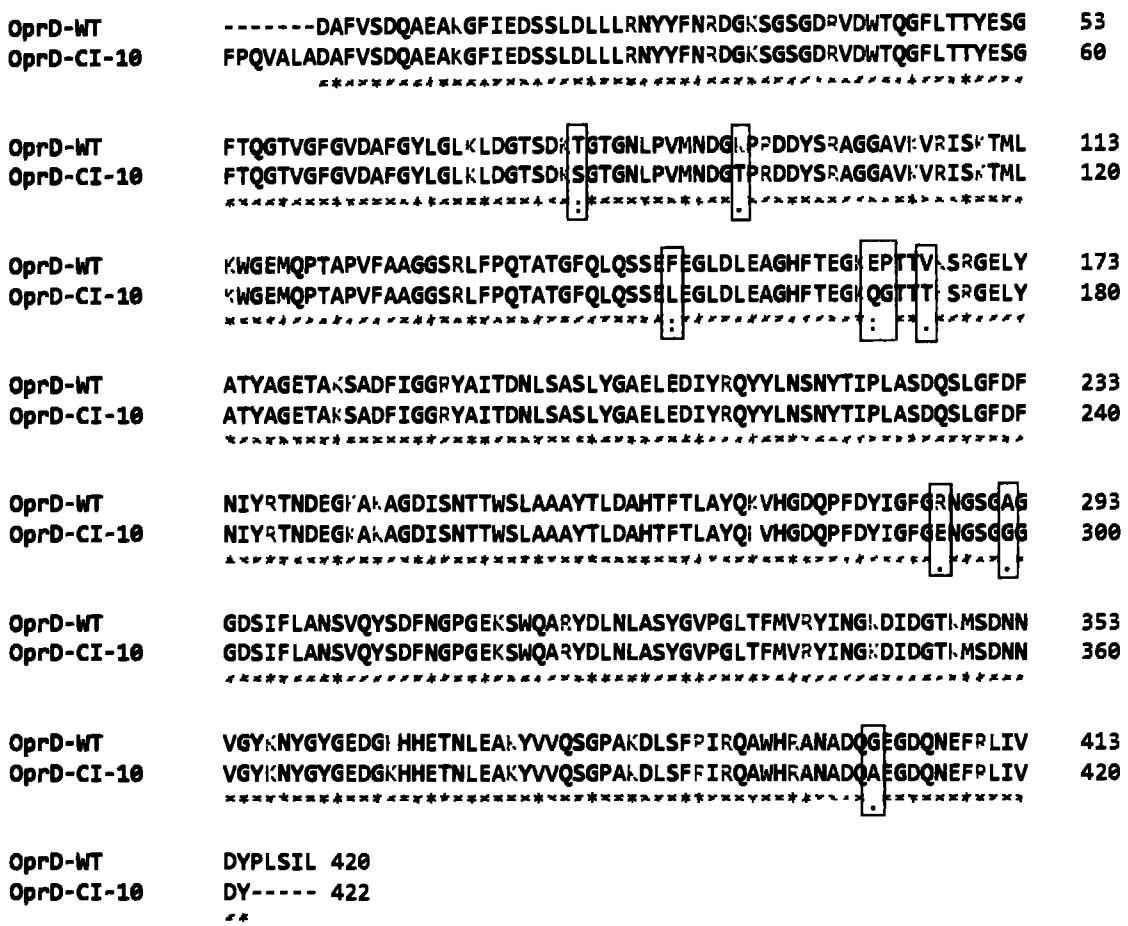

Figure 4.36 Protein alignment results of Clustal omega by comparing OprD-CI-10 and OmpC-WT.

The details of alterations in amino acids of OprD of isolate No 10 in comparison to wild type is given in table 4.21.

Table 4.21 Alteration in the amino acids by comparing OprD-CI-10 and OprD-WT

S.No	Change in Amino Acid
1	Thr 80 Ser
2	Lys 92 Thr
3	Phe 147 Leu
4	Glu 162 Gln
5	Pro 163 Gly
6	Val 166 Thr
7	Arg 287 Glu
8	Ala 292 Gly
9	Gly 402 Ala

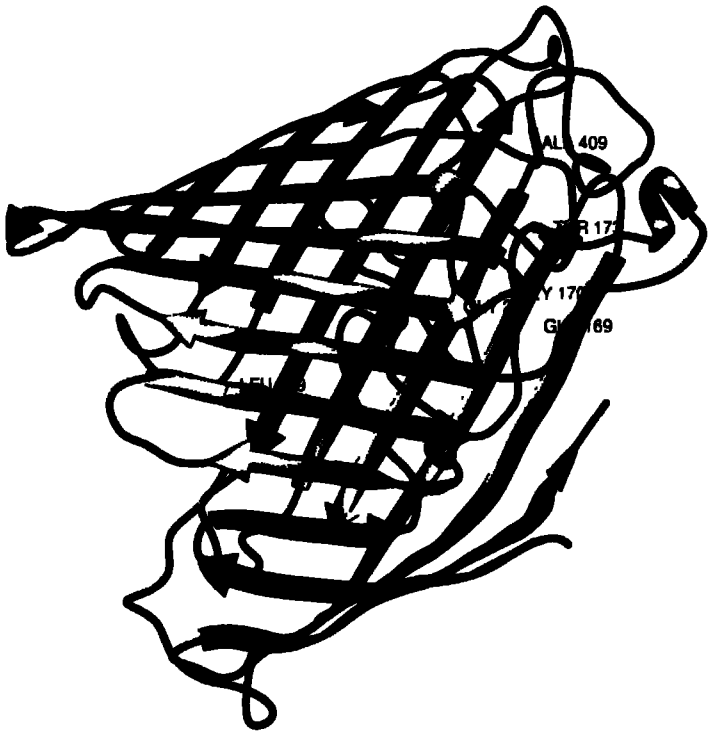

Figure 4.37 CI-10 of P. aeruginosa having OprD mutations at different regions as highlighted in the image.

Chapter 5
Discussion

5 Discussion

The rise of microbial strains that are resistant to antibiotics has alarmed the world and the WHO. This issue is currently spreading like wildfire. Antimicrobial resistance to infectious pathogens rises yearly in the majority of nations. The growth in antimicrobial resistance has been linked to an increase in the use of antibiotics due to a widespread lack of awareness of the need for care in practice.

Antibiotic use in clinical and agricultural contexts leads to the development of antibiotic-resistant bacteria, which pose a severe danger to the treatment of bacterial illnesses. Over the past 20 years, *E. coli*, a Gram-negative bacterium, has become one of the most significant health risks (Padmini *et al.*, 2017) so is the case with *P. aeruginosa*. It has become a global issue that *E. coli* and *P. aeruginosa* are resistant to various drugs at the same time. For controlling and establishing strategies in this area, regional monitoring is essential.

Our study is first kind of lab study where samples were collected from whole Pakistan. Before this, city wise or province wise studies has been done but not an exclusive one, covering all the provinces of Pakistan. For this study clinical isolates of *E. coli* and *P. aeruginosa* were sampled from major cities of four provinces of Pakistan. The isolates were re-cultured in lab and antibiotics from different classes were used to find out their resistance against commonly used antibiotics. Our results revealed Meropenem, Imipenem and Amikacin as the most effective antibiotics against all *E. coli* and *P. aeruginosa* strains.

Doripenem was a more powerful antibiotic to act against *P. aeruginosa* strains with 64 % efficacy. Carbapenems are the most efficient antimicrobials and are regarded as the final line of defense against MDR pathogenic bacteria, according to several research that have been published (Lyman *et al.*, 2015). In research by Ali (2019), which examined the effectiveness of several antimicrobials against strains of *P. aeruginosa*, imipenem and meropenem (carbapenems) emerged as the most efficient ones. Shaikh *et al.*, (2015) and Ameen *et al.*, (2017) earlier reported similar findings. In Uganda, the prevalence of *P. aeruginosa* isolates resistant to carbapenem was 24 % (Kateete *et al.*, 2016), which is lower than the prevalence found in the current investigation Imipenem (40 %) and Meropenem (40 %) and Doripenem (36 %). *P. aeruginosa* is the second-most carbapenem-resistant organism, with a frequency of 60.3 %, according to a study by Cai *et al.*, (2017) in the United States, while Vaez *et al.*, (2017) observed a prevalence of 54 % imipenem-resistant *P.*

aeruginosa in the Iranian population. Mubashir *et al.*, (2021 reported 60 % *P. aeruginosa* resistance against Imipenem in their study. According to research done in Khyber Pakhtun Khuwa 41.7 % of patients showed resistance to Imipenem (Muhammad *et al.*, 2020). Ahmadi *et al.*, 2016 reported Meropenem (2.3 %) and Imipenem (2.9 %), having the highest efficacy rates.

Among fluoroquinolones, CIP was not very effective in *P. aeruginosa* strains in our study (72 % resistance) which is in line with many other studies. Ahmadi *et al.*, (2016) also reported the greatest rates of resistance to Ciprofloxacin (82.5 %). Contrary to our findings, Saleh *et al.*, reported 74.6 % susceptibility for ciprofloxacin (Saleh & Balboula, 2017). Ciprofloxacin showed good activity (74.33 % susceptibility) in study by Ali (2019). Norfloxacin also showed 60 % resistance in our study while Levofloxacin showed 48 % resistance.

In current study resistance to penicillin group of antibiotic ticarcillin was 60 % by *P. aeruginosa* species much higher than the reported values of Ali (2019) who reported 26.20 %. Bavasheh and Karmostaji (2017) reported 34.7 % resistance to ticarcillin. This finding is in close agreement to the report of Llanes *et al.*, who investigated *P. aeruginosa* isolates from cystic fibrosis in France (Llanes *et al.*, 2013).

Aminoglycosides, amikacin and gentamicin showed 36 % and 40 % resistance respectively in the current study which are much higher than other regional studies. According to research done in Khyber Pakhtun Khuwa, 8.3 % of patients showed resistance to Amikacin (Muhammad *et al.*, 2020). A study from Isfahan reported relatively higher resistance to gentamicin (60 %) and Amikacin (70 %) (Golshani, *et al.*, 2012). Ahmadi *et al.*, (2016) also reported the greatest rates of resistance to gentamicin (89.5 %) and Amikacin (77.3 %).

The most efficient antibiotics were Amikacin (72 %), Imipenem (80 %) and Meropenem (84 %) against *E. coli* strains. Iqbal *et al.*, (2021) has reported 71 % meropenem susceptibility rate in *E. coli* species. According to a study by Mubashir *et al.*, (2021), *E. coli* has lower resistance rates to Imipenem (7.4 %) and Amikacin (13.2 %). Sohail *et al.*, (2015) also found reduced *E. coli* resistance to Imipenem (3 %). Jail and Al Atbee (2022) found a substantially lower level of resistance to imipenem in their study conducted in Basrah, Iraq. A study by Sultana *et al.*, (2023) showed that *E. coli* showed greater Amikacin resistance which is contrary to our study where Amikacin showed only 19 % resistance. It has already been declared by world scientists that we are limited to the last resource antibiotic classes, such as polymyxins and carbapenems, as Extended Spectrum β-lactamase (ESBL) and AmpC-producing *E. coli* are more frequently

reported as the cause of severe infections (Mikhayel *et al.*, 2021; Iseppi *et al.*, 2020). In contrast, considerable *E. coli* AK resistance (91 %) was noted in research by Muhammad *et al.*, in 2020.

The highest resistance was shown by Ceftriaxone (96 %), Ampicillin (96 %) and cephradine (96 %), Aztreonam (92 %), Cefepime (88 %) and Ciprofloxacin (85 %) against *E. coli* strains. Same results were reported by Iqbal *et al.*, (2021) where more than 80 % of uropathogenic strains were found to be resistant to Ceftriaxone and Ciprofloxacin. A study conducted in the USA showed same highest resistance against Ampicillin (97.8 %) (Karlowsky *et al.*, 2002). Comparatively lower rates of Ampicillin resistance (55 %) were found in *E. coli* isolates according to a UK investigation by Bean *et al.*, (2008). Alanazi *et al.*, (2018) found high Ciprofloxacin (72.7 %) and lower Ampicillin (42.85 %) resistance in their study (Alanazi *et al.*, 2018). In India, the resistance rates were found as 46 % in Ciprofloxacin, much lower than our results (Manikandan *et al.*, 2011). A study by Sultana *et al.*, (2023) showed that *E. coli* showed greater Ampicillin and Ciprofloxacin resistance. In a study conducted in hospital of Basrah, Iraq, the *E. coli* isolates were found to be highly resistant for ceftriaxone, ampicillin, levofloxacin, aztreonam (Jail and Al Atbee, 2022) which is much like our study.

Gentamicin showed 58 % resistance in our study much closer to the value stated by Manikandan *et al.*, (2011), where the resistance rates were found as 48.8 % in India for Gentamicin. Same was found by Jail and Al Atbee (2022) in Basrah, Iraq where *E. coli* isolates were found to be highly resistant for gentamicin. According to research carried out by Bashir *et al.*, in Pakistan in 2008, *E. coli* showed 47 % resistance pattern against gentamicin.

76 % (n=19) of *P. aeruginosa* samples were MDR in our study. This is a lot higher than the number given by the Pakistan-based research conducted by Ali (2019). From Hamadan West in Iran, Alikhani *et al.*, (2014) discovered 88.7 % MDR clinical isolates of *P. aeruginosa*. In the Brazilian Uberlandia university hospital, Dantas *et al.*, (2017) reported finding 42.7 % MDR isolates of *P. aeruginosa*. MDR isolates (89.87 %) were discovered in a Chinese Burns Unit (Dou *et al.*, 2017). The frequency of *P. aeruginosa* MDR clinical isolates from these nations is somewhat higher than the current reported rate (76 %) from Pakistan. Multi-drug resistant bacteria are a serious issue on a global scale because they raise healthcare costs (Alnour and Ahmed-Abakur, 2017).

In our study, a MAR index value larger than 0.25 was seen in 68 % (n=17) of the *P. aeruginosa* isolates. In contrast to our study, the proportion for the MAR index (54.54 %) is low in a Pakistani study by Ali (2019). A higher MAR index value indicates overuse of antibiotics in a place, which

promotes the development of resistant bacteria (Paul *et al.*, 1997; Adegoke *et al.*, 2016). According to Guvensen *et al.*, (2017), 51.92 % (n=27) of the *P. aeruginosa* isolates from Turkey, had MAR indices greater than 0.2. In an Indian study, 117 individuals were found to have 49 % clinical Gram-negative bacilli with a MAR score more than 0.2. (Bhuvaneshwari, 2017). Imanah *et al.*, (2017) reported 96 samples of *P. aeruginosa* isolated from drains of five hospitals in Nigeria, with MAR indices ranging from 0.33 to 0.89. This indicated a substantial likelihood of bacterial strains that are resistant to several drugs spreading through hospital drains. For *P. aeruginosa* isolates found in clinical specimens and hospital environments in Nigeria, Chika *et al.*, (2017) reported MAR index values of 0.6 to 0.9. This high MAR index, which was reported from Nigerian hospitals, revealed that antibiotics are used excessively in Nigeria. Antibiotic usage restrictions, appropriate management, and surveillance by the government and hospital administration might stop the development of *P. aeruginosa*, a bacterium that is multidrug resistant.

DNA extraction was done for all the samples by using extraction kit and then OmpF, OmpC, OmpW genes of *E. coli* and, OpdK and OprD genes of *P. aeruginosa* were amplified using Polymerase Chain Reaction. Results revealed that no band was visible in 26.92 % of OmpW, 24 % of OprD, 20 % of OpdK, 7.69 % of OmpF and 3.85 % of OmpC samples. After amplification selected PCR products of OmpC, OmpF, OprD and OpdK were sequenced by using Sanger sequencing method. Mutation in OmpC of clinical isolate was determined by using in silico study. For computational analysis of these porins different tools were used such as Clustal Omega, BLASTN, ExPASy, Pyre2 and UCSF Chimera etc.

BLASTN was used to compare our nucleotide sequences with sequences that have already been reported and stored in their database. All our OmpC, OmpF and OprD results matched with the same genes confirming our results. For OpdK, the same result was not obtained, and database showed no result of OpdK in their records, but OprD was seen in the results giving us a hint because OprD protein's overall structure and that of OpdK are extremely similar (Biswas *et al.*, 2008; Biswas *et al.*, 2007). The Phyre2 showed different structural organization of OpdK as compared to OprD.

By comparing protein sequences of our clinical isolated with wild types, various mutations were determined in residues position for all the sequenced samples of OmpF, OmpC, OpdK and OprD. We were not able to make 3D structure of OmpF or OpdK proteins because of presence of stop codons/ truncated protein but 3D models of OmpF and OprD were made up with Phyre2 online

tool. UCSF Chimera was later used to highlight the mutated amino acid regions as compared to WT structures. These amino acids are involved in protein interaction. So, these mutations are considered as damaging to structure and function of outer membrane protein. These mutations may decrease the stability of outer membrane protein. Previous studies revealed that due to decreased in protein stability cause misfolding, aggregation and degradation of protein structure and function (Du *et al.*, 2005).

All the four 3D structures of OprD porins show mutations at the same nine amino acid positions i.e. Thr was replaced by Ser at position 80, Lys by Thr at position 92, Phe by Leu at position 147, Glu by Gln at position162, Pro by Gly at position 163, Val by Thr at position 166 and Arg by Glu at position 287. Previous studies suggested that OmpC play an important role in the transport of various antibiotics such as β-lactams. OmpC deletion increases the MIC of various antibiotics. Furthermore, it's also involved to adapt envelop for stress (Choi and Lee, 2019). Outer membrane protein alteration plays an important role in resistance development against various antibiotics, but various reports describe the diminished expression of porins in clinical isolates (Delcour, 2009).

Some other bacterial strains developed resistance against certain antibiotics such as, β-lactam, tetracycline, cephalosporins and imipenem due to changes occurred in porin channels by mutation (Reygaert, 2018).

The major cause of antibiotic resistance in Pakistan is the overuse of antibiotics in medicine, agriculture, and animal feeds. In Pakistan, antibiotics are freely accessible from pharmacies; street sellers even sell them. Use of antibiotics without a doctor's note may promote the development of bacterial resistance (Ur Rahman *et al.*, 2018). A severe health issue is the spread of MDR *P. aeruginosa* strains worldwide. In order to fight MDR isolates, tight monitoring, limitations on inappropriate antibiotic usage, and adherence to infection control procedures can all be helpful.

Responsible Higher authorities should establish appropriate antibiotic policies, segregation of the sick patients, and stringent sanitary conditions in healthcare facilities and hospitals to avoid the spread of these resistant bacteria in order to address these public health challenges.

The data will be significantly beneficial for devising healthcare policies in relevant areas of Pakistan.

Recommendations

Antibiotic resistance is a critical public health issue that has to be addressed since it has an enormous effect on both individual and societal health. This study suggests that, following the completion of more research, policies be developed for use in hospitals and private clinics to prevent the spread of resistant bacteria and the indiscriminate use of antibiotics. a demand to identify antibiotic-resistant bacterial strains, assess the genetic underpinnings of resistance patterns distributed inside Pakistan, and contrast such patterns with those in other nations.

The Patients' MDR infections need to be under control, and this may be done by employing evidence-based monitoring of therapies for associated bacteria and raising knowledge of the proper use of antibiotics.

The prevalence and severity of infections and drug resistance are still unclear. As a result, it's critical to comprehend the gravity of the genetic elements involved in Gram-negative isolates as well as the risk factors that affect antibiotic resistance in Pakistan. The baseline data from this retrospective study may be utilized to evaluate Pakistan's current level of antibiotic resistance.

Conclusion

Our data shows that most of the pathogenic *Escherichia* and *P. aeruginosa* strains isolated in this study are resistant to Ceftriaxone, Ampicillin, Cephradine, Cefepime while *P. aeruginosa* strains isolated in this study are resistant to Norfloxacin, Ticarcillin, Ceftazidime, Aztreonam and Ciprofloxacin, suggesting that such antibiotic treatment should be suspended following extensive long-term evaluations. To stop the spread of antibiotic resistance in these two clinically significant bacterial genera, inaccurate diagnosis and self-medication should be avoided. The genetic characterization revealed that antibiotic resistance has resulted in mutations and alterations of amino acid sequences in OMP of these bacteria i.e. OmpF, OmpC, OpdK and OprD.

Future prospects

We have only looked at the *E. coli and P. aeruginosa* clinical isolates in this study, but there are other strains as well that can develop antibiotic resistance due to mutations in the genes that code for different porin proteins. This study assists in examining the impact of mutations in the genes that code for porin proteins and structural and functional characteristics of porin protein as well. This study will be useful in the future for the creation of new therapeutic targets and medicines that can combat antibiotic resistance and increase the capacity of all antibiotics to penetrate cell membranes. The biophysical characterization of porins may also be useful for comprehending antibiotic translocation. Furthermore, future studies can also study the non-molecular resistance mechanisms such as antibiotic utilization patterns and the role of environmental factors to understand the issue more comprehensively.

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