Prevalence of Beta Lactamase Antibiotic Resistance Genes (ARGs) in Bacterial Isolates from Different Soils of Pakistan

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Prevalence of Beta Lactamase Antibiotic Resistance Genes (ARGs) in Bacterial Isolates from Different Soils of Pakistan

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Dedicated to mama & baba

to whom I owe all my success and

fulfillments in life.

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Rabia Anwar

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LIST OF ABBREVIATIONS

AMC	Amoxicilline +Clavulanic acid
ATM	Aztreonam
CAZ	Ceftazidime
CFP	Cefoparazone
CRO	Ceftriaxone
CTX	Cephotaxime
DNA	Deoxyribo nucleic acid
ESBL	Extended spectrum b-lactamases
FEP	Cefepime
IPM	Imepenem
M	molar
MDRO	Multiple drug resistant organism
MEM	Meropenim
mm	Mili meter
NCBI	National Center for Biotechnology Information
P	Penicillin
PCR	Polymerase chain reaction
SAM	Ampicilline +Sulbactum
TBE	Tris borate EDTA
TE	Tris EDTA
TZP	Tazobactum+ Piperacilline
ug	Micro gram
uL	Micro liter
UV	Ultraviolet
V	Vancomycin
WHO	World Health Organization

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ABSTRACT

Ever since the discovery of the first antibiotic, 60 years ago, these drugs have been subjected to excessive use and misuse resulting in the emergence of resistant bacteria against all the classes of antibiotics in the current clinical use. We collected isolates from soils of 28 cities across Pakistan and determined their antibiotic sensitivity profile against 14 beta lactam including 3rd & 4th generation cephalosporins, carbapenums and penicillin, vancomycin and tigecyclin by disc diffusion assay and found that more than 81 percent of the bacteria were resistant against 5 or more tested antibiotics. A high prevalence in resistance against 4th generation cephalosporin cefepime 73%, vancomycin (64.7%), Imipenum (30.4%) and the highest in cefexime (91%) were found in the soil samples. In addition, we determined the prevalence of bla CTXM15, bla SHV and bla TEM beta lactamases by polymerase chain reaction and found that 23%, 17% and 96% of the isolated bacterial strains were positive for the three genes respectively. Highest level of resistance was found in the Rawalpindi District and in central Punjab region. On the basis of this study, it is observed that bacteria in the soil samples are resistant against beta lactams, especially to 3rd generation cephalosporin's. However, use of antibiotics in agriculture and husbandry, as well as industrialization seems to cause increased resistance in the environment bacteria.

Chapter 1

INTRODUCTION

Antibiotics are the most widely used drugs since they were introduced for the first time in 1937. Their discovery was a turning point in human history. Antibiotics revolutionized medicine in many respects and countless lives have been saved. However, the decrease in effectiveness of antibiotics has also been observed in recent years due to the emergence of resistance in a large number of bacterial strains. Thus rendering antibiotics ineffective and entering into post antibiotic era (CDC., 2013). Antibiotics are not only used for human health but for animal health care and farming as well. Antibiotics have also been used in agriculture for treating plant infections and increasing their growth (McManus *et al*., 2002; Smith *et al*., 2002; Singer *et al*., 2003; Cabello., 2006).

In developed countries, the increased use of antibiotic in hospitals, community and agriculture have contributed to selection pressure that has sustained resistant strains, forcing a shift to more expensive and more broad-spectrum antibiotics. However in under developed countries the increase in antibiotic use can be attributed to high rates of hospitalization and high prevalence of hospital infections (Laxminarayan R and Heymann DL., 2012).

An estimated 25000 people die every year in Europe from antibiotic-resistant bacteria (ECDC/EMEA., 2013). In the USA in 2005, an estimated 94 000 invasive MRSA infections required hospitalization and were associated with 19 000 deaths (Klevens et al., 2007). A recent report by the US Centers for Disease Control and Prevention conservatively estimated that at least 2 million illnesses and 23 000 deaths a year in the USA were caused by antibiotic resistance (CDC., 2013).

Resistance causing genes have been spread worldwide. The spread of the resistance can be seen via the fact that more than 1000 resistance-related β-lactamases genes have been

identified in different parts of the world, which is ten times more as compared to that in 1990. Antibiotic-resistant gonorrhea was first reported in Vietnam in 1967, (Holmes et al., 2005) after that it was found in Philippines and then in USA as well (Rasnake et al., 2005). NDM enzymes which were reported in 2008, are now found worldwide (Nordman et al., 2011).

In Pakistan, the emergence of pan-resistant bacterial isolates such as *Acinetobacter* spp and carbapenem resistant entero bacteria which causes health-care associated sepsis in hospitals is rendering these infections untreatable (Saleem AF et al., 2010; Perry JD et al., 2011; Khan E et al., 2010). Some other reports have shown that some of the E coli strains associated with urinary tract infections have become resistant to common oral antibiotics like amoxicillin, cefixime, and ciprofloxacin. In India,E *coli* isolated from urine cultures of pregnant women (1st trimesters) showed 75% resistance to ampicillin, 73 % to naladixic acid, and 59 % to co-trimoxazole. Also the proportion of *K pneumoniae* with carbapenem resistance has been increased from 2.4% to 52%.

Microbes develop resistance as a consequence of mutations or due to the selection pressure from millions of tons of antibiotics release in the environment, which gives a competitive advantage for mutated strains. The genes causing resistance are present on chromosomes as well as on plasmids. Due to their presence on r plasmids they are easily transferred to other bacteria via horizontal means.

The risk factors associated with this gene transfer are many besides this horizontal transfer, poor sanitation and hygiene in communities and hospitals, and the increasing frequency of global travel, trade, and disease transmission. Some of the examples of resistant strains include methicillin resistant Staphylococcus aureus (MRSA) USA 300, Escherichia coli ST131 and Klebsiella ST258).

In natural microbial communities besides antibiotics, some other compounds and the condition in the environment add to the selection pressures, giving rise to resistant strains. In soil antibiotics concentration is high due to their production by different strains of other

microbial specie. Most of these antibiotic-producing strains carry genes of antibiotic synthesis pathway and resistance in the same gene cluster (Hopwood, 2007) (Talhan et al., 2007). However, without a certain concentration of antibiotic this selection pressure cannot be exerted, more studies are required to find the exact antibiotic concentrations in soil which can pose this pressure.

Therapeutic use of antibiotics by humans is very less, almost half of the total commercially produced antibiotics. Besides therapeutics, humans are using antibiotics for animal growth, in aquaculture, household pets, and in agriculture. They are also used as biocides in toiletries and in household cleaning products. The large scale disposal of toxic waste products, disinfectants, biocides, metals and other xenobiotic is also contributing in polluting the environment. A study in Hyderabad, India showed that dumping of ciprofloxacin into rivers was around 50 kg a day by pharmaceutical manufacturers (Fick et al., 2009) which not only damages to humans but also populations of insects, birds and animals (Carlson et al., 2009).

Besides these anthropogenic activity other nonhuman applications of antibiotics, and waste disposal, create major environmental reserves of resistance and the organisms that harbor them (Fig. 4) (Doyle., 2006) this has been proved in a study based on genetics of wastewater treatment plants, which showed they are the rich reservoirs of resistant genes and resistant organisms (Schluter et al., 2008) (Szczepanowski et al., 2009).

The mechanism of transfer of resistant genes from one bacterium to another is many. Genes are easily transmitted via horizontal means. Gene transferis mediated by plasmids, bacteriophages, naked DNA or transposons. Some transposons also contain integrons. These are complex transposons that contain a site for integrating different antibiotic resistance genes and other gene cassettes in tandem for expression from a single promoter (Hall R.M et al., 1999). Due to which enhanced resistance is shown by organisms.

Chromosomal genes also undergoes the same mechanism of transfer that is through transformation, this created penicillin-resistant S. pneumonia through the acquisition of genes from the naturally occurring, penicillin-resistant commensal Streptococcus viridans

and the formation of mosaic, penicillin-insensitive, penicillin-binding proteins (Dowson et al.,1994)(Spratt B.G., 1994). Other organisms that are easily capable of integrating naked chromosomal DNA are H. influenzae and A. baumanii.

The most common and the widely spread resistance is conferred via lactamase enzyme against beta lactams class of antibiotics. However, Extended Spectrum Beta Lactams (ESBLs) are lactamase that confer bacterial resistance against a large number of antimicrobial drugs, including; penicillin's, first-, second-, and third-generation cephalosporin, and aztreonam (but not the cephamycins or carbapenem) by hydrolysis of these antibiotics, and are inhibited by lactamase inhibitors such as clavulanic acid.

The first report of plasmid-encoded -lactamases capable of hydrolyzing the extended-spectrum cephalosporins was published in 1983 (Knothe et al., 1983). Other lactamases were discovered afterwards which were closely related to TEM-1 and TEM-2, and had the ability to confer resistance to the extended- spectrum cephalosporin's (Brun-Buisson et al., 1987) (Sirot et al., 1987) .The ESBLs undergo mutations and a single point mutation results in profound change in the enzymatic activity of the ESBLs, thus giving rise to the fact that now they have the ability to hydrolyze even the third-generation cephalosporins or aztreonam.

Third generation antibiotics are considered to be failed to save humans from bacterial diseases. Antibiotics have been one of the pillars allowing us to live longer and live healthier. The spread of resistant genes led us into post-antibiotic era, in which treatment and cure of common infections have become difficult.

Besides all these genetic reasons, factors such as self-medication, misinformation, ignorance, lack of education, and inaccessibility to health care and diagnostic facilities, are also contributing in developing resistance. Poverty also contributes in acquiring infections and poses risk due to issues related to affordability and thus causing early treatment termination.

In south Asia and sub-Saharan Africa, antibiotic resistance possess the highest burden of deaths caused by untreatable bacterial infectious diseases (Awoniyi DO et al., 2009)

.Countries in these regions are endemic with multidrug-resistant pathogens due to poor containment of resistant organisms in hospital and community settings (Zaidi et al., 2005) and inadequate training of prescribers and laboratory personnel (Byarugaba DK., 2004). Steps must be taken immediately to ensure better control of antibiotic release and its proper disposal in the environment.

Keeping in view the severity of the problem, this study was designed which highlights the presence of antibiotic resistant bacteria, multi-drug resistant bacteria, presence of resistance genes in different soils of Pakistan region.

The aims of this study were:

- To isolate bacterial species from different soils of Pakistan.
- To test their antibiotic sensitivity against different classes of antibiotics.
- To find the Percentage abundance of bla-TEM, bla-CTX-M15 and bla-SHV in bacterial isolates showing high resistance.
- To study the trend of resistance with the geographical locations and the corresponding use of antibiotics.

Chapter 2

REVIEW OF LITERATURE

2.1Antibiotic Resistance- A Global Threat

Antibiotics are probably the most widely used and successful family of drugs, being developed, for improving human health. Their introduction in the drugs leads to an impressive increase in life expectancies to the point that, in 1967, the Surgeon-General of the United States of America, William Stewart, stated: 'The time has come to close the book on infectious diseases' (WHO, 2008).

The first effective antimicrobials were introduced in 1937, but soon after it launch the drug faced resistance and its therapeutic use was plagued. Resistant strains of bacteria had been detected even before 1928, when Penicillin was discovered by Alexander Fleming. Later in 1940, before introduction of penicillin as a drug, it was also discovered that an enzyme from bacteria, penicillinase, has antimicrobial activity (Abraham, E.P., et al, 1940).

The use of millions of tons of antibiotics over the past 75 years lead to the selection pressure due to which all disease-causing bacteria have become resistant to antibiotics commonly used to treat them (Abraham, E.P., et al, 1940). WHO's Assistant Director-General for Health Security, Dr Keiji Fukuda, says in a WHO report 2014, The world has entered in a post-antibiotic era, in which common infections and minor injuries which have been treatable for decades can once again kill. Antibiotics have been one of the pillars allowing us to live longer, live healthier, and benefit from modern medicine. Unless we take significant actions to improve efforts to prevent infections and also change how we produce, prescribe and us antibiotics, the world will lose more and more of these global public health goods and the implications will be devastating."

Key findings from the report include (WHO, 2014):

• Resistance to the treatment of last resort for life-threatening infections caused by common intestinal bacteria, Klebsiella pneumoniae–carbapenem antibiotics–has spread to all regions of the world.

• Resistance to one of the most widely used antibacterial medicines for the treatment of urinary tract infections caused by E. coli–fluoroquinolones–is very widespread.

• Treatment failure to the last resort of treatment for gonorrhoea–third generation cephalosporins–has been confirmed in Austria, Australia, Canada, France, Japan, Norway, Slovenia, South Africa, Sweden and the United Kingdom. More than 1 million people are infected with gonorrhoea around the world every day.

• Antibiotic resistance causes people to be sick for longer and increases the risk of death. For example, people with MRSA (methicillin-resistant Staphylococcus aureus) are estimated to be 64% more likely to die than people with a non-resistant form of the infection. Resistance also increases the cost of health care with lengthier stays in hospital and more intensive care required.

2.2 Global epidemiology of Antibiotic resistance

Antibiotic resistance is not restricted to just a certain part of the world rather it is globally spread. In high-income countries, the excessive use of antibiotics in hospitals and agriculture has been the reason for shift to more expensive and more broad-spectrum antibiotics. In low-income and middle-income countries (LMICs), the use of antibiotics has been increased with rising incomes, high rates of hospitalization, and high prevalence of hospital infections (Alaxminarayan R. et al., 2012)

In developing countries, incidence of infections acquired in intensive care units was three times the rate in the USA. Health-care associated infections in neonatal intensive care units in some countries are up to nine times more common than in the USA. This shows the need for antibiotics which ultimately increases the burden of resistance with the rate of health-care associated infections in LMICs.

Resistance has spread worldwide, this can be seen with the distribution of resistance genes, such as Entero bacteriaceae producing extended-spectrum β-lactamase (ESBL), NDM-1, and Klebsiella pneumoniae carbapenemase (KPC) (Nordman P et al., 2011), indicates the ease with which resistance can spread 72% of viable K pneumoniae isolates from South Africa showed ESBL production Between July 2010 and August 2011 (19).

In India a study was conducted between 2004-2007, in which E coli isolated from urine cultures of pregnant women in their first trimesters showed highest resistance to ampicillin, naladixic acid, and co-trimoxazole, as 75%, 73%, and 59%, respectively (22)

In Pakistan, Acinetobacter spp and enterobacterial pan-resistant bacterial isolates causes sepsis in hospitals which is rendering these infections untreatable. (16-18) similarly 50–60% of community-acquired Gram-negative pathogens such as E coli associated with urinary tract infections have become resistant to common oral antibiotics (eg, amoxicillin, cefixime, and ciprofl oxacin).

The rapid evolution of bacterial resistance is clear in the case of β-lactamases class of antibiotics. Nearly 1000 resistance-related β-lactamases that inactivate these antibiotics have been identified, a tentimes increase since before 1990 (Davies et al., 2010).

2.3 The high burden of resistant infections

Antibiotic resistance posing a high burden on treating infections and is probably concentrated in three major categories:

1. Longer duration of illness and higher rates of mortality in patients with resistant infections,
2. Increasing costs of treatment for resistant infections,
3. And inability to do procedures that relies on effective antibiotics to prevent infection.

An estimated 25 000 people die every year in Europe from antibiotic-resistant bacteria (ECDC/EMEA., 2009). In the USA in 2005, an estimated 94 000 invasive MRSA infections required hospitalization and were associated with 19 000 deaths (Kelvens RM et al., 2007). A recent report by the US Centers for Disease Control and Prevention conservatively estimated that at least 2 million illnesses and 23 000 deaths a year in the USA were caused by antibiotic resistance (CDC, 2013).

Bacterial resistance is not only related to infections caused by bacteria but renders inability to do other interventions such as surgery, transplantation, and chemotherapy (Laxminayaran R. et al., 2007).

Without effective antibiotics, 30–40% of patients having total hip replacements would have a postoperative infection, with a case-fatality rate of roughly 30% (Smith R, Coast., 2013) this problem is not endemic in certain areas but it is problem for every country.

2.4 Emergence of super resistance and super bugs

Excessive use of antibiotics for treating human infections have led bacteria to evolve as a Multidrug-resistant (MDR) bacteria- also named as super bug. For example, MDR M. tuberculosis. The term "superbugs" refers to the bacteria which undergoes multiple mutations and has increased morbidity and mortality due to endowing high levels of resistance to the antibiotic classes. Superbugs are omnipresent in the biosphere. Due to which rate of MDR infection increases. Currently, the most notorious superbug is the Gram-positive organism Staphylococcus aureus. The discovery of methicillin (the first designer anti resistance antibiotic) in 1959 were thought defense against the penicillinases, but the appearance of methicillin-resistant S. aureus (MRSA) within just 3 years led to the basics of having more potent variants of drug resistance.

2.5 Origin of resistance and factors determining the emergence of resistance

2.5.1 Genetic basis

a. Genetic Jugglery
b. Intrinsic Resistance
c. The Resistome

a. Genetic Jugglery

Plasmid encoded genes for -lactamase enzymes are probably the most widely distributed and the random mutations of the genes encoding the enzymes have given rise to enzymes with modified properties and also with increased extended spectra of resistance (Gniadkowski, M.2008). TEM, a beta lactam coding gene has spawned a huge tribe of related enzyme families, providing ample proof of this adaptability. Another example, CTX-M, a new extended- spectrum -lactamase are highly successful at transmission and are a global phenomenon and threat (Fig. 1) (Hawkey,P.M., et al 2009).Epidemics of these r genes with efficient HGT and rapid mutational radiation are very difficult to control.

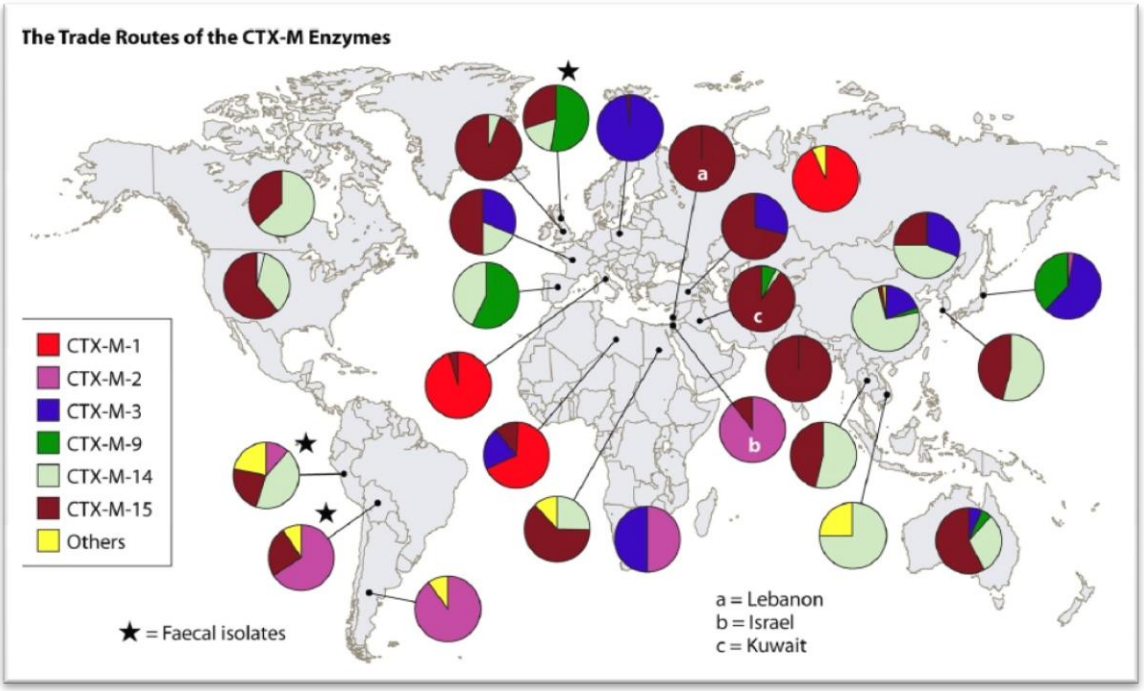

Figure 2.1: Global Prevalence of CTX-M15

b. Intrinsic Resistance

Intrinsic resistance refers to the resistance due to the existence of genes in bacterial genomes, i.e., proto- or quasi-resistance. Due to latest technologies and genome wide studies it is possible to detect potential/intrinsic gene functions in bacteria that may lead to resistance phenotypes in clinical situations. For example, a common genetic route to enhanced antibiotic resistance is gene amplification, notably for resistance to the sulfonamides (Kashmiri, S.V.S., 1975) and trimethoprim (Brochet et al., 2008).

c. The Resistome

The environmental antibiotic resistome includes the population of r genes in environment. Different environments would have different r genes and thus different type of resistances. Novel resistance mechanisms, as well as many mechanisms related to those found in pathogens, were identified by Wright and colleagues they screened a collection of morphologically distinct spore-forming actinomycetes (including many known antibiotic-producing strains) against 21 different antibiotics they quantify the r genes/phenotype density in the environment. A significant number of strains were resistant to an average of 7 or 8 antibiotics; they were naturally multidrug resistant.

2.5.2 Anthropogenic Activities

The role of humans in the antibiotic pollution in the environment cannot be ignored. Since 1940 a large amount of antibiotics for human use have been manufactured, used clinically, released into the environment, and widely disseminated, which ultimately provides selection and maintenance pressure for populations of resistant strains in all environments. It can be estimated that many millions of metric tons of antibiotic compounds have been released into the biosphere over the last half-century while a small proportion of antibiotic is produced naturally by antibiotic-producing strains in their (Gottlieb D., 1976).

Besides use as a therapeutic agent for humans, antibiotics have been used for other purposes as well. These are as follows:

(i) Growth promotion/prophylactic use in animals;

(ii) therapeutic/prophylactic use in humans;

(iii) therapeutic/prophylactic use in aquaculture;

(iv) therapeutic/prophylactic use in household pets;

(v) Pest control/cloning for plants and agriculture;

(vi) use as biocides in toiletries and in hand care and household cleaning products;

(vii) And culture sterility, cloning, and selection in research and industry.

Numerous types of anthropogenic activities create major environmental reserves of resistance (Fig. 2) (Doyle, M.P.2006) and, of virulence genes, including antibiotic use in agriculture and aquaculture, other nonhuman applications of antibiotics, and waste disposal (Moura et al., 2010). Because the r genes resides on transmissible plasmids thus providing ready sources of resistance determinants and are transferred easily.

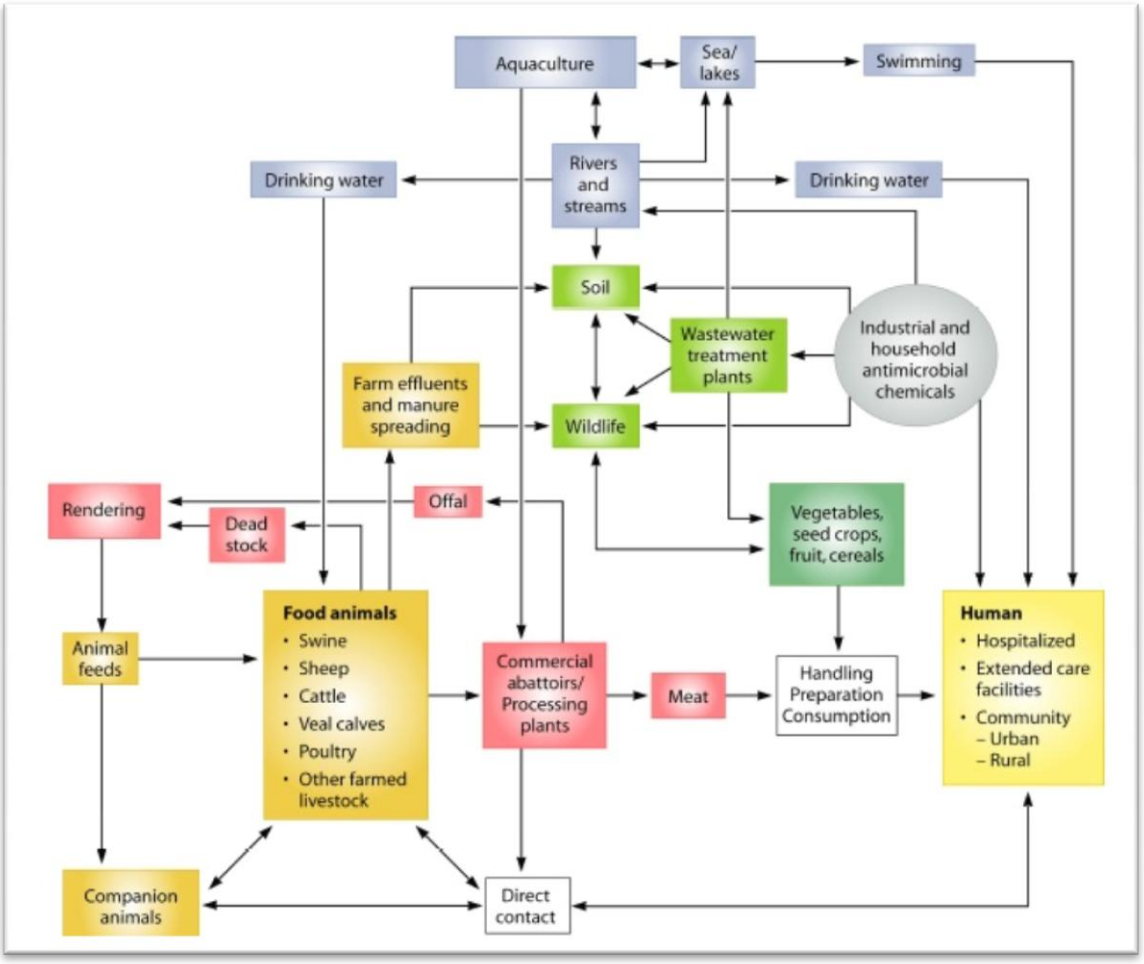

Figure 2.2: Anthropogenic routes leading to antibiotic resistance

2.6 Mechanism of antibiotic resistance

Bacterial growth inhibition by an antibiotic is achieved when the antimicrobial efficiently interacts with its target. There are two mechanisms of resistance

1. Passive mechanisms of resistance
2. Active mechanisms of resistance

In **passive mechanism** of resistance the antibiotics acts on bacterial envelopes and are dependent on cellular enzyme for their activation (Barlow, M., 2002). Therefore in this case Mutations in the genes coding for transporters, targets, or proteins activating the pre-antibiotic can resistance (Baquero et al., 2008)

Another mechanism of resistance, **active mechanism**, can be through modification by antibiotic inactivating enzymes which causes the reduction in the amount of active antibiotic thus causing resistance. This type of resistance can be easily spread, either by clonal expansion or by HGT (Benveniste, R., 1973).

In addition to these antibiotic resistance mechanisms there are other ways of developing resistance as well like the determinants of basic processes of bacterial metabolism, have shown to be involved in conferring susceptibility to antimicrobials (Boucher, H. W., et al., 2009)

2.7 Ecological role of antibiotics and antibiotic resistance

Natural environment is saturated by the presence of r genes which has raised many questions as to why the bacteria are under the constant pressure of developing resistance. It might be possible that they are under constant exposure to a variety of toxins. Or there might be other sources, both natural and artificial, which add to the concentration of r genes in the environment. Like products from plants, antibacterial compounds from insects and fungi, and general organic decay. Plants naturally produce many compounds that have antibacterial activities.

Besides natural sources there are many products of human contamination that trigger the antibiotic production like chemicals from petroleum industry, solvents, the products and waste of industrial processes, garbage, Heavy metals etc. To tackle this issue of toxins, bacteria form the multivalent pumping systems that prevent intracellular accumulation of structurally diverse bactericidal and bacteriostatic substances (Piddock, L. J. 2006) (Poole, K. 2010). Its example is demonstrated by tetracycline-producing organism Streptomyces rimosus (Petkovic et al., 2006) it possess multiple efflux systems (Mendez., 2001).

The studies of the genome sequences have confirmed that the strain which has the ability to produce antibiotic has also the mechanisms of developing resistance (Chater, K. F. et al., 1985). However these resistance mechanisms are for self-protection (Hopwood, D. A. 2007). Anti-bacterial compounds have no role in growth rather they are produced after exponential phase. That's why they are called as "secondary metabolites. An antibiotic selection does not necessarily leads to antibiotic resistance. Antibiotic resistance is highly pleiotropic in character.

These Pleiotropic interactions can be due to pathways that are being disturbed in cells; disturbance could be an alteration in the concentration of a protein or disfunctioning of an enzyme could lead to adjustments in processes concerned with whole networking of microbial community (Vallino, J. J. 2003).

Like a simple mutation in ribosomal protein genes leading to antibiotic resistance have a number of extra ribosomal effects like mistranslation, temperature sensitivity, phage propagation which ultimately influence cell function. Different selective pressures may lead to mutations that coincidentally confer a level of antibiotic resistance.

Investigators' are now trying to find out the role of r genes besides antibiosis. Because of the ubiquity of r genes in the environment, it is estimated that it is not only involved in resistance but it might have other roles in bacterial signaling as well, it could be between bacteria and other organisms in the environment (fungi, plants, insects, and even human and animal hosts.

2.8 Soil antibiotic resistome

Resistance has been reported in soil harboring bacteria for decades. However, the possible reason of similar resistance mechanism by clinical pathogens and soil inhabiting organisms were not clearly defined until the 1970s. However a study was conducted later in 1973, in which mechanisms of aminoglycoside resistance were found similar for both soil bacteria and clinical pathogens (Benveniste R., 1973). Since then, various studies have been conducted and identified molecular mechanism and protein homology between determinants in soil actinomycetes and those in clinically important strains. Vancomycin, clinical drug of last resort is the best example in this regard.

This phenomenon of resistance has also been studied in Non-antibiotic producing soil bacteria in both actinomycetes and non-actinomycetes, as evidenced in the case of

vancomycin resistance in Streptomyces coelicolor and Paenibacillus spp., respectively (Hong H., 2002) (Guardabassi et al ., 2004) .Besides culturable bacteria resistance in unculturable organisms was also determined by Riesenfeld et al but it was more challenging (Reinsenfeld CS., 2004). This kind of challenge can be circumvented by creating a functional metagenomic library (Handelsman J., 2004) in which cloned genomic fragments can be expressed from DNA isolated directly from soil and selecting

for resistance. As a whole, it is important to consider resistance of both culturable and non culturable organism in soil resistome.

2.9 Resistance Gene Transmission- Horizontal transmission

Evolution is a common phenomenon in bacterial life. Similarly gene transfer through horizontal means is also an important aspect of its life and this process has continued since billions of years ago. This phenomenon has helped bacteria to develop and transfer the mechanisms of resistance for their survival in environment rich in antibiotics.

Genome sequence analyses of environmental microbes revealed that they are replete with plasmids—mostly large and often carrying multigene pathways responsible for the biodegradation of xenobiotic molecules, such as the polychlorinated phenolic compounds that have been used and distributed widely since the days of the industrial revolution.

The horizontal gene transfer is commonly mediated by plasmid mediated transmission. Plasmids contain the accessory genetic elements, which are found in bacteria and are capable of transferring resistance to other bacteria (Norman et al., 2009). The existing processes of gene acquisition, transfer, modification, and expression are expanding and accelerating in the modern biosphere due to the increasing pollution of environment by the antibiotics.

Horizontal gene transfer is mediated by:

- Transduction
- Transformation
- Conjugation
- Or through cell-cell fusion

• **Transduction:** It is process of transferring genes by bacteriophages. It is common in S. aureus (Skurray, R. A. et al., 1997). Bacteriophages carrying antibiotic r genes have been identified in resistant bacteria isolated from environment and in hospital isolates. They are frequently seen as phage “fingerprints” flanking genes encoding resistance or virulence on different vectors.

• **Conjugation**: Gene transmission by conjugation has also been studied and seen in experiments. Experiments have shown that transmission by conjugation is several times greater in nature than any other mechanism of gene transfer (Sorenson, S. J., 2005). Recent studies have demonstrated diverse antibiotic r genes in the human gut micro biome.

• **Transformation**: Some bacteria directly acquire DNA from its environment. Streptococci, meningococci, and their related genera, exchange both virulence and pathogenic genes via direct acquisition, i.e transformation (Feil, E. J.et al., 1999).

• Bacterial **cell-cell fusion** takes place in complex mixed microbial communities, such as those found in biofilms (Gillings, M. R, et al., 2009).

Besides all these processes it is also important to identify the intermediate steps involved in the transfer of resistant gene from clinical to environment or from environment to clinic. The pathway from an environmental gene to a clinical r gene is not known, studies are required to study this whole chain.

2.10 Antibiotics affect microbial communities in soil

Small bio- active molecules, like quinolones, phenazines, bacteriocins and pheromones exist widely in nature, and they each possess a range of biological functions. These molecules are secreted as anti-bacterial substances that affect the growth of other microbes in their surroundings.

Some of the evidences have been collected from environment in different studies, which revealed that these small bio active compounds function as antibiotics.

A study was conducted on fungus-growing ant system, in which a bacteria residing on ant surface protects fungus from its parasite, Escovopsis sp. (Currie, C. R., et al., 1999).

Another phenomenon in microbes is quorum-sensing which is shown in a wide range of microbial species (Henke, 2004). In this microbes produce specific bioactive compounds that activate biochemical pathways at low concentrations, in one or more target organisms. Some auto inducers are also produce having high antibiotic activity and induce changes in eukaryotic host organisms or tissues (Kravchenko, V.V et al., 2008).

The production of an antibiotic is associated with the presence of genes encoding one or more self-protection processes; antibiotic biosynthesis gene clusters always encode one or more potential resistance proteins that are either specific to the compound being made (for example, they modify the compound or target) or multi-functional (for example, efflux systems).

Some other types of genes have been identified which has a role in causing resistance in addition to the known antibiotic resistance genes. These genes encode proteins for cellular functions but also show antibiotic resistance phenotypes.

In E. coli, for example, 4,000 random single-gene knockouts were screened for hypersensitivity to antibiotics (Tamae, C. et al. 2008) (Duo, M., Hou (2008). Knockouts out of them showed increased sensitivity to at least 1 of 7 antibiotics (Tamae, C. et al. 2008).

Similar studies were performed in Pseudomonas aeruginosa (Breidenstein, E.B., et al (2008) and Acinetobacter baylyi (Gomez, M. J. 2006), revealing that there is little overlap between the intrinsic resistomes of different organisms. This reveals the possible targets for antibiotic resistance and genes in a single organism that can contribute to the overall environmental antibiotic resistome.

2.11 Effect of antibiotics from different sources on soil bacteria

2.11.1 Antibiotics used by humans

Antibiotic are the drugs that are most commonly being used by people across the world. And due to excessive use it poses selection pressure on bacteria that ultimately contributes to resistance. The problem lies in the fact that most of the antibiotics are misused either by physicians uncertain of a diagnosis of a disease or in the treatment of self-limiting bacterial or viral infections. This problem has global implications because it affects both developed or developing countries in somehow or other in the same way.

In high-income countries, patients with resistant infections can turn to more expensive, newer-generation antibiotics, but in developing countries, where infectious diseases are common and the burden is high, patient undergoes second-line treatments.

According to data of the European Surveillance of Antimicrobial Consumption Network (ESAC-Net)-2010, the consumption of antibiotic varied from 11•1 to 39•4 (ECDC., 2012).

Carbapenems consumption has been tremendously increased in 15 of 19 countries, including Egypt, India, and Pakistan, which are low income countries, according to the data between 2007 and 2010. Prescribed use of antibiotics fell by 17% between 1999 and 2010. However, states in southeast USA continued to consume more than twice the amount of antibiotics per person as compared to population of Pacific Northwest and New England.

Worldwide, antibiotic consumption is on the rise (fig.3), it could be due to many reasons, one of it could be, use of un-prescribed antibiotics, according to data 19–100% of antibiotics are used without prescription (Morgan DJ., 2011). Physicians also need to adequately screen the patients and recommend drugs carefully.

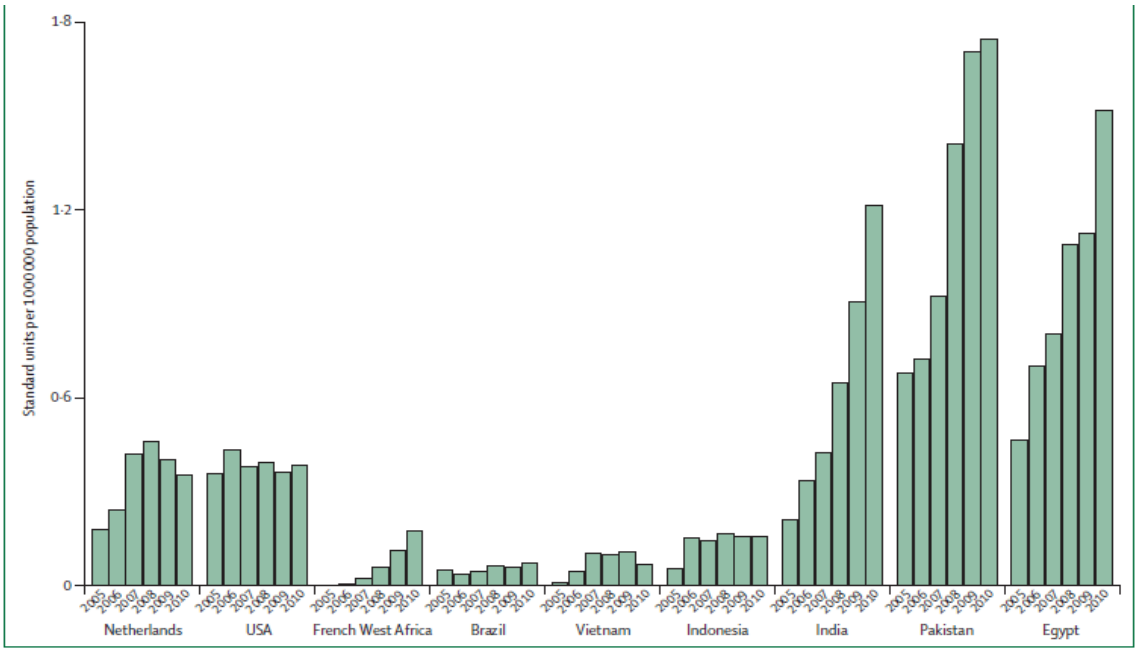

Figure 2.3: Worldwide consumption of antibiotic

2.11.2 Antibiotics used in veterinary industry

Veterinary medicines are used worldwide for therapeutic use and to increase production in animal husbandry which specifically includes antibiotics. The extent of use of antibiotics for animal use can be seen by the fact that In USA alone, the quantity of antimicrobials used for food animals is approximately fourfold greater than for human use (Maron,D. et al.(2013). Since the antibiotic classes being used in food-producing animals and in human medicine are almost the same, this increases the risk of infections in both animals and humans by the emergence and spread of resistant bacteria (WHO, 2014).

A number of antibiotics are also excreted in the urine by the animals as the primary source or as metabolites which might act as active metabolite depending upon the pharmacokinetics process in the animals (Boxall, A.B. et al. (2004) (Sarmah, A.k.et al. (2006).

The bacteria present in manure also harbor antibiotic Resistant Genes, which are enriched in the animals during antibiotic therapy and in the manure during storage, and these genes and genetic elements are spread to agricultural soil along with manure (Heuer, H. et al. 2012). In addition to veterinary antibiotics, heavy metals are also found in manure which can further contribute in the abundance of antibiotic resistance in bacterial populations in that agricultural soil.

The addition of antibiotics in soil via manure application causes alteration in structural composition and functionality of microbial communities (Figure 4). A study was conducted in which the abundance and diversity of nitrifying microbes was tested by application of manure from SDZ treated pigs. The numbers of taxa that were significantly affected by SDZ concentration and the highest number were shown at highest SDZ concentration. Antibiotics present in manure greatly affect the structure and function of soil bacterial communities com-pared to manure without antibiotics.

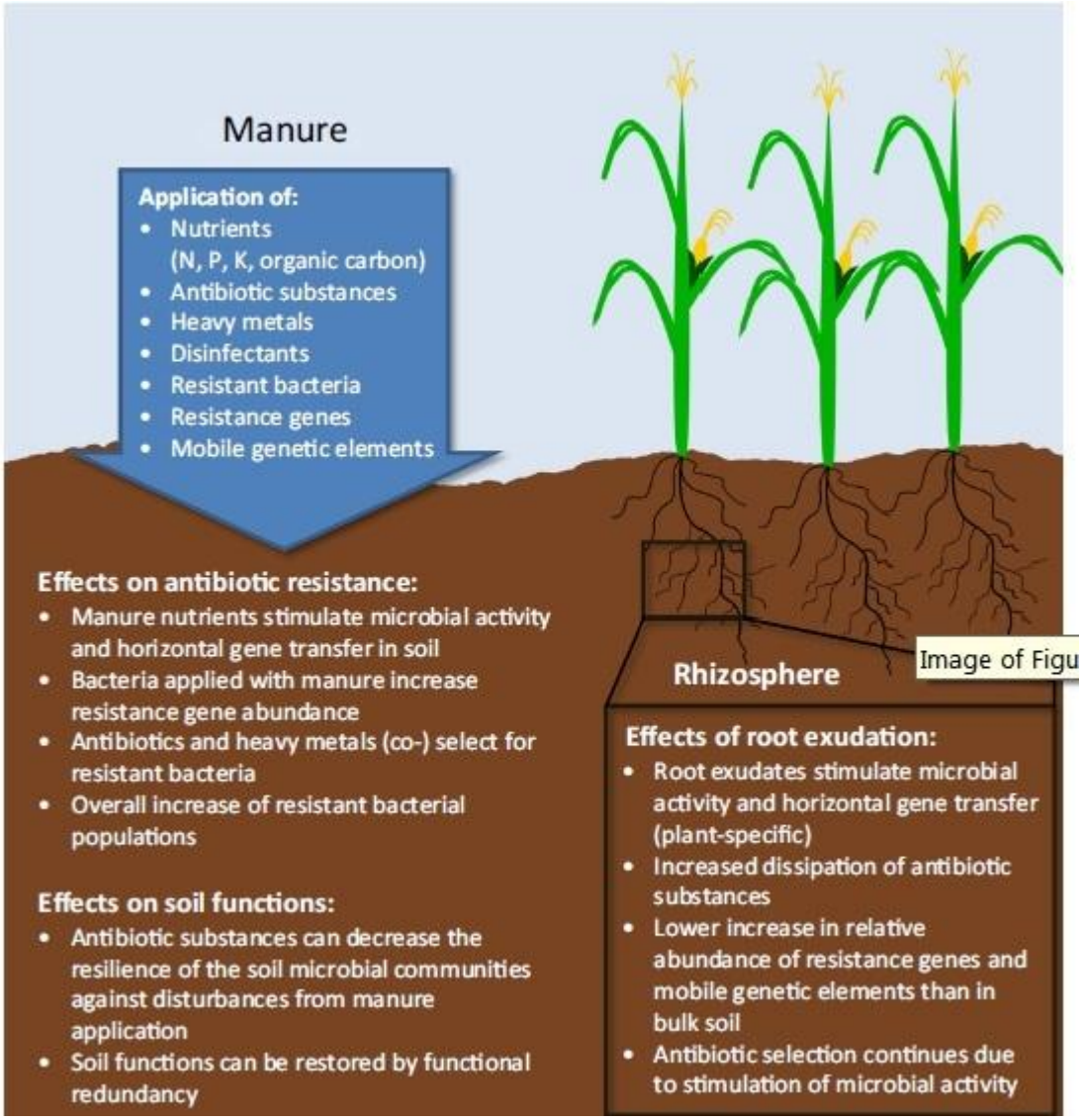

Figure 2.4: Effect of antibiotics on soil microbial communities

2.11.3 Antibiotics present in industrial waste

An antibiotic from industrial waste water ultimately adds to the soil resistome. There it interacts with soil particles (and sediments) which favors them in a way that soil particles causes the delay in the biodegradation and ultimately becomes the reason for long term sustenance of drug in the soil environment. Also there are certain chemicals in the soil which have association with antibiotic For example; humic acid. It is involved in complexes of antibiotics [5]. Presence of heavy metals (as methyl-mercury) also aggravates this process of association and formation of antibiotic complexes. Like Aluminum and iron oxides causes alteration in surface charge. Due to which different types of ciprofloxacin-surface complexes are formed [5] by changing the reactivity of fluoroquinolones during soil and water interaction. Besides this alteration of pH or ionic

strength in water or in soil might also contribute in antibiotic–soil–water interactions, producing different types of antibiotic release (dissolution) from soil particles.

2.12 Different antibiotics used in the treatments

Many life threatening infections are caused today by bacteria like community acquired infections (CAIs), urinary tract infections (UTIs), nosocomial infections (NI), nosocomial pneumonia (NP intra-abdominal infections (IAIs), Pediatric bacterial meningitis, septicaemia, neutropenia, and pelvic inflammatory diseases (Plosker et al., 1998; Lamb et al.,2002; Baughman, 2009; Chaudhuri et al., 2011).

Many different classes of antibiotics have been used for the treatment since the discovery of antibiotics, and to overcome the issues of increasing resistance, derivatives of established antibiotics are trialed and used in antibiotic therapy. (Coates et al., 2002).

2.12.1 B-Lactam antibiotics

a. Cephalosporins

Infections caused by Gram-negative and Gram-positive bacteria are most commonly treated by cephalosporins. The cephalosporins are divided to different classes, depending upon the order of their discovery, 1st, 2nd, 3rd, 4th and 5th generations. In 1945, 1st generation cephalosporin was introduced and it killed bacteria by disrupting the cell wall component; peptidoglycan. (Butler and Buss, 2006). Third generation cephalosporins are among the most widely used subclass of antibiotics and is administered to treat hospital acquired infections caused by Enterobacteriaceae e.g. K. pneumoniae and Escherichia coli. Third generation cephalosporin includes cefotaxime, ceftazidime, and ceftriaxone

b. Carbapenems

Carbapenems is another class of antibiotic drug and it is used to treat the bacteria having resistance against extended spectrum cephalosporins. It is derived from the antibiotic thienamycin which is a natural product of the Gram-positive bacterium Streptomyces cattleya. This class of β-lactams includes meropenem, imipenem, ertapenem, and doripenem.

Carbapenems are classified into two groups:

Group 1: comprises antibiotics that have limited antibacterial activity against non-fermenters Gram-negative bacteria such as ertapenem.

Group 2: includes antibiotics recommended to treat nosocomial infections and are active against non-fermenters

2.13 Classification of β-lactamases

The importance of the antibiotics penicillins and cephalosporins to treat infectious diseases has led to the focus on exploring the characteristics of enzymes produced by bacteria that hydrolyze these antibiotics. Many bacteria are able to exhibit a new approach to withstand antibiotics, more specifically β-lactams. This is frequently noticed by the insertion of new nucleotide sequences in the genetic context of a particular antibiotic resistance gene or bychanging of one or more nucleotides in the nucleotide sequence that lead to different amino acid sequences e.g. TEM group of β-lactamases. Consequently, this may result in a different substrate hydrolysis profile that can lead to a higher level of antibiotic resistance. However, a decrease in antibiotic hydrolysis may also be observed. By 2009 more than 500 unique protein sequences for β-lactamases had been reported (Bush and Jacoby, 2010). B-Lactamases have been classified in two ways, the first classification is Ambler classification based on the classification of β-lactamases according to their primary structure (Ambler, 1980), while Bush, Jacoby, Medeiros classification is based on functional characteristics of β-lactamases (Bush et al., 1995).

2.14 Extended spectrum β-lactamases (ESBLs)

ESBLs are a group of enzymes, often found on plasmids, are able to hydrolyze and confer resistance to penicillins, cephalosporins, monobactams and oxyimino-cephalosporins except carbapenams, rendering carbapenems as the last choice for treating infections. (Bush, 2010b). Cephalosporin includes cefotaxime, ceftazidime, ceftriaxone, cefuroxime and cefepime. A number of antibiotic resistance genes that confer resistance to antibiotics such as aminoglycosides and trimethoprim/sulphamethoxazole are also found on the same plasmids.

According to the ESBLs classification the class A β-lactamases (Ambler class), include blaSHV and blaTEM that have evolved from e.g. blaSHV-1 and blaTEM-1 encoding genes due to point mutations occurring on or out the β-lactamase active site (Paterson and Bonomo, 2005).

Among the wide spread ESBLs are the CTX-M enzymes, up till now over 120 CTX-M type ESBLs have been discovered (Bauernfeindet al., 1990), http://www.lahey.org/studies/other.asp#table1).

2.14.1 Carbapenemases

Carbapenems enzymes have been classified into three classes according to Ambler classification; class A, B and D. The Class A and D are known as serine carbapenemases and Class B are called MBLs (Walsh, 2010). They are hydrolysed by carbapenemases produced by Gram-negative bacteria such as members of Enterobacteriaceae and non-fermenters.

2.15 Diversity of ESBL genes- Asia

In 1988, isolates of Klebsiella pneumoniae from China which contained SHV-2 were reported (Jacoby, G.A et al 1988)). Further reports of other SHV-2-producing organism in China occurred in 1994 (Cheng, Y., 1994). In reports comprising limited numbers of isolates collected

In 1998 and 1999, 30.7% of Klebsiella pneumoniae isolates and 24.5% of Escherichia coli isolates were ESBL producers (Bell, J D, 2002). In a major teaching hospital in Beijing, 27% of Escherichia coli and Klebsiella pneumoniae blood culture isolates collected from 1997 through 1999 were ESBL producers (Du, B, Y, Long et al, 2002). Of isolates collected from Zhejiang Province, 34% of Escherichia coli isolates and 38.3% of Klebsiella pneumonia isolates were ESBL producing (Yu, Y., W, Zhou et al., 2002). National surveys have indicated the presence of ESBLs in 5 to 8% of Escherichia coli isolates from Korea, Japan, Malaysia and Singapore but 12 to 24% in Thailand, Taiwan, the Philippines,and Indonesia. Rates of ESBL production by Klebsiella pneumoniae have been as low as 5% in Japan (Lewis, M, T., 1999) (Yamaguchi, K., D. Mathai, 1999) and 20 to 50% elsewhere in Asia. Newly described SHV-type ESBLs have recently been reported from Taiwan and Japan (Chang, F, Y et al 2001).

However, the appearance of CTX-M ESBLs in India (Karim, A, L 2001) and China (Chanawong, A., F. et al 2002), and more frequent reports of outbreaks of infection with CTX-M-type ESBLs in Japan (Komatsu, M, N. Ikeda, 2001), Korea (Pai, H., et al., 2001), and Taiwan (Yu, W. L., et al 2002)), raise suspicions that these may indeed be the dominant ESBL types in Asia. Plasmid-mediated non-TEM, non-SHV ESBLs, showing homology to the chromosomal -lactamases of Klebsiella oxytoca (Toho-1 and Toho-2), have been detected in Japan (Ishii, Y., et al., 1995). A new non-TEM, non-SHV ESBL (VEB-1) has been reported from Thailand and Vietnam (Girlich, D., et al., 2002).

2.15.1 SHV

Among all ESBLs, SHV is the most abundantly found gene (Jacoby, G. A. 1997). SHV refers to sulfhydryl variable and its designation is due to its inhibition by SHV by p-chloromercuribenzoate, which is substrate-related and is a variable (Sykes, R. B, 1982). (This activity was never confirmed in later studies with purified enzyme.)

In 1983 an isolate from Germany was discovered, Klebsiella ozaenae, it has a B-lactamase which efficiently hydrolyzed cefotaxime, and to a lesser extent ceftazidime (Knoth, H. et al, 1983).

Sequencing of this gene showed that this B-lactamase differed from SHV-1 by a single point mutation and has gained extended-spectrum properties. It was designated as SHV-2. Within the time of 15 years, after the discovery of this enzyme, organisms harboring SHV-2 were found in every inhabited continent (Paterson, D. L., 2003), the possible reason for this could be the selection pressure from third-generation cephalosporins in the first decade of their use was responsible. SHV-type ESBLs have been detected in a wide range of Enterobacteriaceae and outbreaks of SHV-producing Pseudomonas aeruginosa and Acinetobacter spp. have also been reported (Huang, Z, M et al., 2004).

2.15.2TEM

All kind of TEM ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 was first reported in 1965, from an Escherichia coli isolate from a patient, named Temoneira from Athens (Hence it is designated as TEM) (Datta, N., and P. kontomichalou., 1965). TEM-1 has a very low activity against extended-

Spectrum cephalosporins but it is able to hydrolyze ampicillin at a greater rate as compared to carbenicillin, oxacillin, or cephalothin. It is inhibited by clavulanic acid.

TEM-2 has almost the same hydrolytic profile as TEM-1, but differs from TEM-1 by having a more active native promoter and by a difference in isoelectric point (5.6 compared to 5.4).

TEM-3 differs from TEM-2 by two amino acid substitutions (Sougakoff et al., 1988). It may not have been the first TEM-type ESBL. Over 100 TEM-type -lactamases have been described, of which the majority are ESBLs. TEM-type enzymes which are less susceptible to the effects of B-lactamase inhibitors have negligible hydrolytic activity against the extended-spectrum cephalosporins and thus are not considered as ESBLs.

However, there are certain TEM -lactamases that maintain the ability to hydrolyze third-generation cephalosporins as well as inhibitor resistance. These are referred to as complex mutants of TEM (CMT-1 to -4) (Sirot, D., et al., 1997). TEM-AQ, a recently discovered TEM-derived enzyme, has been found in Italy (Perilli, M., et al., 1997). Detailed analysis has shown that this enzyme has an amino acid deletion and several amino acid substitutions which make it a unique TEM-related enzyme.

2.15.3 CTX

In 1987, isolates of Klebsiella pneumonia were detected in France which harbors a novel plasmid-mediated –lactamase named as CTX-1 (Brun-Buisson et al., 1987). It was named CTX because of its hydrolytic activity against cefotaxime.Those organisms which produces CTX-M-type -lactamases typically have cefotaxime MICs in the resistant range (64 g/ml), while ceftazidime MICs were in susceptible range (2 to 8 g/ml).

However some CTX-M-type ESBLs have shown to hydrolyze ceftazidime and confer resistance against this cephalosporin as well (MICs as high as 256 g/ml) (Baraniak et al., 2002). Aztreonam have shown variable MICs. CTX-M-type -lactamases hydrolyze cefepime with high efficiency (Tzouvelekis, L, S et al., 2000), and cefepime MICs are higher than observed in bacteria producing other ESBL types (Yu, W, L., et al 2002). Tazobactam exhibits an almost 10-fold greater inhibitory activity than clavulanic acid against CTX-M-type -lactamases (Bush, K et al., 1993)). Some organisms have both CTX-M-type and SHV-type ESBLs or CTX-M-type ESBLs and AmpC-type -lactamases, which may alter the antibiotic resistance phenotype (Yan, J. J., et al., 2000).

The numbers of CTX-M-type -lactamases have been increasing and are detected in every continent .In comparison with other ESBLs like TEM and SHV-type it has shown 40% or less identity (Alobwede, et al., 2003).

In Western Europe and North America, CTX-M-type -lactamases have previously appeared to be infrequent (De Champs et al., 2000). However, in recent years, a number of authors have reported the advent of CTXM- type ESBLs in these regions (POIREL, et al., 2001). CTX-M ESBLs were predominantly found in three geographic areas: South America, the Far East, and Eastern Europe. Widespread findings of CTX-M-type ESBLs in China and India, it could be speculated that CTX-M-type ESBLs are now actually the most frequent ESBL type worldwide.

The prevalence of the enzymes in isolates of community-acquired diarrhea raises speculation that oxyimino cephalosporins available outside the hospital (such as ceftriaxone) may be important. Interestingly, identical -lactamases have been discovered in widely separated parts of the world (for example, CTX-M-3 has been discovered in Poland and Taiwan), suggesting independent evolution of these enzymes (Yan,J. J., et al., 2000).

Chapter 3

MATERIALS AND METHODS

3.1 Sample collection

Ethical approval for this study was obtained from Institutional Review Board (IRB) Atta Ur Rahman School of Applied Biosciences (ASAB) NUST. Soil samples were taken from 26 cities across the country. Aseptic plastic bottles were used for sample collection and werecarried to the laboratory in an ice box and kept in a refrigerator at 4 °C until analysis.

3.2 Sample processing

The obtained soil samples were diluted upto 1000 times, and spread on nutrient agar plates with varying amounts of salt concentrations. The plates were incubated (Memment, Schwabach, Germany) at temperatures ranging from 30 to 60 degrees over a period of 18 hours to 5 days, and bacteria were isolated based on the morphology.

3.3 Antimicrobial susceptibility testing

Antibiotics used in this study are given in table 3.1 and include Beta Lactams, Vancomycin and Tigecyclin. All the antibiotics were obtained from oxoid (Mosa ji traders).

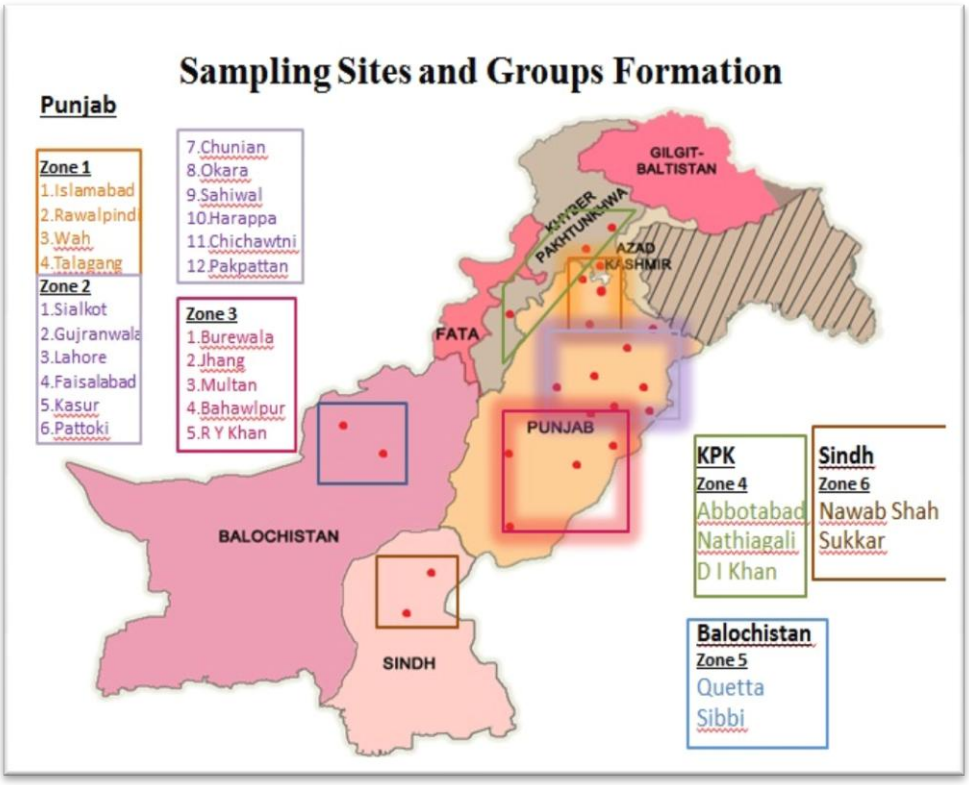

Figure 3.1: Map showing the cities from which the soil samples were obtained.

Table 3.1: Antibiotics used in the study.

Antibiotics	Concentrations (ug)
BETA LACTAMS	
Cephalosporins	
Cefpirome	30
Cefepime	30
Cefixime	5
Ceftizoxime	30
Cefotaxime	30
Ceftriaxone	30
Cefoperazone	75
Ceftazidime	30
Monobactam	
Aztreonam	30
Penicillin	
Amoxycillin/Clavulanic Acid	30
Piperacillin/Tazobactam	110
Ampicillin/Sulbactam	20
Penems	
Meropenem	10
Imipenem	10
GLYCOPEPTIDE	
Vancomycin	30
TETRACYCLINES	
Tigecycline	15

The Kirby Bauer's disc diffusion method (Bauer *et al.*, 1959) was performed for the antibiotic susceptibility testing ,Clinical And Laboratory Standard Institute CLSI guidelines were followed for the selection of media, inoculums turbidity, and preparations of media plates along with the application of discs and the interpretations of zone of inhibition.

Different colonies were suspended in sterile saline and prepared to a turbidity of 0.5 McFarland turbidity standards. Suspension was inoculated on the media plate with the assistance of sterile glass spreader. Antibiotic discs were applied using sterile forceps. Zone of inhibition around the tested antibiotics was measured and interpretations were made using the breakpoints elaborated in the CLSI guidelines (Cockerill, 2011).

3.4 Molecular detection of antibiotic resistance genes:

3.4.1 DNA extraction:

Bacterial template DNA was extracted from colony of overnight streaked plate. The colony was picked with sterile pipette tip and suspended in 100 ul of NF water in PCR tube and mixed thoroughly to form a turbid solution. The tube was then given a heat shock of 20 minutes at 85 degrees, and was then spinned for 3-4 minutes until pellet formed. The supernatant was then collected in a separate sterile tube and the pellet was discarded. The supernatant contained the bacterial chromosomal and plasmid DNA.

3.4.2 Determination of DNA concentration:

Concentrations of DNA was obtained by measuring the optical density (OD) at 260 nm using 1/100 equals 50 μg/ml DNA dilution in quartz cuvette (Sam brook et al., 1989). The purity of nucleic acid was indicated by the ratio of two readings i.e., one taken at 260 nm and the other taken at 280 nm wavelengths. The measurement of DNA quality is based on the fact that absorbance (A) at 260 nm is twice than that at 280 nm, if the solution contains pure DNA. In case of any contamination like protein, there is some additional OD which decreases the absorbance ratio between 260 and 280 nm. Pure nucleic acid samples would have an A_{260}/A_{280} ratio of 1.8.

3.4.3 Primer designing:

Primers for bla SHV,bla TEM, bla CTX-M15, Van A and ribotyping were obtained as reported in the previous papers (Dallenne *et al.,* 2010; Fang *et al.,* 2007; Chaudry *et al*., 2013). The sequence of primers used for the PCR amplification of the antibiotic resistance genes are given in the table 3.2.

Table 3.2: Primer sequences and amplicon size.

Target gene	Primer code	Sequence 5' 3'	Tm °C	GC %	Product size
bla -SHV	Bla-SHV-F	CTT TAT CGG CCC TCA CTCAA	60.4	50	237
	Bla-SHV-R	AGG TGC TCA TCA TGGGAA AG	60.4	50	
bla-TEM	Bla-TEM F	CGCCGCATACACTATTCTCAGAA TGA	64.6	46	445
	Bla-TEM- R	ACGCTCACCGGCTCCAGATTTAT	64.6	52	
bla –CTX-M15	Bla-CTX-M 15 –F	AGGCAGACTGGGTGTGGCAT	64.5	60	445
	Bla-CTX-M 15 R	TTACCCAGCGTCAGATTCCG	62.4	55	
Van-A	Van-A-F	GAGGAGCATGACGTATCGGTA	54.4	40	936
	Van-A-R	CGATCAAGCGGTCAATCAGT	54	42	
Ribotyping	RS1	AAACTCAAATGAATTGACGG			445
	RS3	ACGGGCGGTGTGTAC			

3.4.4 PCR amplification:

All DNA samples were subjected to PCR amplification by using the primer designed for that purpose. Different profiles were optimized respectively. Same recipe was used for preparing master mix in PCR tubes (Axygen^{R,} California, USA), total reaction mixture volume was 25ul contain ,2mM DNTPs,10x PCR buffer,50mM $MgCL_2$, 50 pmol of each primer, 5ul DNA sample 1unit of

thermostable Taq DNA polymerase (Fermentas, USA) and the final volume was adjusted with the help of nuclease free water. The reaction mixture was centrifuged for thorough mixing using microcentrifuge (Edison, New Jersy, and USA) then PCR was performed in Swift^{TM} MaxPro thermal cycler (Applied Biosystem, Foster city, USA).

3.4.4.1 PCR profile for bla SHV, CTX-M15:

Initial denaturation was given at 94 ºC for 4 minutes followed by 35 cycles of denaturation at 94 ºC for 45seconds, annealing at 56^{o}C for 45 seconds and extension at 72ºC for 2 minutes. The reaction was ended with a final extension of 10minutes at 72ºC and kept on hold at 4ºC until tubes were taken out of the cycler.

3.4.4.2 PCR profile for bla TEM:

Initial denaturation was given at 94 ºC for 4 minutes followed by 35 cycles of denaturation at 94 ºC for 45seconds, annealing at 55^{o}C for 45 seconds and extension at 72ºC for 2 minutes. The reaction was ended with a final extension of 10minutes at 72ºC and kept on hold at 4ºC until tubes were taken out of the cycler.

The PCR product was visualized afterwards on 2% 1XTAE agarose gel, stained with ethidium bromide and visualized under a UV transilluminator.

3.4.4.3 PCR profile for Ribotyping:

Initial denaturation was given at 95 ºC for 5 minutes followed by 35 cycles of denaturation at 95 ºC for 45seconds, annealing at 51^{o}C for 45 seconds and extension at 72ºC for 1minute. The reaction was ended with a final extension of 5 minutes at 72ºC and kept on hold at 4ºC until tubes were taken out of the cycler.

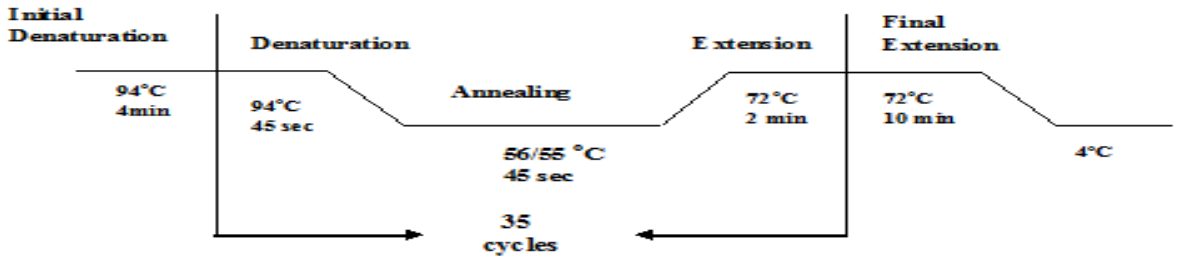

Figure 3.2: PCR cycle

3.4.5 Agarose Gel Electrophoresis

To analyze the PCR products, 2% agarose gel was prepared in 1X TAE (In 990ml distilled water, 1M Tris HCl (10ml): PH 8.0 and 400ul of 0.5 EDTA was added) and was run in the same buffer composition. For making gel, 0.8 g agarose was dissolved in 40 mL of 1X TAE by heating in microwave oven. The gel mixture was cooled to ~60 °C and upon cooling, 5 µL of ethidium bromide (0.5µg/ml) was added to stain the gel. For analysis, 7 µL of PCR product was run on the gel along with 1X loading dye. The gel was subjected to electrophoresis (Wealtec, Sparks, USA) run at constant current 60 mA for half an hour to resolve the DNA under electric field. After half an hour the voltage was increased up to 80 volts for complete resolution of the product. The gel was visualized under UV transilluminator (Biometra , Goettingen, Germany). The resulting gel was photographed by Gel Documentation System (Wealtec Dolphin Doc Sparks, USA) (S/N470883). The PCR product was stored at -20 °C for further use.

3.4.6 Sequencing:

The genes were sequenced and checked for their homology with other reported bla genes, Ribotyping and VanA.

3.5 Controls:

Controls used in our study were HBV DNA and *E. coli* clinical samples.

Chapter 4

RESULTS

4.1 Isolation of bacteria from the Environment

Bacteria were isolated from the soil samples by serial dilution of the samples. Colonies were obtained by pouring all the dilutions on nutrient agar plates and incubation of plates for 24 hours to 7 days. The bacteria were distinguished based on the colony morphology characteristics and restreaked. The colony characteristics that were considered were colony size, shape, margins, texture, opacity, elevation, pigmentation and form.

4.2 Identification of representative population by Ribotyping

A representative population of 13 isolates was identified by ribotyping. The 16S rDNA was amplified and the amplicon of approximately 470 bp was sequenced in both orientations (Figure 4.1). The result sequence was analyzed in Basis Local Alignment Search Tool (BLAST), and the bacteria were identified based on sequence homology (Figure 4.2).

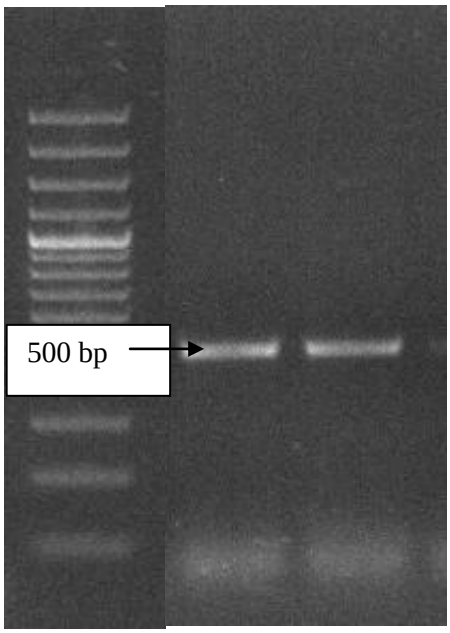

Figure 4.1: PCR amplification of 16SrRNA. Lane 1 shows 100 bp ladder, while 2-5 show amplicons of approximately 470 bp.

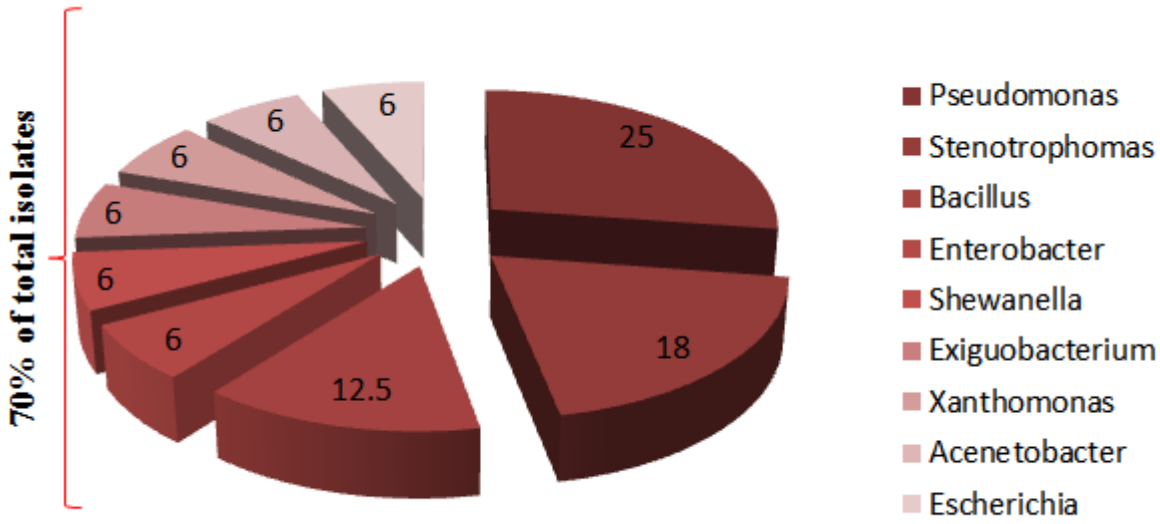

Figure 4.2: Prevalence corresponds to 13 major morphological forms and represents about total of 70 % isolates.

4.3 Sensitivity Test

A total of 174 isolates from 26 cities across the country were subjected to sensitivity testing against 16 antibiotics by disc diffusion method (Figure 4.3). The antibiotics consisted of eight 3rd and 4th generation cephalosporins, 3 combination betalactams and lactamase inhibitors, a monobactam, two carbapenums, vancomycin and tigecylin. The highest resistance was found against aztreonam (monobactam) and cefexime (3rd generation cephalosporin) being 94.2% and 91% respectively (Figure 4.3).

Figure 4.3 Disc Diffusion Test to check the resistance of bacteria against antibiotics.

4.3.1 Percentage of resistant samples against corresponding antibiotics

The results of disc diffusion show the highest level of resistance against Aztreonam that is 94.2%. Among cephalosporin's, the highest resistance is shown

by Cefixime (CFM) that is 91% and the lowest is shown by Cefoperazone, which is 21.7 %. Amoxycillin/ Clavulanic Acid (AMC) is a combination disc shows 77% resistance which is highest than other combination antibiotics. In carbapenums, imipenum showed 26.47 % whereas resistance against meropenum was lower, being 14% (Figure 4.4).

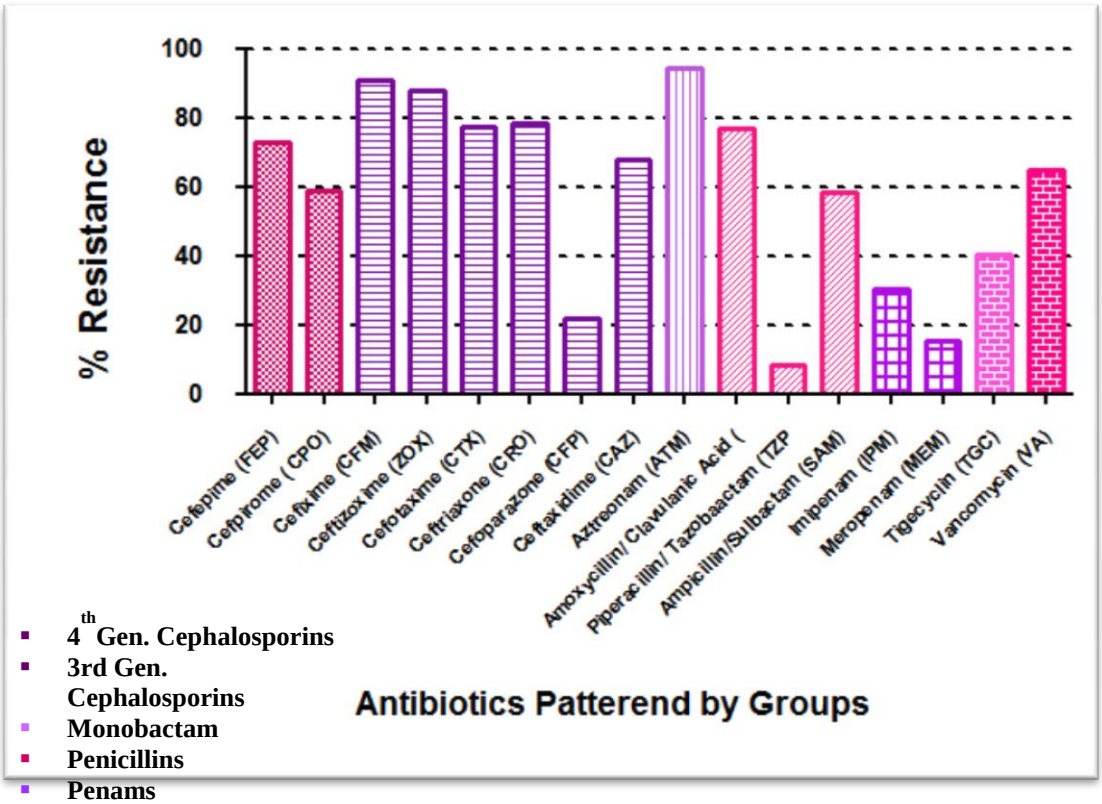

Figure 4.4: Figure shows the percentage of resistance found against the 16 antibiotics tested. Highest percentage of resistance was found to be 94.2% against Aztreonam.

4.3.2: Resistant Breakdown by number of antibiotics

Greater than 10 percent of the isolates were resistant against 10 and 11 antibiotics each, while the number of isolate resistant against 3, 4 and 16 were 4.8, 2.7 and 0.0 percent respectively. Greater than 50 percent of the isolates were resistant against 8-13 antibiotics (Figure 4.5).

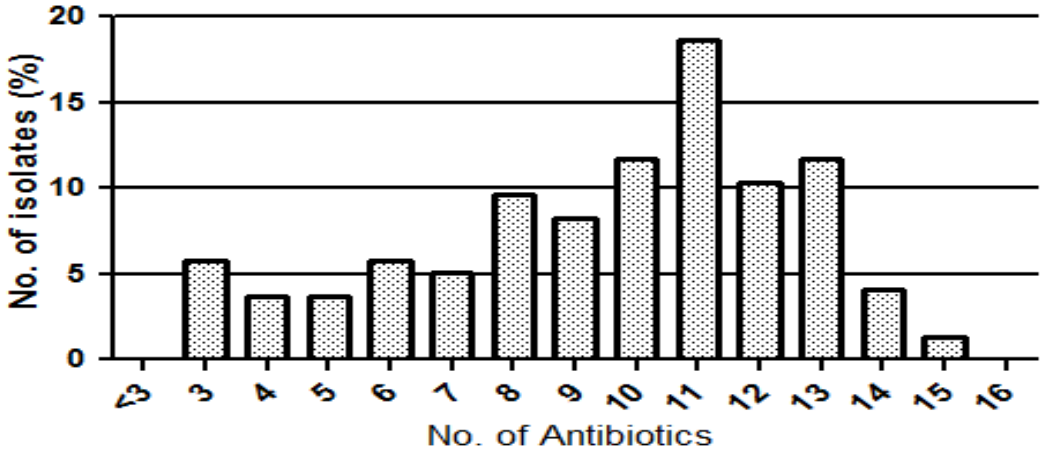

Figure 4.5: Percentage of total isolates tested vs number of antibiotics to which isolates demonstrated resistance.

4.3.3 Calculation of MAR Index

The Multiple antibiotic resistance indexes (MAR Index) for the environmental isolates of each city was calculated by using the formula:

The MAR index for a sample site or area = the number of antibiotics to which all isolates were resistant/

(Number of antibiotics tested X number of isolates).

MAR value greater than 0.2 indicates high exposure to antibiotics. 24 out of 36 cities showed MAR value greater than 0.2, with Sahiwal & Rawalpindi showing the highest (0.77) and the lowest being that of Chunian (0.14) (Figure 4.6).

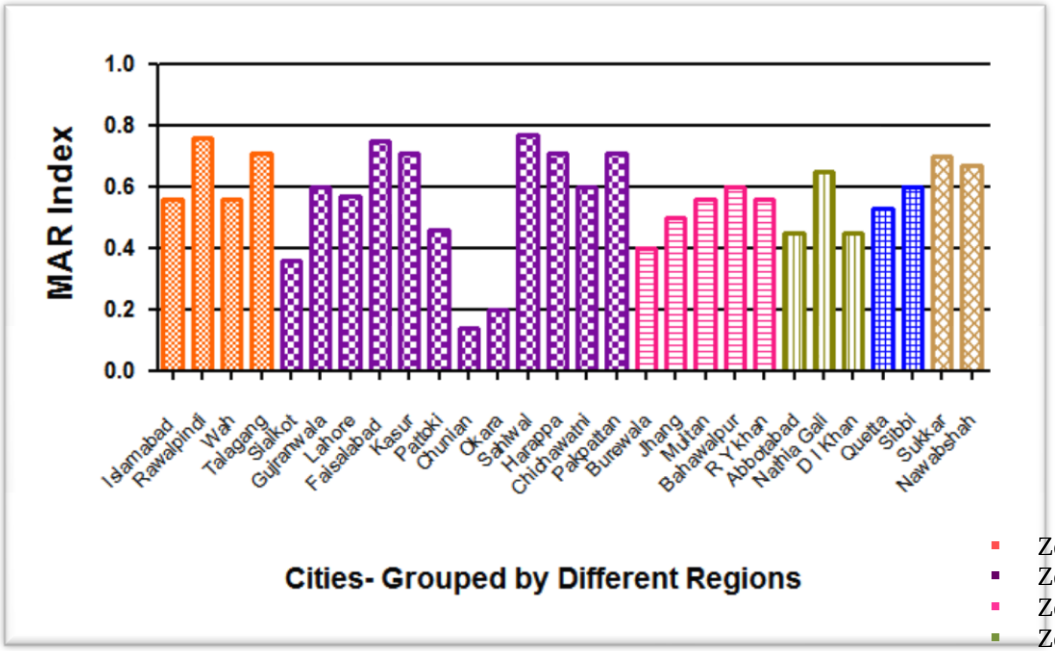

Figure 4.6: Figure shows the MAR values for various cities; the highest was found to be 0.77 for Sahiwal and Rawalpindi.

4.3.4 Resistance in Cephalosporins- A comparison

Resistance against 3rd generation Cefexime (91 %) was found to be the highest, followed by that of 3rd generation Ceftizoxime (87.8) and 4th generation. The lowest was found to be that of cefoparazone (21.7%), which is a 3rd generation cephalosporin (Figure 4.7).

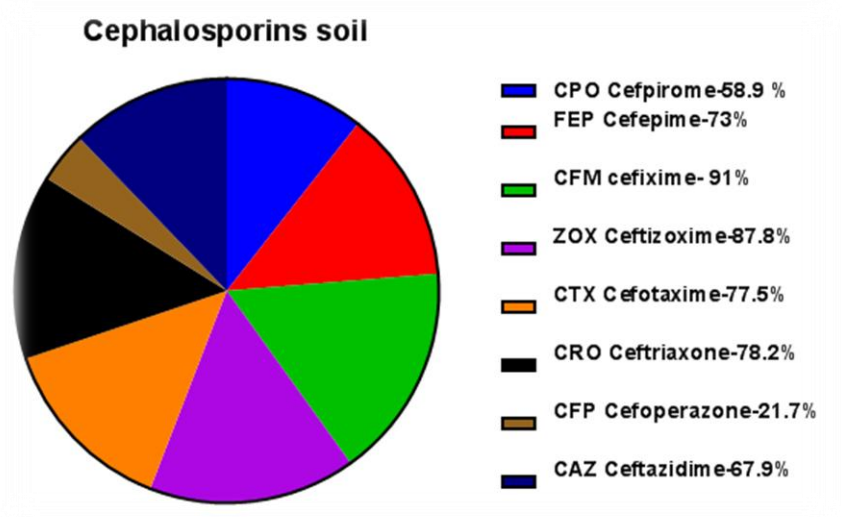

Figure 4.7: Pie chart showing the relative resistance against 3rd and 4th generation cephalosporins.

4.3.5 City Wise resistance against Vancomycin

The overall prevalence of resistance against vancomycin was found to be 56.2%. All the bacteria isolated from Faislabad, Sahiwal, Harrapa Kasur and Rawalpindi was resistant against vancomycin. The lowest percentage of sensitivity against the antibiotic was found in Sialkot, Burewala and Pattoki which is 20% (Figure 4.8).

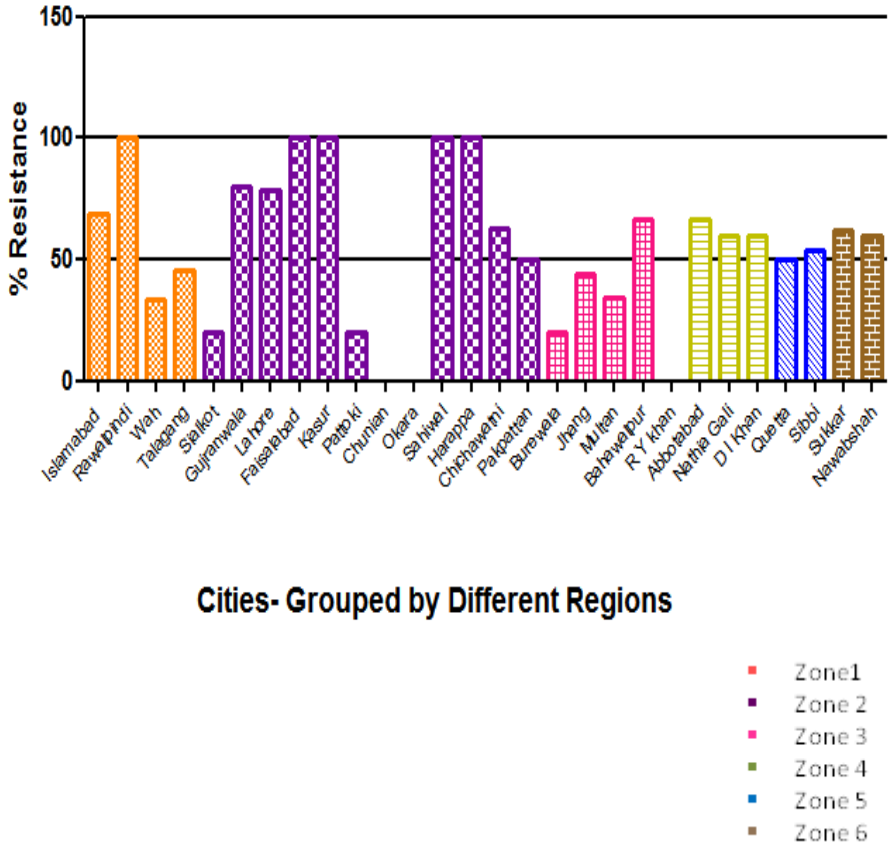

Figure 4.8: Figure showing the prevalence resistance against vancomycin in various cities of Pakistan.

4.3.6 Resistance against Tigecyclin

Resistance against Tigecyclin was found to be one of the lowest in all the 16 antibiotics (19.7 %). Among cities, Rawalpindi and Nawabshah showed the highest (100%) (Figure 4.9).

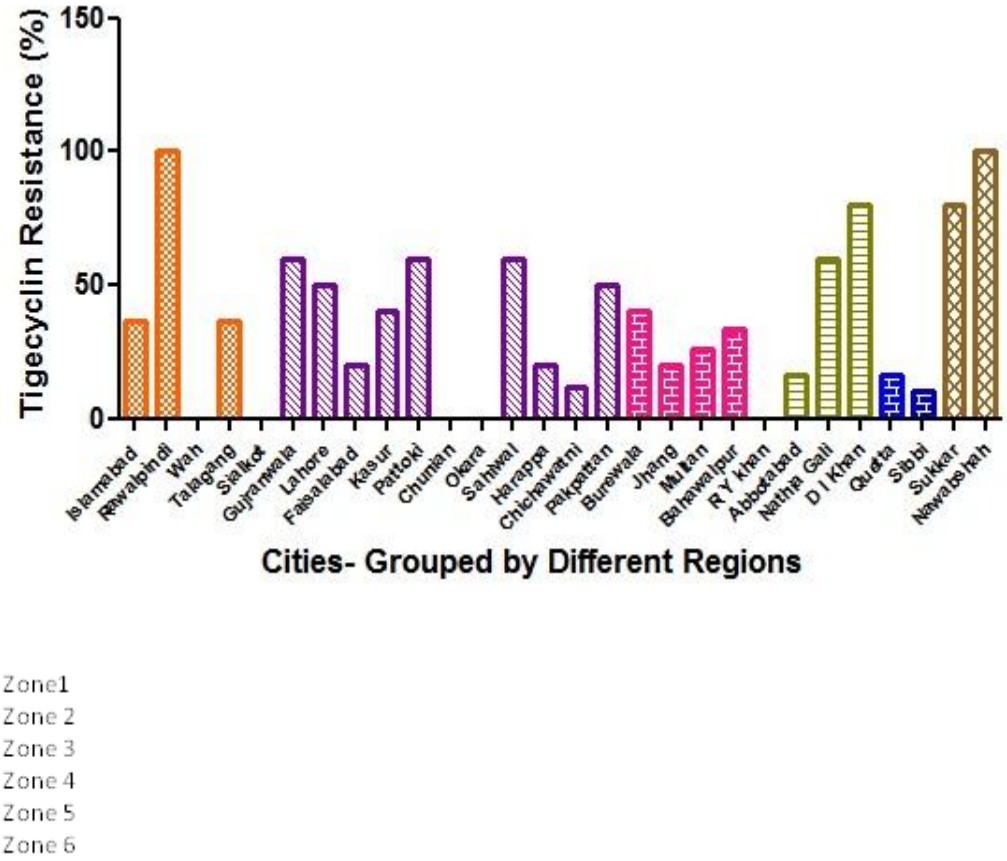

Figure 4.9: Figure shows the resistance percentages against Tigecyclin in the 28 tested cities.

4.3.7 Resistance against Carbapenums

Two carbapenums namely Imipenum and Meroenum were tested for sensitivity in Bacteria isolates. Imipenum (26.47%) showed higher resistance than that of Meropenum (14%). (Figure 4.10).

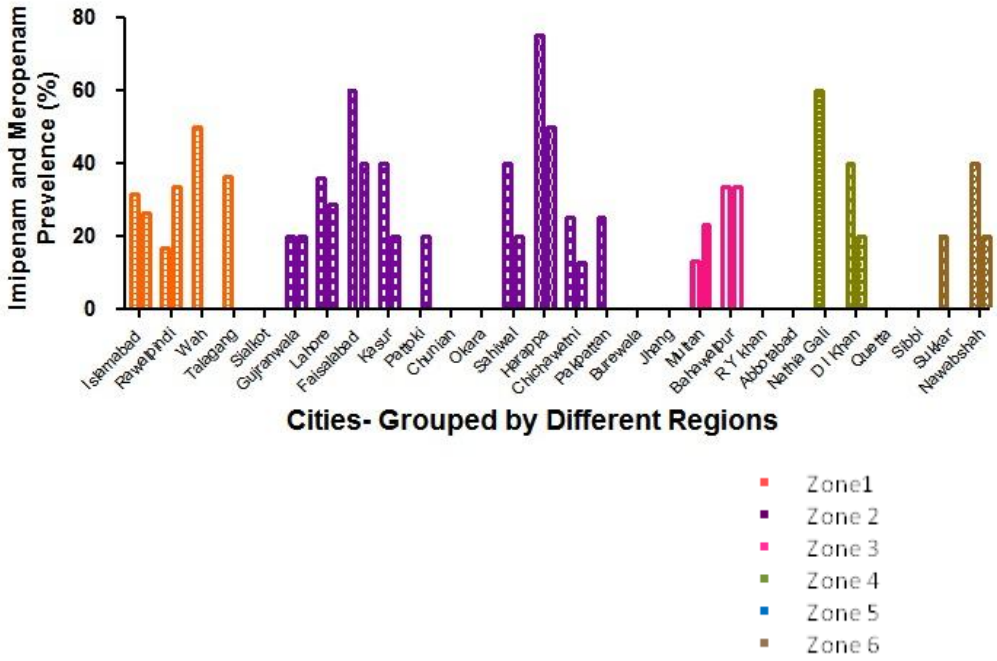

Figure 4.10: Figure shows the relative resistance of Imipenum and Meropenum in different cities.

4.3.8 Resistance in Different types of soil of Islamabad region

Resistance was compared in different soil sources from Islamabad region. The residential area of Rawalpindi region showed the maximum index of Antibiotic resistance as compared to the soils from hospital and other regions of the city.

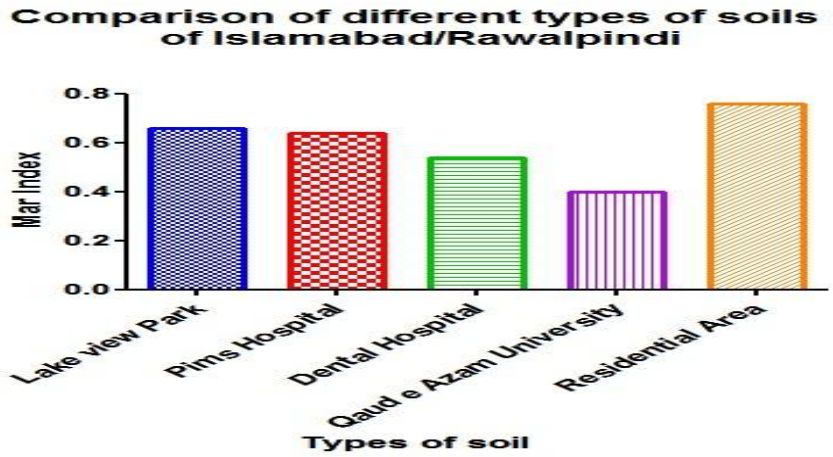

Figure 4.11: Figure shows the relative resistance of different areas of Islamabad.

4.4 Molecular detection of Resistant Markers

4.4.1. bla-CTXM-15

Bla CTXM-15 was amplified using PCR, and the resultant band was observed at 445bp with 100bp DNA marker. The overall prevalence was found to be 23% (Figure 4.13).

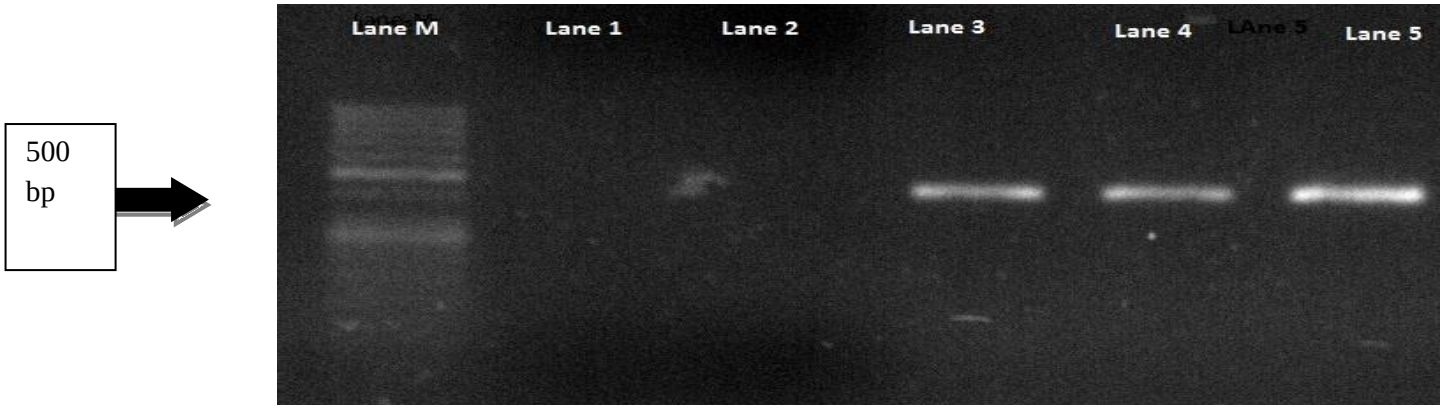

Figure 4.12: PCR Amplification of bla CTXM-15 gene, lane M 50bp ladder; lane 1 negative control; lane 2 reagent control; lane 3,4,5 gene expression in bacteria.

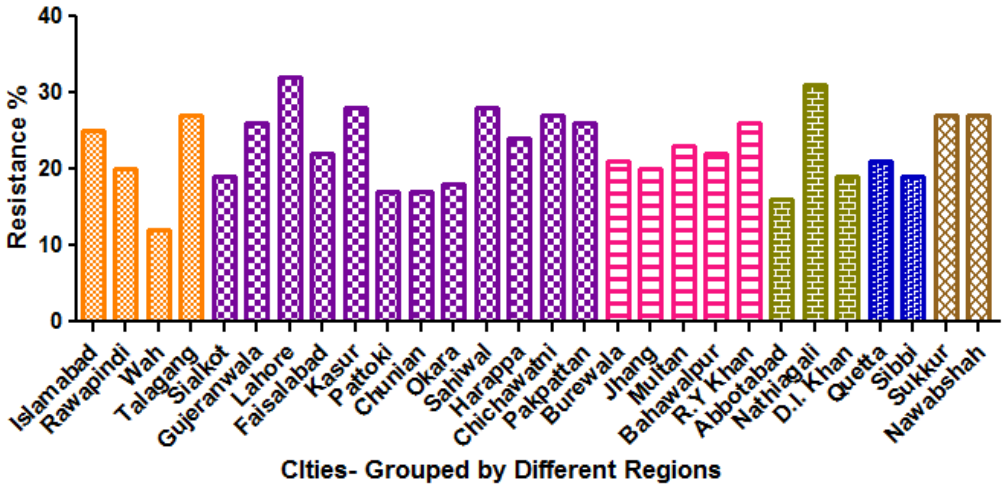

Figure 4.13: Prevalence of bla CTXM15 gene in different cities of Pakistan.

4.4.2: Molecular Detection of bla-TEM

bla TEM was amplified using PCR. The resultant bands were observed at 445 bp compared with 50bp ladder. 96% of the samples were found to be positive for blaTEM (Figure 4.15).

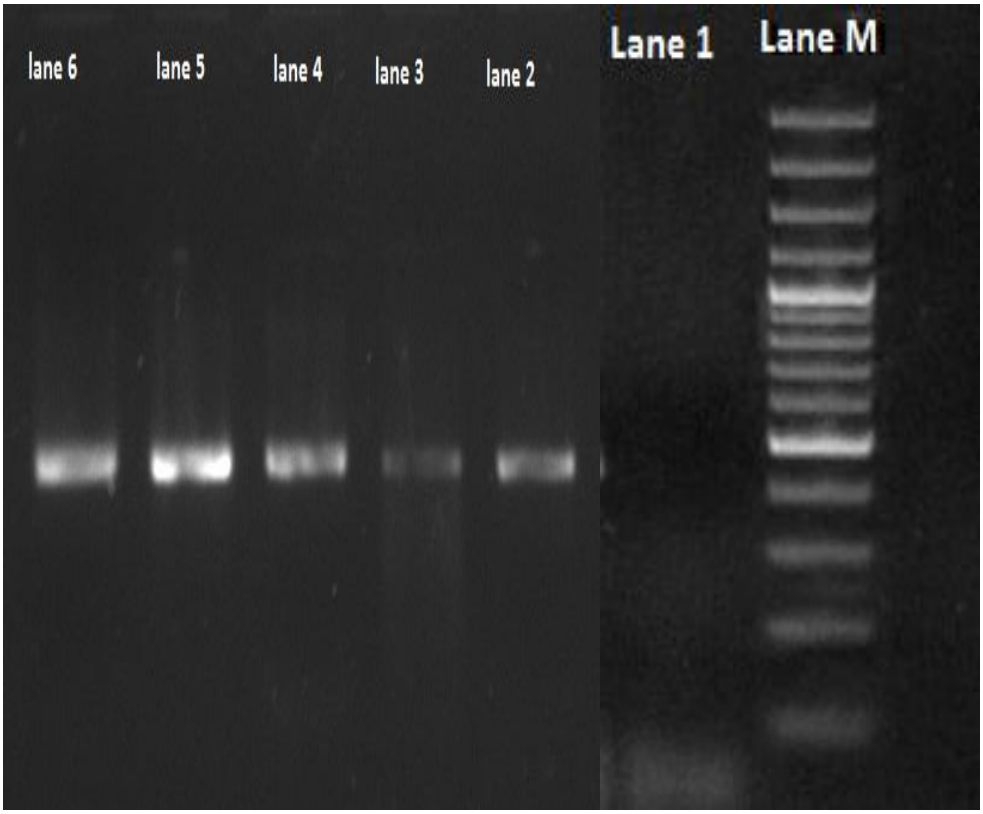

Figure 4.14: PCR Amplification of bla TEM gene, lane M 100bp ladder; lane 1 negative control; lane 2-6 gene expression in bacteria.

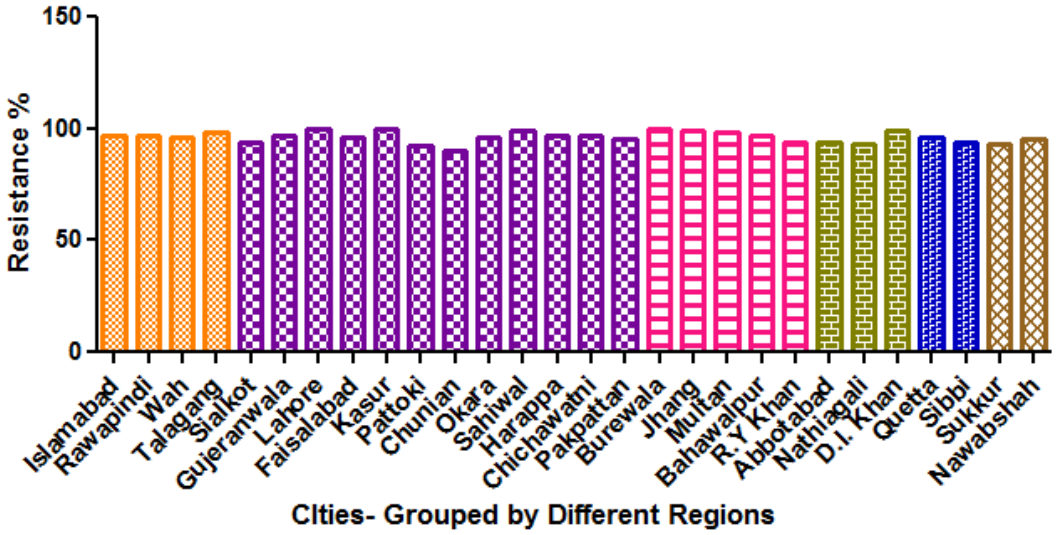

Figure 4.15: Prevalence of bla TEM gene in different cities of Pakistan.

4.4.3: Molecular Detection of bla-SHV

bla SHV was amplified using PCR, and the resultant band was observed at 237bp when compared with 50 bp ladder. The prevalence of the gene was found to be 17% (4.17).

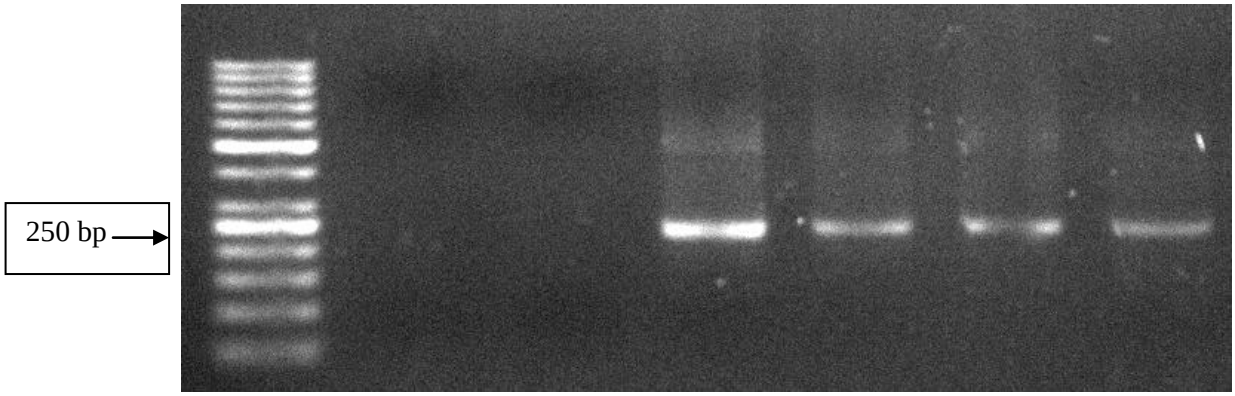

Figure 4.16: PCR Amplification of bla SHV gene, lane M 50bp ladder; lane 1 negative control; lane 2 reagent control; lane 3-6 gene expression in bacteria.

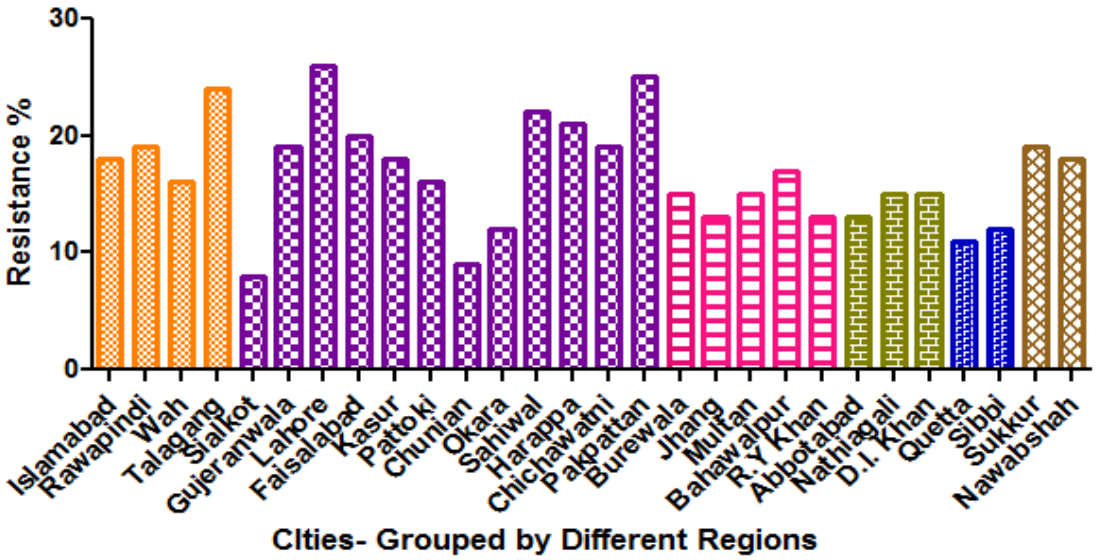

Figure 4.17: Prevalence of bla SHV gene in different cities of Pakistan.

4.4.4 Overall prevalence of Beta Lactamases

Bla TEM was found to be the most prevalent beta Lactamase whereas the prevalence of bla SHV and bla CTXM15 was found to be 17% and 23 % respectively (Figure. 4.18).

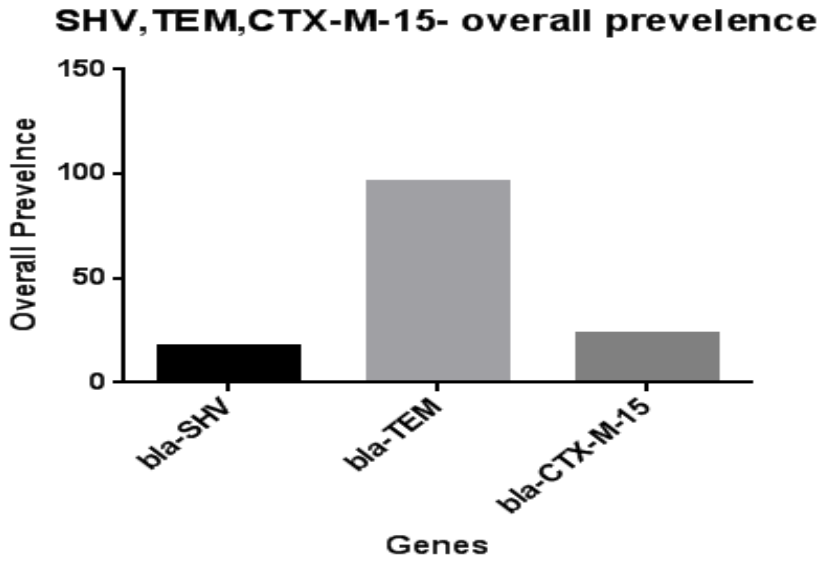

Figure 4.18: Overall prevalence of bla SHV, bla CTXM15 and bla TEM in bacterial isolates from water. The greatest are that of bla TEM being 96%.

4.5 PhylogeneticAnalysis of genes

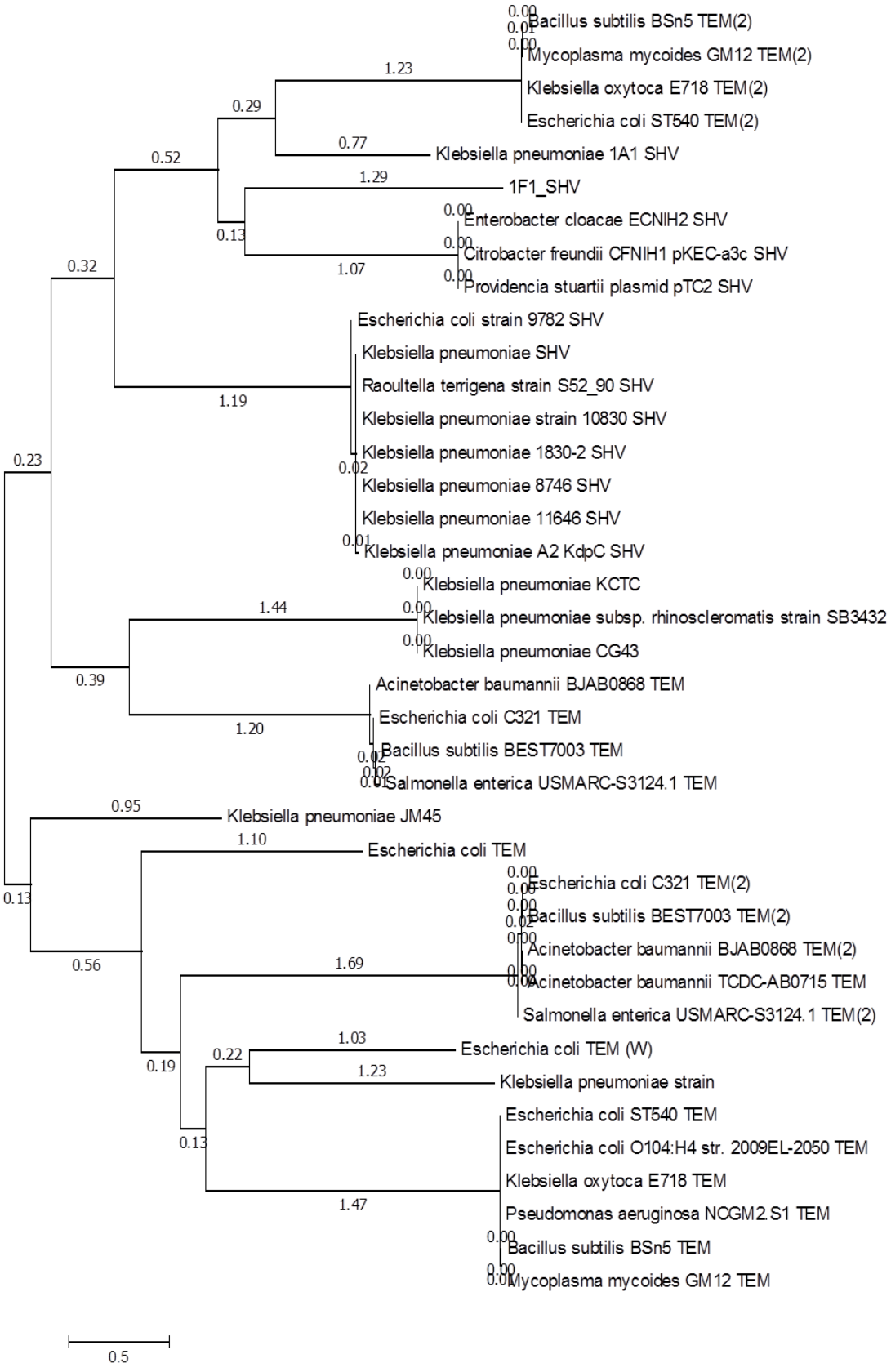
Bacillus subtilis BSn5 TEM(2)
Mycoplasma mycoides GM12 TEM(2)
Klebsiella oxytoca E718 TEM(2)
Escherichia coli ST540 TEM(2)
Klebsiella pneumoniae 1A1 SHV
1F1_SHV
Enterobacter cloacae ECNIH2 SHV
Citrobacter freundii CFNIH1 pKEC-a3c SHV
Providencia stuartii plasmid pTC2 SHV
Escherichia coli strain 9782 SHV
Klebsiella pneumoniae SHV
Raoultella terrigena strain S52_90 SHV
Klebsiella pneumoniae strain 10830 SHV
Klebsiella pneumoniae 1830-2 SHV
Klebsiella pneumoniae 8746 SHV
Klebsiella pneumoniae 11646 SHV
Klebsiella pneumoniae A2 KdpC SHV
Klebsiella pneumoniae KCTC
Klebsiella pneumoniae subsp. rhinoscleromatis strain SB3432
Klebsiella pneumoniae CG43
Acinetobacter baumannii BJAB0868 TEM
Escherichia coli C321 TEM
Bacillus subtilis BEST7003 TEM
Salmonella enterica USMARC-S3124.1 TEM
Klebsiella pneumoniae JM45
Escherichia coli TEM
Escherichia coli C321 TEM(2)
Bacillus subtilis BEST7003 TEM(2)
Acinetobacter baumannii BJAB0868 TEM(2)
Acinetobacter baumannii TCDC-AB0715 TEM
Salmonella enterica USMARC-S3124.1 TEM(2)
Escherichia coli TEM (W)
Klebsiella pneumoniae strain
Escherichia coli ST540 TEM
Escherichia coli O104:H4 str. 2009EL-2050 TEM
Klebsiella oxytoca E718 TEM
Pseudomonas aeruginosa NCGM2.S1 TEM
Bacillus subtilis BSn5 TEM
Mycoplasma mycoides GM12 TEM
0.5

Figure 4.19: Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method .The optimal tree with the sum of branch length = 18.82690921 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 39 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 166 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Chapter 5

DISCUSSION

Antibiotics revolutionized medicine in every aspect and have saved countless lives. Not only its been in use for saving the lives of humans but they are being excessively used in animal and plant growth as well. Almost fifty to ninety percent of the drugs or their primary metabolites are excreted rapidly into the environment (Michael and Bester, 2006; Hammesfahr et al., 2008).

Excessive use and release of antibiotics is creating problems worldwide especially it contributes to the emergence of resistant strains. Bacteria usually become resistant when they are exposed to sub lethal doses of the antibiotic. Control of antibiotic resistant bacteria is only possible by limiting their use or by more sensible use. A recent database lists the existence of more than 20,000 potential resistance genes (r genes) of nearly 400 different types, and these are mostly found in the genomes of bacteria (Liu and Pop, 2009). Antibiotic resistance development is not just a local public health issue but includes broader environmental influences, which are amplified by international travel and global trade in foodstuffs.

Soil is considered as one of the major reservoir for containing a large and diverse microbial population and also a source of antibiotic resistance (Vanessa et al., 2007).The microbes present in soil are involved in enzymatic degradation of compounds, including antibiotics for their own growth and proliferation (Jenkinson and Ladd, 1981). Native soil bacteria are not resistant however the resistance genes can easily be transferred due to the addition of resistant bacteria from clinical settings in the soil.

Studies have shown that how multi drug resistance is developed and transferred starting from any clinical setting, and then ending up in soil resistome (Nwosu, 2001;Popowska et al., 2012; Cytryn, 2013) (Nelson and Cox, 2008).

Some soil microorganisms like actinomycetes naturally produce antibiotics. These natural antibiotics are degraded by different bacteria's in soil and serve as a source of nutrients and energy. More studies are required in order to find out the role of soil

bacterial communities in creating antibiotic resistant bacterial strains and what measures are required for prevention and control of resistance.

Keeping these parameters in mind and seeing the developing threat of antibiotic resistance

This study was designed in which different types of soils were collected from different cities of Pakistan covering all the provinces. The bacterial isolates were tested against 16 different types of antibiotics including penicillin's, 1st, 2nd and 3rd generation cephalosporin, penams and Vancomycin.

The highest resistance percentage was shown against aztreonam i.e 94.2%. Among cephalosporins the highest percentage 91% is shown by cefixime. Fig.4.3.These both are third generation medicines. Their resistance percentages are evidence of presence of multi drug resistant bacteria in the environment. Almost 50% of the total isolates were resistant against 8-13 antibiotics.

A tool has been devised to measure the extent of resistance in a population at a specific location known as Antibiotic resistance Index (ARI). It is used to differentiate between low and high risk resistance bacterial contamination sites and to compare the resistance level of isolates across different areas. MAR Index of 0.200 is used to differentiate between low and high risk contamination suggested by Krumperman. If ARI value is ≤ 0.2 then it shows the contamination is less, values greater than 0.2 show high contamination with antibiotics (1983).

We calculated MAR index for all the cities. The highest index is of Sahiwal and Rawalpindi region i.e 0.77. Kasur, Harappa, Talagang, Faisalabad, Thar and Bahawalpur also showed index of greater than 0.6. These are pretty much high values indicating the threating situation in Pakistan and especially in Punjab region. The reasons for this high prevalence could be many; it could be due to over crowdedness, over population, excessive use of drugs for therapeutics as well as in dairy, agriculture and livestock. Besides it there could be a possibility of presence of bacteria that live on antibiotic substrate, as studied by Dantas, that soil bacteria subsisting on antibiotics are a substantial addition to the antibiotic resistome in terms of both phylogenetic diversity and prevalence of resistance (Dantas et al., 2008; Forsberg et al., 2012).All this along with other factors contributes in soil resistome of the environment.

We also find out the molecular prevalence of Beta lactam genes. The molecular marker of bla-CTXM15, bla-SHV, bla-TEM genes were used to detect theirpresence in environmentally isolated resistant bacteria. The highest prevalent gene is bla-TEM having 96% prevalence , bla-CTXM15 shows 23% and bla-SHV shows 17 % prevalence among resistant bacteria isolated from soil. The same genes were detected in clinically infectious Ecoli by using the same markers of detection. The presence of antibiotic resistance genes in the different soils indicates that soils are reservoir of multiple antibiotic-resistance machinery, and antibiotic resistance determinants harbored by soil-dwelling microbesand human pathogens (Davies, 1994; D'Costa et al., 2007; Forsberg et al., 2012).

TEM and SHV seemed to play an important role in conferring resistanceto the β-lactams antibiotics, and are widely distributed in the isolatessubsisting on neomycin. The TEM subset of β-lactamases is presentin the genomes of many soil-dwelling isolates, as well as Gram-negativebacteria that are associated with human infectious diseases(e.g. Burkholderia pseudomallei, Bordetella bronchiseptica, and Delftiaacidovorans) (Nyberg et al., 2007; Rosenau et al., 2000; Endimiani et al.,2007).

The lateral transfer ofgenes among soil bacteria encoding the enzymatic machinery responsible for subsistence on synthetic antibiotics could introduce novel antibiotic resistancemechanismsso far not observed elsewhere. Because such occurrences seem to occur rarely depends on several factors, of whichone is the difficulty of reproducing an ecological niche similar to what bacteria experience in soil.

Chapter 6

CONCLUSION

Soil has the largest and diverse bacterial community. It has large pool of undiscovered antibiotic resistance and degradation genes. The ecological and health impacts of this have still to be fully explored. However a high prevalence of resistance was shown in areas which are densely populated, like soil from Adiala, Rawalpindi showed the highest resistance percentage. Also in agricultural soils of cities of Punjab the resistance was higher including Pakpatan, Harrappa, Sahiwal and Talagang, indicating the link of antibiotic use for animal growth and in agriculture with resistance development. Resistant organisms disseminate from humans to environment and vice virsa via various environmental pathways, including foodstuffs, animal wastes, agriculture, livestock and water sources. In our samples 23% of the sample population was found to be multi drug resistant with 100% of isolates from Rawalpindi exhibiting MDR. Beta Lactamases were found to be prevalent in all cities with the highest percentages found for blaTEM Sequence analysis of TEM and SHV genes with the reported clinical and environmental strains shows high similarity suggesting the possible horizontal transfer between them. A One Health approach is needed to address all the different contributions that assist in the development and dissemination of antimicrobial resistant organisms. The presence of antibiotic resistant bacterial strains and genes in soil may provide both barriers and opportunities for research in antimicrobial drug discovery and microbial ecology.

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