Beta-lactamases Dissemination amongst Multidrug-resistant Pathogenic *Enterobacteriaceae* Isolated in Pakistan and the United States

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I would like to dedicate my thesis to all those who

helped me directly or indirectly in fulfilling my desire

to earn a PhD

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
AR	Antibiotic Resistance
ATCC	American Type Culture Collection
AmpC	AmpC β-Lactamase
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CDC	Centers for Disease Control and Prevention
CLSI	Clinical Laboratory Standards Institute
CREs	Carbapenem Resistant *Enterobacteriacae*
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum β-lactamase
EPEC	Enteropathogenic *E. coli*
EIEC	Enteroinvasive *E. coli*
EAEC	Enteroaggregative *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
FDA	Food and Drug Administration
KCTC	Korean Collection for Type cultures
KPC	*Klebsiella pneumoniae* carbapenemase
LB	Luria-Bertani
M	Molar
MATE	Multidrug and toxic compound extrusion

MDR	Multidrug resistant
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
mL	Milli-litre
MLST	MultiLocus Sequencing Typing
mM	Milli-molar
NCBI	National Centre of Biotechnology Information
NDM-1	New Delhi metallo-β-lactamase
OMP	Outer membrane protein
ORF	Open reading frame
PABN	Phenyl alanine arginyl b-naphtylamide
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicllin binding protein
PCR	Polymerase Chain Reaction
QRDR	Quinolone Resistance-Determining Regions
RND	Resistance-Nodulation-Division
rpm	Revolutions per minute
RND	Resistance nodulation division
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TBE	Tris-borate-ethylenediaminetetraacetic acid
TE	Tris-EDTA
UPEC	Uropathogenic *E. coli*
UTI	Urinary Tract Infection
WGS	Whole Genome Sequencing

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ABSTRACT

Increasing resistance to modern antimicrobials in *Enterobacteriaceae* is a global health concern. Carbapenem resistant *Enterobacteriaceae* (CRE), encoding carbapenemases such as New Delhi Metallo-β -lactamase-1 (NDM-1) or *Klebsiella pneumoniae* Carbapenemase (KPC), are emerging threats. CREs harboring NDM are highly prevalent in the Indian subcontinent, while KPC isolates are globally distributed. Accurate genomic-based diagnostics of these and other multidrug resistant (MDR) pathogens could enable more rapid and efficacious treatment of bacterial infections. In this study, whole genome sequencing (WGS) was performed on 78 isolates of *Enterobacteriaceae* recovered from clinical samples in Pakistan and U.S.A. For each isolate high-resolution phylogenetic determination, antibiotic resistance and virulence gene detection, antibiotic susceptibility profile prediction, and antibiotic susceptibility phenotype validation was performed. Highly related bacteria were identified in Pakistani and U.S. samples harboring identical β-lactamases, and it was found that total antibiotic resistance gene carriage was not significantly different between locations. Though KPC was only identified in U.S. isolates and NDM-1 was identified in Pakistani isolates only, overall β-lactamase genotypes did not correlate with phylogeny or point of origin. When all sequenced plasmids carrying KPC or NDM-1 available on NCBI were compared, including plasmids from U.S. and Pakistani isolates, plasmids carrying either carbapenamase were similarly diverse in sequences. The presence of NDM-1 in diverse plasmid backbones and widely distributed pathogenic bacterial strains suggests that it is poised for global dissemination. WGS has a high potential for accurate detection of susceptibility profile of MDR pathogens such as CREs carrying carbapenemases.

Chapter 1

INTRODUCTION

1.1 ANTIBIOTIC RESISTANCE

Antibiotic resistance is globally regarded a major threat to human health. It restricts not only treatment options but also increase the risk of hospitalization and severity of infection. This challenge of resistance is consistently increasing with time. According to the World Health Organisation's report (WHO, 2014), the ever increasing resistance to the latest antimicrobial drugs has threatened the prevention and treatment options available for controlling infections caused by parasites, fungi, virus and bacteria. The world seems to enter in the post-antibiotic era- in which minor injuries, like a scratch on the skin will lead to death, a devastating reality of 21st century not far away from our lives.

Since the discovery of antibiotics in the 1930s, several antibiotic classes were developed for treatment of infectious diseases (Chopra *et al.,* 1997). Although antibiotics have been very effective in combating variety of human infections, still infectious diseases cause significant mortality, not because of the newly emerging diseases but because of drug resistant pathogens (Poirel *et al.,* 2008; WHO, 2007). This overwhelming resistance to antimicrobial drugs has put at risk the practice and future of modern medicine (Levy and Marshal, 2004). Multi-drug resistant (MDR) pathogens have been isolated from diverse habitats of environment, animals and humans (Hammerum and Heuer, 2009). The distribution and magnitude of antibiotic resistance encountered in bacteria is directly linked to the consumption of antibiotics. Several other contributing factors are substandard living; poor economic conditions,

and genetics of bacteria (Butler *et al.,* 2006; Moreno *et al.,* 2009). Several reports have confirmed the overuse of antibiotics and the emergence of resistance in bacteria (Davies and Davies, 2010). Global estimates of drugs usage show that nearly 50% of antimicrobials are inappropriately used (Dellit *et al.,* 2007). Most common inappropriate uses of antibiotics include their prescription for viral infections, such as common cold. In November 2013, an extensive survey was conducted by European Union regarding the proper use of antibiotics, in which more than 50 % of people in Europe thought that antibiotics kill viruses, and about 40 % was thinking they can be used for treating common cold and flu (EC, 2013). Such overwhelming misconceptions about antibiotics in general public lead to their inappropriate use (CDC, 2014).

Antibiotics are very widely used in animal husbandry, agriculture and aquaculture. However there is no comprehensive data available on their total consumption (Kummerer, 2009), but several careful estimates of non-therapeutic uses of antibiotics proves that their consumption only in livestock is eight times the amount used in human medicine (Mellon *et al.,* 2001).

Antibiotic resistance is an evolutionary process, every time antibiotics are used they create selective pressure on bacteria, few evolve and become resistant. These resistant bacteria then multiply and disseminate the resistance trait to other bacteria through various means. Resistance mechanisms have been associated mainly with horizontal gene transfer of antibiotic resistance genes (ARG) present on integrons, plasmid and transposons (Carattoli, 2009; Sherley *et al.,* 2004).

Antibiotic resistance is not just restricted to certain part of the world rather it is globally prevalent. In high income countries, the leading cause of resistance is the

overuse of antibiotics in agriculture. While in low income countries resistance generally spread through propagation of resistant bugs in hospital infections (Laxminarayan and Heymann, 2012).

Two types of resistance mechanisms have been reported in bacteria, intrinsic resistance and extrinsic resistance. Intrinsic resistance refers to the inherent ability of bacteria to resist the effects of antimicrobials through its unique structural and functional characteristics. Intrinsic resistance in bacteria is often caused by their membrane permeability and extensive efflux pumps that restrict either the entry of drugs into the cell or push them out of the bacterial cell, respectively (Nikaido, 2001). In contrast, acquired resistance is caused by mutations in bacterial DNA or acquiring genes via HGT (Thomas and Nielsen, 2005). Horizontal gene transfer (HGT) is the process where portion of DNA from one bacterium is transferred to another bacterium in non-reproductive process. This is done by transformation, conjugation, or transduction. HGT is regarded a very important contributory factor in bacterial evolution making them adapt to new conditions.

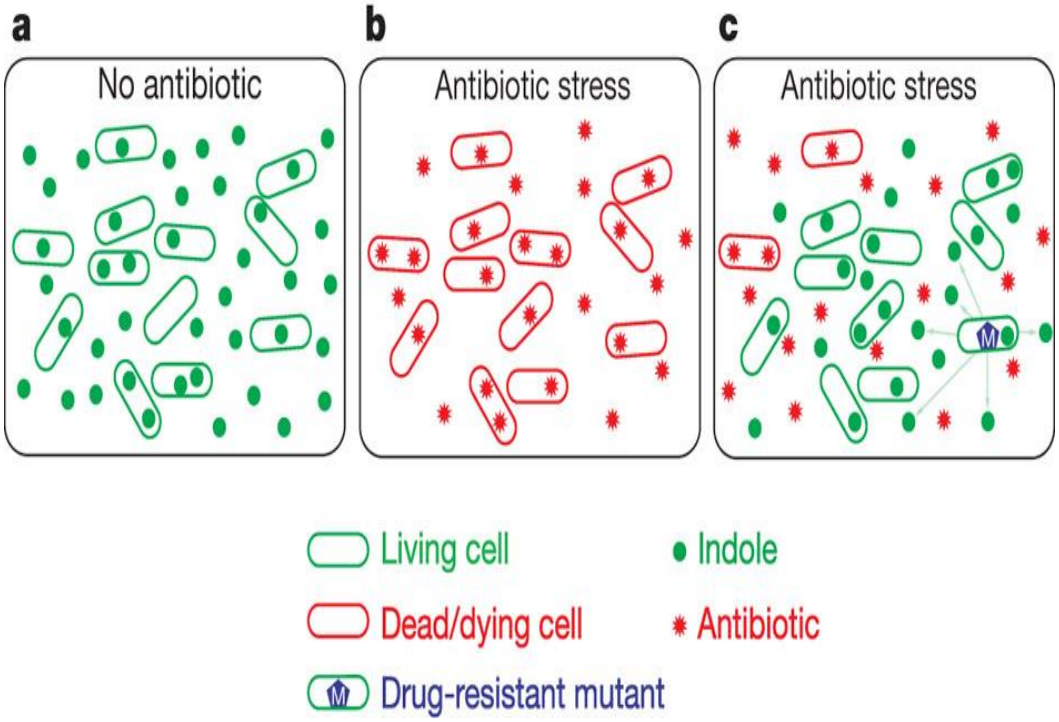

Figure 1.1: A population-based antibiotic-resistance mechanism

A population of bacteria under antibiotic stress is shown in the figure. **a**, In the absence of antibiotic, bacterial cells produce indole. **b**, Under antibiotic stress, bacterial cells stop producing indole and eventually die. **c**, Drug-resistant mutant is able to produce indole even in the presence of antibiotics. This indole helps the vulnerable cells in bacterial population to survive the antibiotic stress, through several antibiotic-tolerance mechanisms.

Adapted from (Lee *et al*., 2010)

1.2 *ENTEROBACTERIACEAE*

Enterobacteriaceae is a very important family of bacteria and is believed to have the most frequently encountered pathogens in microbiology (Corry *et al.,* 2003). Although many members of *Enterobacteriaceae* constitute normal flora of human body, but there are members which are of great concern such as *Escherichia coli* and *Klebsiella pneumoniae*. Infections of *Enterobacteriaceae* can be because of bacteria that normally live harmless in the body or by bacteria that enter from the environment. *Enterobacteriaceae* members generally are the most common cause of urinary tract infections in humans but they also cause pneumonia, sepsis, infections of wounds and of the intestines.

1.2.1 *Enterobacteriacae* and Resistance

Enterobacteriacae that constitute normal flora of the body have been found as reservoir for antibiotic resistance genes (Walker *et al.,* 2001). Beta-lactam resistance genes have been identified in commensal microbiota (Bailey *et al.,* 2011; Ehlers *et al.,* 2009; Sommer *et al.,* 2009). Sommer *et al.* (2009) investigated antibiotic resistance genes in the guts of healthy humans. They identified several genes that were quite different from antibiotic resistance genes isolated from pathogenic bacteria. On the other hand, they also found several other genes that were exactly similar to those of pathogens. They identified a penicillin resistance gene *bla*TEM-1 that has also been reported in several pathogenic Gram-negative bacteria.

Acquired resistance in *Enterobacteriacae* is an emerging problem of greater concern. Horizontal gene transfer via conjugation has been found the main mechanism for acquired antibiotic resistance in *Enterobacteriaceae* (Barlow, 2009). Plasmids are extra-chromosomal circular DNA molecule most often involved in the

conjugative transfer of resistance genes. In plasmids antibiotic resistance genes are usually present within special genetic structure called integrons (Partridge *et al.*, 2009). Integrons have been reported to carry several types of resistance genes for different classes of antibiotics. Several classes of antibiotics exist in clinical medicine, of them β-lactam antibiotics are common and effectively used against *Enterobacteriaceae.*

1.3 BETA-LACTAM ANTIBIOTICS

β-lactam antibiotics constitute penicillin and its derivatives, cephalosproin, monobactam and carbapenem. They represent one of the most widely used groups of antibiotics owing to their cheaper availability, efficacy and safety profile. β-lactams are bactericidal in action and inhibit cell wall synthesis in bacteria. They target the penicillin-binding proteins (PBPs), proteins involved in crosslinking of the peptidoglycan units within the cell wall. β-lactam antibiotics are widely used in human medicine for curing a broad range of bacterial infections caused by variety of Gram-negative and Gram-positive bacteria.

1.3.1 Mechanism of Resistance to Beta-Lactams

Three main mechanisms of resistance to antibiotics exist in bacteria. One important and widespread mechanism is the production of enzymes that degrade the structure of antibiotics. Other mechanisms of resistance include modification of the antimicrobial target site in bacteria, reduced permeability of antibiotics into the cell, and forced efflux of drugs outside of bacterial cell (Wilke *et al.*, 2005). However, the most prevalent mechanism is through β-lactamase enzymes, which degrade β-lactam ring of antibiotics and make them unable to bind with PBPs (Livermore, 1995). By late 2009, more than 890 variants of β-lactamases were on the record (Bush and

Jacoby, 2010). β-lactamases are important from clinical perspectives because genes coding for these enzymes have the ability to spread across bacterial communities through horizontal gene transfer. A variety of such transferable genes have been described in clinical environments. Most important of them are *bla*NDM, *bla*KPC, *bla*CTX-M, *bla*TEM, *bla*SHV, and *bla*AmpC. Among the *bla* genes, the most common are the *bla*CTX-M, *bla*SHV and *bla*TEM, which have been globally reported from clinical and non-clinical settings. Several variants of TEM (n=150), SHV (n=120) and CTX-M (n=80) have been reported from worldwide (Jacoby and Bush, 2009).

TEM β-lactamase was identified in 1965 in *E. coli* (Datta and Kontomichaloum, 1965). TEM represents one of the most common β-lactamases in *Enterobacteriaceae.* Although TEM was originally reported and predominantly present in *Enterobacteriaceae,* however they have also been reported in other Gram-negative bacteria (Bradford, 2001). SHV β-lactamases were identified in 1972 (Pitton, 1972), and have been found in a variety of *Enterobacteriaceae,* but most commonly identified in *K. pneumoniae* (Tzouvelekis and Bonomo, 1999). However, several outbreaks of SHV have also been reported from other species such as *P. aeruginosa* and *Acinetobacter* spp. (Huang *et al.,* 2004; Poirel *et al.,* 2004)**.**

CTX-M β-lactamase was first reported in 1986 in Europe (Matsumoto *et al.*, 1988). CTX-M is also an important group of β-lactamases (ESBLs) that are rapidly spreading among *Enterobacteriacae* worldwide. Variants of TEM, SHV, and CTX-M hydrolyse a broad range of penicillins and extended spectrum cephalosproins and known as extended spectrum β-lactamases (ESBLs). Recently, an increasing prevalence of ESBLs has been encountered worldwide (Jacoby and Medeiros, 1991).

Specific synthetic inhibitors were developed to inhibit the β-lactamases. These inhibitors are coupled with β-lactams and act in synergistic manner. The inhibitors inactivate β-lactamases, and β-lactam drug reach their target in bacteria. However, all β-lactamases such as NDM, KPC, OXA and AmpC types are not inhibited by these inhibitors and such β-lactamases pose even greater threats to the health today.

1.3.2 Classification of Beta-Lactamases

β-lactamases are classified either by their molecular structure or by their function. These two classification schemes are the Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification respectively. The Ambler molecular classification places all β-lactamases into 4 groups (A, B, C, D) based on their amino acid sequence similarity (Ambler, 1980). The Bush-Jacoby-Medeiros classification is based on the hydrolytic spectrum of function of all the β-lactamases. In the Ambler molecular classification classe A, C and D β-lactamases are known as serine β-lactamases because they have a serine in their active site. The class B β-lactamases are known as metallo-β-lactamases, as they have a zinc ion in their active site (Paterson *et al.,* 2005). Originally β-lactamases had only two groups, serine β-lactamases (class A) and metallo β-lactamases (class B). But when a new class A β-lactamase was identified that had a different amino acid sequence, it was placed in a new group, and named as class C (Jaurin and Grundstrom, 1981). Similarly, when other ß-lactamases were discovered that had serine in their active sites, and had different sequences than class A and class C, they were placed in a new group and were named as class D (Ouellette *et al.,* 1987). The class A β-lactamases constitute a large family of β-lactamases. The most common members of class A β-lactamases are TEM, SHV and CTX-M β-lactamases, but there are also several other enzymes in this

class. Class A β-lactamases are the most commonly encountered β- lactamases (Bush *et al.,* 1995).

1.3.3 Extended Spectrum Beta-Lactamases (ESBLs)

Extended spectrum β-lactamases is the term given to class A β-lactamases that hydrolyse variety of penicillins and advanced generation cephalosporins. Variants of TEM, SHV, and CTX-M have been reported with such extended spectrum activities. TEM β-lactamases are most frequently reported in *E. coli,* in which they were originally identified, and *K. pneumoniae*, however they have also been extensively reported in other bacteria (Bradford, 2001). SHV β-lactamases are commonly identified in *K. pneumoniae* (Tzouvelekis and Bonomo, 1999). In 1986, a novel β-lactamase was discovered in *E. coli* that was resistant to cefotaxime, and was named as CTX-M (Matsumoto *et al.,* 1988). The *bla*CTX-M gene variants share 40% sequence similarity to *bla*SHV and *bla*TEM, while SHV β-lactamases share about 68% sequence homology of amino acids with TEM (Tzouvelekis and Bonomo, 1999).

ESBLs are frequently encountered in resistance to 3rd generation cephalosporins in *Enterobacteriaceae*. Genes for ESBLs are generally present on plasmids along with other resistance genes for other classes of antibiotics such as aminoglycosides, sulfonamides and fluoroqinolones (Paterson, 2006). Therefore, resistance to multiple drugs is increasing among ESBL producers (Livermore *et al.,* 2008).

Although resistance is emerging to β-lactam antibiotics, but their efficacy, safety profile and cheap availability still make them the most widely used group of antimicrobial drugs, covering over 65% of the global antibiotic market (Poole, 2004).

1.4 ANTIBIOTIC RESISTANCE EPIDEMIC

The global antibiotic resistance (AR) epidemic is blooming in both community and hospital settings. One factor facilitating the spread of AR is the ability of many bacteria to exchange AR genes with each other through horizontal gene transfer (HGT) (Thomas and Nielsen, 2005). This has allowed globally disseminated pathogens to combine the most effective AR genes from different parts of the world into multidrug resistance plasmids, which themselves spread between strains. Characterizing the set of plasmids driving this expansion of AR has proven difficult, because the plasmids themselves are constantly being changed by recombination and transposases (Hawkey and Jones, 2009).

Broad-spectrum empiric antibiotic therapy is frequently administered to patients with suspected infection prior to the availability of susceptibility testing results to direct more specific therapy, which typically requires at least two days following specimen collection (Didelot *et al.,* 2012). Expedient identification of optimal antibiotic therapy regimens can dramatically improve patient outcomes (Kumar *et al.,* 2006). Rapid administration of appropriate therapy also facilitates antibiotic stewardship, reserving the use of broad-spectrum antibiotics only for cases in which they are absolutely necessary.

Whole genome sequencing (WGS) of pathogens has been proposed as a relatively rapid method for predicting antibiotic resistance, while simultaneously providing virulence prediction and high-resolution phylogenetic information for real-time epidemiology, which may be especially important in the hospital setting (Didelot *et al.,* 2012; Bertelli *et al.,* 2013). Indeed, WGS analyses have shown success in comparative genomics and genomic epidemiology of pathogenic bacteria (Grad *et al.,*

2014; Leopold *et al.*, 2014; Snitkin *et al.*, 2013) and in diagnosing infections from rare pathogens (Wilson *et al.*, 2014). However, the majority of reports describing the use of WGS to predict antimicrobial susceptibility have focused on pathogenic bacteria from Western Europe (Hasman *et al.*, 2014; Stoesser *et al.*, 2014; Gordon *et al.*, 2014), which are less frequently broadly resistant to antibiotic agents compared to phylogenetically similar isolates recovered from the Indian subcontinent. Antibiotic usage selects for both HGT of AR genes and clonal spread of resistant bacteria, and globalization and international travel enable pathogen dissemination to distant geographies (Hawkey and Jones, 2009; Cuzon *et al.*, 2010). Accordingly, it is likely just a matter of time before the most resistant MDR pathogens from any location will spread and become responsible for clinical infections worldwide. Therefore, the analytical performance characteristics of new diagnostic technologies such as WGS must be evaluated on MDR isolates from geographies with high AR burdens.

1.5 CARBAPENEM RESISTANCE IN *ENTEROBACTERIACEAE*

Carbapenem is the newest class of β-lactam-based antibiotics, and they are often considered as drugs of the last resort against MDR pathogens. The Centers for Disease Control and Prevention (CDC) named carbapenem resistant *Enterobacteriaceae* (CRE) as one of the three most urgent MDR threats today (CDC, 2013). In the *Enterobacteriaceae*, β-lactam resistance, including carbapenem resistance, is primarily mediated by enzymatic degradation of the antibiotic by plasmid-borne β-lactamases. Two such β-lactamase subclasses are now especially problematic: *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi Metallo-β-Lacatamase-1 (NDM-1). KPC, first identified in the U.S. in 2001 (Yigit *et al.*, 2001), has become endemic in several geographically non-contiguous areas of the world,

including the U.S., Israel, Greece, South America, and China (Munoz-Price *et al.,* 2013). NDM-1 was first described in 2008 (Yong *et al.,* 2009), and was reported as predominant carbapenemase in Pakistan (Kumarasamy *et al.,* 2010). Although NDM-1 has also been isolated in most clinical specimens, the patient has had an epidemiological link to the Indian subcontinent (Dortet *et al.,* 2014).

Aims of the study

Looking at prevalence of NDM-1 in the Indian subcontinent, it is surprising that NDM-1 has not spread more quickly to other regions. Possible explanations for the apparent restricted distribution of NDM-1 include 1) a difference in the genetic context of NDM-1 that reduces its propensity for HGT, 2) an ecological barrier that prevents pathogens in the Indian subcontinent from intermingling with other pathogens around the world, 3) the relatively short time since the first reports of NDM-1, or 4) effective surveillance and containment procedures at hospitals worldwide that prevent the spread of NDM-1 expressing pathogens.

Looking at the nature of NDM-1, and searching for other resistance determinants in bacteria, this study was designed with the following objectives:

- To compare and investigate the differences in clinical resistome in bacteria from different geographies using whole genome sequencing
- To correlate the phylogenetic relationship of the resistance determinants between bacteria of distant geographies
- To investigate carbapenemase determinants and disseminations in *Enterobacteriaceae*
- To investigate and identify any significant variations in the genetics of resistance determinants

Chapter 2

REVIEW OF LITERATURE

2.1 ANTIBIOTICS RESISTANCE IN *ENTEROBACTERIACEAE*

Alexander Fleming discovered antibiotics in 1930. However the term "Antibiotic*"* was used for the very first time in 1942 for substances that could inhibit the growth of other microorganisms (Waksman, 1947). Today antibiotics are one of the most extensively used drugs worldwide for controlling bacterial diseases. Antibiotics have saved millions of lives since their first use in human medicine. However, the blessings of antibiotics have been limited by resistant bacteria (Rossolini and Thaller**,** 2010). Antibiotics resistant bacteria develop tolerance to antibiotics in such a way that antibiotics are no more effective to kill them. This resistance phenomenon is linked to the misuse and overuse of antibiotics (Gangle, 2005). The indiscriminate use of antibiotics has made generations of bacteria resistant and this resistance is globally recognized a constant threat to human race (Walker *et al.,* 2009). The prevalence and spectrum of resistant bacteria has substantially increased globally including Europe (Harbarth *et al.*, 2001; Fluit *et al.*, 2001), United States (NNIS, 2000) and the developing world (Blondeau and Tillotson, 2000).

The emergence and quick replicating resistance in *Enterobacteriaceae* to all classes of available antibiotics make the treatment options difficult (Paterson, 2006). Several members of *Enterobacteriaceae* are posing greater health threats to humans today. Among them multidrug resistant *E. coli* and *K. pneumoniae* are very commonly encountered both in hospital and community acquired infections. Besides,

other members like *Enterobacter* spp., *Salmonella* spp. *and Shigella* spp. are responsible for significant mortality and morbidity in less developed countries.

E. coli often causes severe infections of the bloodstream (Kennedy *et al.,* 2008), and is most frequently encountered a causative agent of urinary tract infections. Resistance in *E. coli* to fluoroquinolones, third and fourth-generation cephalosporin antibiotics is on the rise and quite often these MDR variants are reported in the community acquired infections (Laupland *et al.,* 2008; Mesa *et al.,* 2006). The common cause of spread of MDR *E. coli* is contaminated food and water (Corpet, 1988). However these resistant *E. coli* are not limited to community acquired infections, many nosocomial cases of MDR *E. coli* have also been reported worldwide. Very recently Jafri *et al.* (2014) reported 65% MDR *E. coli* in clinical samples. A gradual increase was seen in the prevalence of MDR *E. coli.* For example, in a 12-year study (1971–1982) on the susceptibility of clinical *E. coli* isolates, negligible resistance was seen to several antibiotics (Atkinson and Lorian, 1984). In contrast, in another study (1997–2007) on *E. coli* isolates recovered from urine specimens, an increasing pattern in resistance was observed to various classes of antimicrobial agents including quinolones, sulfonamides, and β-lactam/β-lactamase inhibitor combinations (Blaettler et al., 2007). Similarly, Kronvall (2010) studied resistance pattern of *E. coli* in Sweden for covering a period of 30-years (1979–2009), and noticed a substantially increasing trend in resistance to ampicillin, trimethoprim/sulfamethoxazole and gentamicin. Significant resistance was seen to the antibiotics augmentin and gentamicin (87.5 and 77.5 % respectively), while imipenem showed least resistance (32.5%).

K. pneumoniae frequently causes infections of respiratory tract, urinary tract, gastrointestinal tract, blood and skin. *Klebsiella* spp. have intrinsic resistant to penicillins and can acquire resistance to all generations of cephalosporins through plasmid-mediated ESBLs (Bouza and Cercenado, 2002). ESBL producing *K. pneumoniae* are very difficult to treat because the genes encoding ESBL enzymes often co-exist with other genes which confer resistance to many other important groups of antibiotics such as sulfonamides, aminoglycosides, chloramphenicol, and tetracycline (Al-Zahrani and Akhtar, 2005). *Enterobacteriaceae* harboring ESBLs are very commonly recovered in health care settings. Among them *E. coli* and *K. pneumoniae* are very frequenntly encountered pathogens, which also show resistance to multiple drugs (Paterson, 2006).

Prevalence of MDR *K. pneumoniae* strains varies in different regions. Falagas *et al.,* (2009) reported13.3% prevalence of ESBL *K. pneumoniae* in Europe, 7.5% in North America, and 44% in Latin America. Among Asian countries, Iran had 77% prevalence (Mehrgan *et al.,* 2010), China 51% (Xiong *et al.,* 2002) and India 58% (Jain *et al.,* 2007). In Pakistan different studies show varying prevalence at different hospital set ups. A study conducted on *K. pneumoniae* isolated from hospitalized patients at Pakistan Institute of Medical Sciences (Shah *et al.,* 2003), 35 out 50 isolates (70%) were ESBL positive. Similarly, Afridi *et al.,* (2011) has reported that 84.6% of *K. pneumoniae* isolates had Extended Spectrum Beta-lactamase enzymes, which are responsible for resistance to many β-lactam enzymes including third generation cephalosporins.

Enterobacter spp. are widespread throughout the environment and also carried by humans and are well-recognized community and nosocomial pathogens. Generally

Enterobacter spp. have AmpC β-lactamases which are constitutively expressed (Paterson, 2006), and render resistance to ampicillin, amoxicillin, amoxicillin-clavulanate, first-generation cephalosporins, and cefoxitin (Bouza and Cercenado, 2002). Overproduction of AmpC β-lactamases is pre-dominantly involved in resistance to third and fourth generation cephalosporins in *Enterobacter* spp. (Paterson, 2006).

Salmonella spp. are Gram-negative bacteria live the intestines of mammals, birds, and reptiles. They can live for long time in soil, water and food (Angulo *et al.*, 2000). *Samonella* spp. cause various diseases, typhoid fever and salmonellosis are frequent and serious among them. The antibiotics chloramphenicol and ampicillin have been used in treatments for *Salmonella* infections since long (Shanahan *et al.*, 2000). But recently ciprofloxacin has been in use and is the most prescribed drug for these infections now (Angulo *et al.*, 2000). However, siginificant level of resistance has been reported to ciprofloxacin. Rahman *et al.* (2014) studied the susceptibility pattern of ciprofloxacin in *S. typhi* isolates from six countries in the Middle East and Central Asia. They collected 968 *S. Typhi* isolates from Egypt, Pakistan, Jordan, Qatar, Uzbekistan and Iraq between 2002 and 2007. Most of the strains were MDR and were resistant to ciprofloxacin, chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole. MDR strains were substantially more prevalent in Iraq (83%) and Pakistan (52%) compared with the other countries studied (13-52%).

Shigella spp. cause several intestinal diseases. *Shigella flexneri* is the cause of shigellosis-the most frequently encountered and communicable of bacterial dysenteries. Every year over 164 million people are infected with shigellosis resulting in over 1 million deaths, in developing countries and children (Jennison and Verma,

2004). *Shigella dysenteriae* also often causes severe intestinal infections and mortality rate attributed to *S. dysenteriae* can rais up to 20% (Ashkenazi *et al.,* 2003). *Shigella* species have been frequently reported globally as causative agents of diarrhea and is often attributed for substantial mortality and morbidity in countries with poor healthcare infrastructure including Pakistan (Khalil *et al.,* 1998). Resistance to antibiotics has been reported in *Shigella* spp. As early as in 1965 resistance to tetracycline, chloramphenicol, and streptomycin were reported to *Shigella*. Since then, resistance in *Shigella* continues to increase against various antimicrobials. A study reported from Israel shows that resistance to tetracycline was increased from 23% in 1992 to 87% by 2000 in *Shigella* spp. (Ashkenazi *et al.,* 2003).

2.2 *ENTEROBACTERIACEAE* AND RESISTANCE TO BETA-LACTAMS

Extended spectrum β-lactamases give resistance against variety of β-lactam antibioics including penicillins, cephalosporins (e.g., ceftazidime, cefotaxime, ceftriaxone and cefepime), monobactam (e.g., aztreonam), but are inhibited by carbapenems (e.g; meropenem, imipenem) and cephamycins (e.g; cefoxitin, cefotetan). ESBLs are generally inhibited by inhibitors of β-lactamases e.g., clavulanic acid, but now days many ESBL strains have been found that are resistant to commercially available β-lactam/β-lactamase inhibitors combinations. These resistant strains are often also resistant to fluoroquinolones. ESBLs- the plasmid mediated β-lactamases was first reported in 1983 (Knothe *et al.,* 1983). Since then they have been found and described worldwide (Paterson and Bonomo, 2005).

Enterobacteriaceae that produce these plasmid mediated β-lactamases is an increasingly growing threat worldwide, resulting in significant mortality and

morbidity (Paterson and Bonomo, 2005). ESBLs have been most commonly reported in *K. pneumoniae* and *E. coli*. Owing to the fact that *K. pneumoniae* and *E. coli* are natural resident of gastrointestinal tract, most frequent ESBL infections are urinary tract infections.

Almost all *Enterobacteriaceae* (except *Salmonella* spp.) produce chromosomally encoded β-lactamases which are responsible for intrinsic resistance to β-lactams (Susić, 2004). However, intrinsic resistance in *Enterobacteriaceae* is proportional to the expression of genes. *E. coli* and *Shigella* spp. produce very little AmpC β-lactamases, and are susceptible to ampicillin and other β-lactams (Baylis *et al.,* 2011). Similarly, *Enterobacter* spp. and *Serratia* spp. produce small amounts of inducible AmpC β-lactamases that pose resistance to ampicillin, co-amoxiclav and first-generation cephalosporins (Susić, 2004). Resistance to quinolone antibiotics in *Enterobacteriaceae* is usually because of mutations in chromosomes which results in alterations of the target enzymes of the drug. However, quinolone resistant plasmids, carrying the qnr genes, have also been reported (Paterson, 2006). Generally drug-resistant *Enterobacteriaceae* are inhibited by carbapenems, and carbapenems are considered preffered antimicrobials agensts in empiric therapy against MDR infections of *Enterobacteriaceae* (Paterson, 2006).

Carbapenem are the last line antibiotics but bacteria are getting resistant to them too by producing carbapenemases. Carbapenemases are type of β-lactamases that degrade the newest group of β-lactams, the carbapenem. In addition to carbapenem inhibition, these enzymes also inhibit almost all clinically available non β-lactams (Queenan and Bush, 2007). *Enterobacteriaceae* that are resistant to carbapenems, are known as carbapenem resistant *Enterobacteriaceae* (CRE). Two

types of CRE are increasingly becoming problematic in healthcare settings. One is KPC (*Klebsiella pneumoniae* carbapenemase), and other is NDM (New Delhi Metallo-β-lactamase). KPC-producing *Enterobacteriaceae* were reported first time in 2001 from a patient in North Carolina, United States (Yigit *et al.*, 2001). Soon after, many outbreaks of KPC-producing bacteria were reported from other parts of United States (Bratu *et al.*, 2005). KPC harboring bacteria have been reported from worldwide. A novel metallo β-lactamases of sub-continental origin NDM-1 was first identified in 2008 in a Swedish tourist to India when he was hospitalized in New Delhi and diagnosed with an untreatable infection (Yong *et al.*, 2009), and was reported as predominant carbapnemase in Pakistan, India and Bangladesh (Kumarasamy *et al.*, 2010). NDM-1 harbouring bacteria have been found resistant to all available antibiotics which are in clinical use. NDM-1 has also been reported from UK (Kumarasamy *et al.*, 2010) and Japan (Yuasa, 2010). Carbapenems are very important in the treatment of MDR *Enterobacteriacae*, and resistant to carbapenems were very rare, however the plasmid mediated KPC and NDM-1 have made *Enterobacteriacae* an alarming threat.

2.3 MECHANISMS OF RESISTANCE TO ANTIBIOTICS

2.3.1 Intrinsic Resistance

Bacteria resist the action of antimicrobials by two mechanisms, i.e; intrinsic resistance and acquired resistance. Intrinsic resistance refers to the inherent ability of bacteria to resist the effects of antimicrobials through its unique structural and functional characteristics. Intrinsic resistance in bacteria is often caused by their membrane permeability and extensive efflux pumps that restrict either the entry of drugs into the cell or push them out of the bacterial cell, respectively (Nikaido, 2001).

2.3.1.1 Decreased permeability

Porins are transport proteins in in bacterial cell membrane which restrict the influx of antimicrobials into the cell, and are widely accepted one of emerging problems of bacterial resistance (Pagès *et al.,* 2008; Vila *et al.,* 2007).

Antimicrobials enter bacterial cells either by passive transport via diffusion or through active transport. Hydrophilic antibiotics like, β-lactams and chloramphenicol penetrate bacteria through porins. While many other antimicrobials like, aminoglycosides enter bacteria self-promoted uptake. Besides β-lactams, most widely used antibiotics fluoroquinolones penetrate bacteria through porins, and size, copy number or selectivity or these porins can greatly influence the diffusion of fluoroquinolones entry into bacteria (Dzidic *et al.,* 2008; Nikaido, 2003; Denyer *et al.*, 2002; Hancock *et al.,* 2002; Dé *et al.,* 2001; Chevalier *et al.,* 1999)

2.3.1.2 Efflux pumps

Efflux pumps are transport proteins extensively found in cell membrane of most bacteria and are involved in the efflux of toxic molecules from bacterial cell. Efflux pumps have been reported in all studied bacterial cells (Webber and Piddock, 2003). Efflux pumps are mostly chromosomally encoded but genes for these pumps have also been found on plasmids and suggest their potential of dissemination across bacteria populations (Webber and Piddock, 2003). Efflux pumps are mostly non-selective for molecules and are capable to expel variety of drugs out of bacterial cells, thus make bacteria simultaneously resistant to multiple drugs (van Veen and. Konings, 1997). Efflux pumps are adaptation strategy of bacteria to survive them against the toxic effects of antimicrobials. Efflux pumps have been found involved in resistance to various classes of antibiotics, like; macrolides, tetracyclines and

fluoroquinolones (Findlay, 2011). Different efflux pumps have different mechanisms for the removal of toxic substances (Webber and Piddock, 2003), and have been classified into five families based on their mechanism and substrate specificity (*Dzidic et al.*, 2008). The efflux proteins families are known as; ATP binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the multidrug and toxic compound extrusion (MATE) family, and the small multidrug resistance family SMR family.

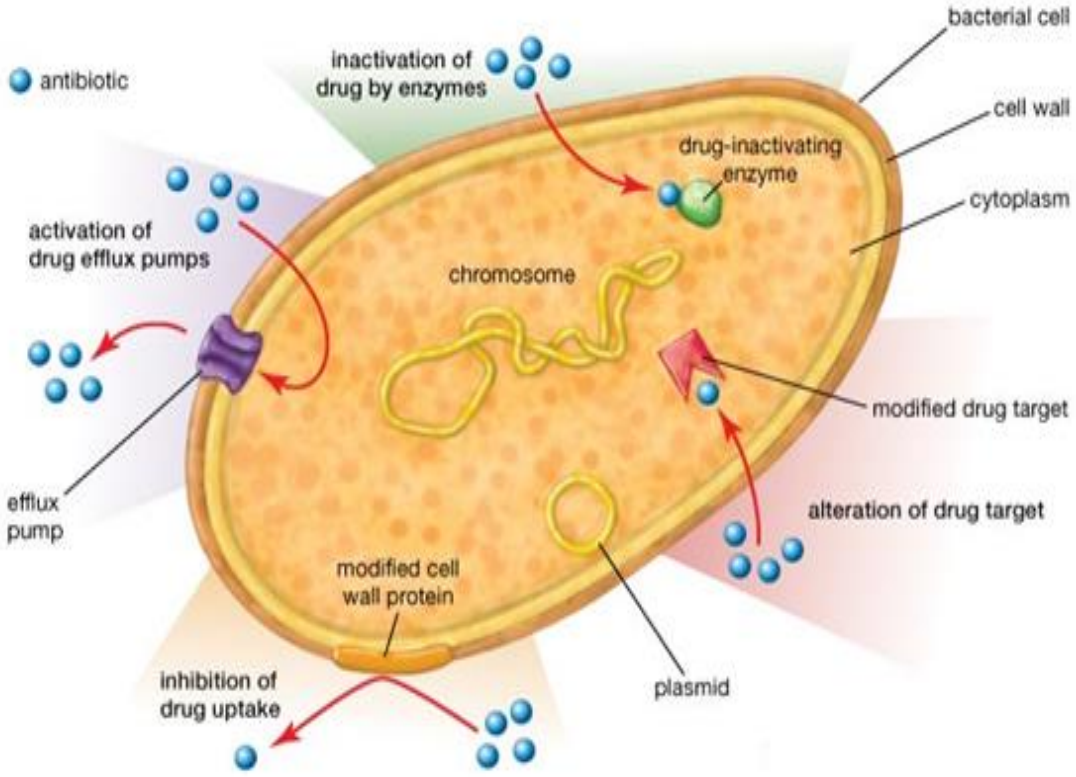

Figure 2.1: Mechanism of antibiotic resistance

There are multiple mechanisms of antibiotics resistance in bacteria. Examples include drug efflux pumps that remove drug from the cell, drug modification by bacterial enzymes, the drug targets modifications, and the restriction of drug uptake into the cell.

(Adapted from Encyclopaedia Britannica, Inc., 2009.)

2.3.2 Acquired Resistance

Acquired resistance in bacteria is the condition when susceptible strains mutate or borrow the resistant genes from other bacteria and start resisting the antimicrobials compounds to which they were previously susceptible. Once resistant is acquired, it vertically passes on to daughter cells or can also be shared with other susceptible bacteria, subsequently creating a population of resistant bacteria. Once bacteria become resistant, resistance can never be revered. Bacteria that live in the intestinal tract can readily exchange resistant genes among themselves (Finlay and Falkow, 1997). Such sharing of resistant genes leads to a huge problem when commensal bacteria borrow those genes and transform into pathogens (Manges *et al.,* 2001). Various extensive pangenomic studies have proved the exchange of resistant genes within the body among Gram-negative *Enterobacteriaceae* (Stecher *et al.,* 2012)

2.3.2.1 Mutation

One mechanism of acquired resistance found in bacteria, is mutation in the target site of antimicrobials. The modified site is no more detected by the antimicrobial compound. Bacteria either become resistant to antibiotics through mutation spontaneously or aquire resistance genes from other bacteria through horizontal gene transfer (HGT) (Toprak *et al.,* 2012; Lee *et al.,* 2010; Levy and Marshal, 2004).

Spontaneous mutations in bacteria are changes in the sequence of DNA that codes for a particular structure in bacterial cells that may lead to the formation of a modified structure. A single base change in the DNA may lead to incorporation of a different amino acid in the respective proteins, and which consequently brings

changes in the structure or function of that protein. Most often these modified structures lose affinities for the antimicrobials, which were effective before. Such types of resistance have been widely reported. Examples of spontaneous mutation in single base pair are commonly found in bacteria for antimicrobial drugs such as quinolones and rifampicin (Ruiz, 2003; Yee *et al.,* 1996). However, sometimes multiple mutations are needed in the DNA to confer resistance multiple classes of antibiotics (Toprak *et al.,* 2012; Lozovsky *et al.,* 2009; Dzidic *et al.,* 2008; Weinreich *et al.,* 2006; Huovinen *et al.,* 1987). The rate and frequency of mutations required to make bacteria resistant to antibiotics also vary for different antibiotics. For example, mutations required for resistance to antimicrobials like rifampin, streptomycin, and nalidixic acid are very common as compared to vancomycin (Dzidic *et al.,* 2008; Perreten *et al.,* 1997).

Mutations, sometimes, are spontaneous and are results of errors in replications, but most often antibiotic pressure cause bacteria to mutate and adapt to the stress. Such types of mutations occur in particular genes responsible for the survival of bacteria (Findlay, 2011). For example, excessive fluoroquinolones use and the *gyrA* gene mutations have been found associated in resistance to fluoroquinolones in *Enterobacteriaceae* (Weigel *et al.,* 1998).

E. coli susceptible strains were found resistant to the antibiotic rifampicin after many spontaneous mutations occurred in the absence of rifampicin (Severino*v et al.,* 1993; Jin and Gross, 1988). Similarly, many spontaneous mutations in *mycobacterium tuberculosis* are responsible for multidrug resistance (Musser, 1995). Resistance to streptomycin in the absence of any aminoglycoside modifying enzymes has also been

attributed the spontaneous mutations in the 16S ribosomal RNA genes, which modifies the ribosome- the target of streptomycin (Musser, 1995).

2.3.2.2 Horizontal Gene Transfer

Horizontal gene transfer is the exchange of genes between different genomes. Comparative genomics has demonstrated that a very large portion of bacterial genomes have been shared through horizontal transfer (Koonin *et al.,* 2001). Antibiotic Resistant Gene (ARG) transfer between bacteria was first described as early as 1959 in a publication from Japan (Ochiai *et al.,* 1959). Today, horizontal gene transfer is considered the main reason for resistance to antibiotics among bacterial communities (Gyles and Boerlin, 2014; OECD, 2010; Kay *et al.,* 2002; Koonin *et al.,* 2001; Nielsen, 1998), and is thought to be responsible for the exchange of hundreds of ARG between diverse bacterial species (Barlow, 2009). Horizontal gene transfer (HGT) in pathogenic bacteria has conferred resistance to several classes of antibiotics and is the primary reason for worldwide epidemics of hospital and community infections (Warnes *et al.,* 2012). ARG are often carried by large self-transmissible plasmid, and they are often linked to transposons, integrons and resistance islands (Dzidic and Bederkovic, 2003). Such mobile DNA elements use various mechanisms of recombination to get integrated into the recipient bacteria genomes (Normark and Normark, 2002).

2.3.2.3 Mobile genetic elements

Mobile genetic elements are generally described in two categories. First category includes genetic elements that are capable of horizontal transfer between different bacteria such as plasmids and bacteriophages, and second category comprise those DNA fragments that can move and re-arrange themselves within the same

genome, such as transposons and integrons (Bennett, 2008). All these mobile genetic elements have caused this widespread dissemination of resistance genes to commonly used antibiotics such as β-lactams, aminoglycosides and fluoroquinolones (Bennett, 2008).

2.3.2.3.1 Bacteriophages

Bacteriophages are type of viruses that infect bacteria. Bacteriophages can transfer ARG from one bacterium to another through a process known as transduction. During transduction a lysogenic bacetriophage infect susceptible bacteria and incorporate bacterial resistant genes during replications and packaging into its own genome. Subsequent infection of another bacterium by same phage results in the transfer of gene. Transduction was first demonstrated for penicillin resistance genes in staphylococci in 1958 (Garrod and O'Grady, 1971).

Bacteriophages generally have a limited host range. However, their common prevalence in diverse environmental conditions makes them efficient candidates for horizontal gene transfer (HGT) (Gangle, 2005). It was proved in recent past that phage mediated HGT events are much more prevalent (Brabban *et al.*, 2005; Cangelosi *et al.*, 2004). Several reports suggest that phages are involved in ARG sharing through mobilization or general transduction (Banks *et al.*, 2004; Schmieger and Schicklmaier, 1999; Blahova *et al.*, 1993; Hyder and Streitfeld, 1978). Colomer-Lluch *et al.* (2011) demonstrated the transfer of *bla*TEM and *bla*CTX genes through transduction. They further found that the host bacteria became resistant to ampicillin after transfection. They concluded from their study that phages are reservoirs of resistance in the environment. Reports about the ARG transfer via HGT are on the

rise (Muniesa *et al.,* 2013). Phages are also capable of carrying integrons, transposons and plasmids (Findlay, 2011).

2.3.2.3.2 Transposons

Transposons, also called as 'jumping genes', can move around within the genome by a process known as transposition. Transposons are identified by inverted repeats sequences at both ends of the elements (Findlay, 2011). Several types of resistant transposons are described on basis of structure and mechanism of transpositions (Bennett, 2005). Transposon differs from insertion sequences, because insertion sequences do not carry any genes while transposons by definition carry genes and changes the phenotype of the cell, for example a transposon carrying antibiotic resistance genes confers resistance to a particular antibiotic(s) (Bennett, 2008). Within bacterial cells transposons are capable of exchanging gene transfer between chromosomal DNA and plasmid.

2.3.2.3.3 Integrons

Integrons are another class of mobile genetic elements that capture and transfer genes, such as antibiotic resistance genes, across bacterial genomes by site-specific recombination. Integrons can be present on chromosomes or on plasmids. Integrons have two important characteristics that make them different from transposons. First, transposons have repeat sequences at their both ends, but integrons do not have such repeats; and secondly, integrons possess a site specific integrase gene as those found in bacteriophages but they do not have the enzymes involved in transposition (Boucher *et al.,* 2007). Gene cassettes are genetic elements that carry the ARG or genes for other virulence factors (Roe and Pillai, 2003). ARG cassettes have been reported for several classes of antibiotics including chloramphenicol,

trimethoprim, aminoglycosides β-lactams, and quinolones; and many gene cassettes have been reported for each of these classes (Boucher *et al.,* 2007). So far more than 40 different types of gene cassettes have been reported (Recchia and Hall, 1995). Among the recently found important ARG cassettes are those that carry carbapnemases such as imipenemase (IMP) and Verona integron-encoded metallo-β-lactamase) VIM (Nordmann and Poirel, 2002; Lauretti *et al*., 1999).

Three distinct classes of integrons have been described. Each of the integrin class shows the distinct characteristics of integron i.e; gene for integrase, a receptor site for gene cassette and a having promoter for the genes carried on the gene cassettes (Hall, 1997). Class 1 comprises most of the integrons reported in clinical isolates (Bunnett, 1999).

2.3.2.3.4 Plasmids

Plasmids are extra-chromosomal DNA ring, capable of independent replication within the cell. Plasmids have been reported in trasnfer of antibiotic resistance against several classes of antibiotics in variety of bacteria (Svara and Rankin, 2010; Furuya and Lowy, 2006). The most common mechanism of ARG dissemination via plasmid is conjugation (Huddleston, 2014; Norman *et al.,* 2009). Conjugation is the process of bacterial mating via a specialized membrane structure a pilus, or pore that makes a passage for the transfer of plasmids between conjugating bacteria (Huddleston, 2014). Much of the bacterial genomes plasticity is linked to the exchange of gene contents through plasmid (Treangen and Rocha, 2011; Nelson *et al.,* 2010; Hehemann *et al.,* 2009). Plasmid transfer via bacterial conjugation is certainly fueling the emergence of super ARG bugs (Johnson *et al.,* 2010; Paulsen *et al.,* 2003).

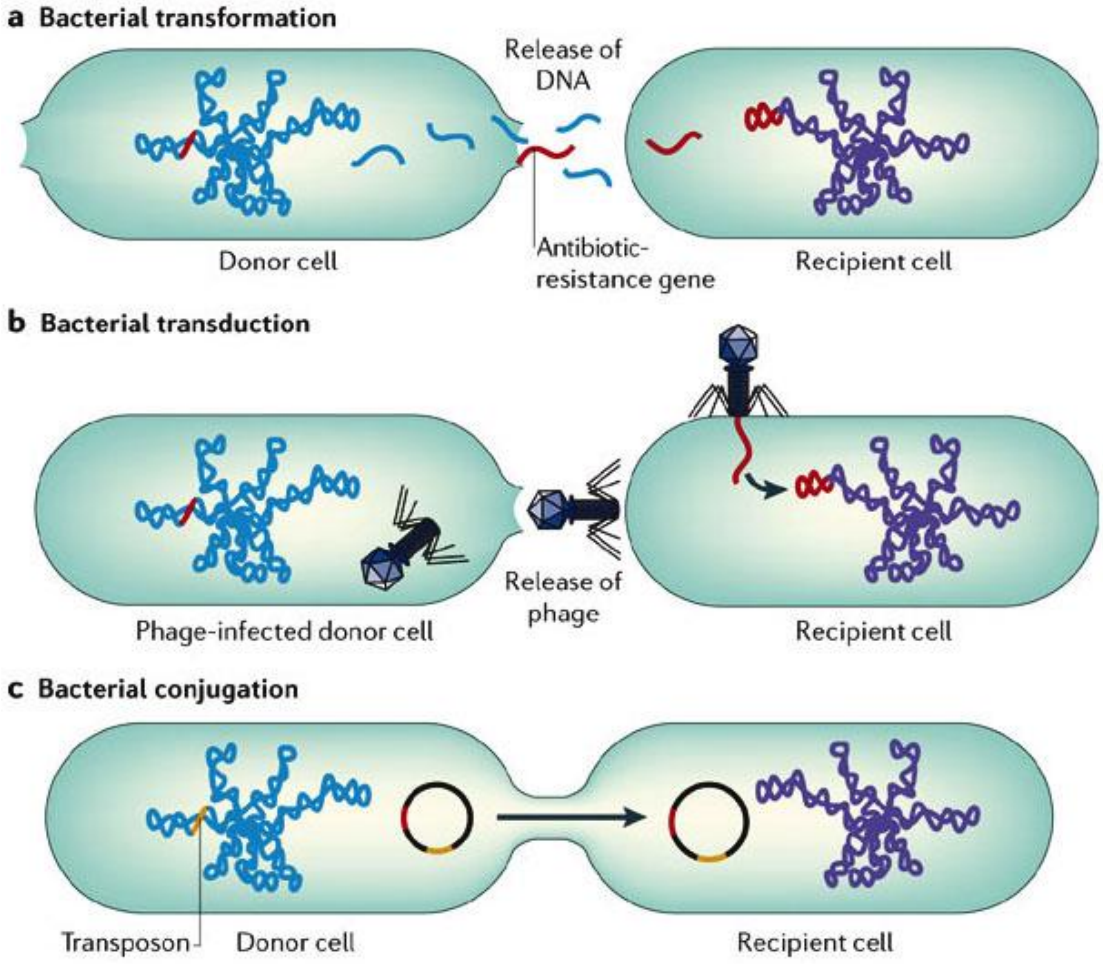

Figure 2.2: Horizontal Gene Transfer in Bacteria

Three common mechanisms of HGT in bacteria. Transformation is the uptake of DNA (having antibiotic resistance genes) from the ruptured bacterial cell by live bacterium. Conjugation is the transfer of resistance genes between bacteria through plasmid. Transduction is the transfer of antibiotic resistance genes from one bacterium to another through lysogenic bacteriophages.

Adapted from (Furuya and Lowy, 2006).

2.4 SPREAD OF RESISTANCE AND BETA-LACTAMASES

All β-lactamases inhibit the β-lactam ring of antibiotics found in penicillins, cephalosporins, monobactams and carbapenems. Class A, C, D enzymes have serine in their active site and knows as SBLs (Serine β-lactamases). SBLs inhibit in penicillins, cephalosporins and monobactams classes of antibiotics. Class B β-lactamases have zinc in their active site and are called as metallo-β-lactamases (MBLs). MBLs hydrolyse penicillins, cephalosporins and carbapenems but not the monobactams (Gupta, 2007; Ambler, 1980).

ß-lactamases are one of the most widely disseminated and clinically important classes of AR genes (Davies and Davies, 2010). The dissemination of β-lactamases may be chromosomal or plasmid mediated, or even mediated by transposons such as TEM 1 and TEM 2 (Heritage et al, 1992). β-lactamases may be carried by integrons (Poirel *et al.,* 2000). Genes for β-lactamase SHV is found on the chromosome of *K. pneumonia* (Babini and Livermore, 2000; Haeggman *et al.*, 1997). However, SHV gene may also be plasmid mediated (Preston, 2004).

The TEM 1 enzyme was first reported in *E. coli,* isolated from a patient named Temoniera, hence named as TEM 1. The plasmid and transposons mediated characteristics of TEM, made it widely prevalent across bacterial communities. The second commonly found plasmid mediated β-lactamase in *K. pneumonia* and *E. coli* is SHV. The SHV1 β-lactamase is chromosomally encoded in *K. pneumoniae* but is generally plasmid mediated in *E. coli* (Tzouvelekis and Bonomo, 1999).

Most of the pathogenic bacteria of the family *Enterobacteriaceae* have at least one chromosomal β-lactamase (Forssten, 2009). Chromosomal β-lactamases normally confer low level of resistance to the host because they are mildly expressed.

Chromosomal β-lactamases generally making bacteria resistant to penicillins only, while resistance to other β-lactam groups such as cephalosporins or/and carbapenems are plasmid mediated (Sanders, 1987). Chromosomally encoded AmpC β-lactamases have been found to confer resistance to carbapenem in porin deficient strains (Jacoby, 2009). AmpC are typically found on chromosome, however, they have also been reported from plasmid (Findlay, 2011). Integrons have been reported in the acquisition of genes encoding AmpC type β-lactamases in plasmids (Philippon *et al.,* 2002). Some carbapenemases like Verona integrons encoded (VIM) and imipenem (IMP) are also found within integrons (Gupta, 2007).

2.5 CLASSIFICATION OF BETA-LACTAMASES

β-lactamases are increasing in number with the passage of time. More than 1000 β-lactamases (BL) have been reported in Gram-negative bacteria (Marsik and Nambiar, 2011). Clinically, the most important β-lactamases include extended spectrum β-lactamases (ESBLs), AmpC β-lactamases, *Klebsiella pneumoniae* carbapenemases (KPC), and the metallo-β-lactamases (MBLs) (Bush *et al.,* 2011). Beta-lactamases are generally broadly placed into two main groups; those that contain a non-metal ion serine, at their active site, known as serine β-lactamases, and those enzymes which require zinc as a co-factor, known as metallo β-lactamases (Jacoby and Munoz-Price, 2005). β-lactamases are also classified into four molecular classes; A, B, C and D, on basis of their nucleotides composition. These classes can also be further grouped into distinct functional classes on basis of their inhibition profiles (Bush *et al.,* 1995).

2.6 THE CLINICALLY MOST IMPORTANT BETA-LACTAMASES

2.6.1 Class A β-lactamases

Class A β-lactamases are involved in the most commonly encountered resistance in bacterial communities (Bush *et al.,* 1995). The class A β-lactamases is a large family of β-lactamases, most common of them is TEM, SHV and CTX-M.

2.6.1.1 TEM β-lactamases

TEM β-lactamases are one of the most common β-lactamases in *Enterobacteriaceae*. Although TEM was originally reported and predominantly present in *Enterobacteriaceae*, however they are also frequently reported in other Gram-negative bacteria (Bradford, 2001). Hundreds of TEM-type β-lactamases have been identified, and majority of them possess the ESBL activity (Paterson and Bonomo, 2005). ESBL are popular for inhibition of extended spectrum cephalosporins. ESBL phenotype of TEM is typically created by point mutation at positions 104, 164, 238, and 240, but usually ESBLs have been found to have more than a single mutation (Bradford, 2001). More than 150 variants of the *bla*TEM gene that encodes TEM β-lactamases have been identified (Jacoby and Bush, 2009). TEM β-lactamses also inhibit sometimes the β-lactamase inhibitors. Various studies have documented that a single base mutation at position 244 of the *bla*TEM make it resistant to inhibitor (Delaire *et al*., 1992). Such types of TEM variants were first named inhibitor resistant TEM (IRT) (Bonomo and Rice 1999). IRT β-lactamases have been reported various species *Enterobacteriaceae* (Bradford, 2001).

2.6.1.2 SHV β-lactamases

SHV stands for sulphydryl variable and the first representative of this group, SHV-1, was reported in 1972 (Pitton, 1972), while a variant of SHV-1, SHV-2 was reported in Germany in 1980s (Kliebe *et al*., 1985).

SHV β-lactamases have been found in a variety of *Enterobacteriaceae.* The SHV-type ESBLs most prevalent in clinical isolates as compared to other types of ESBLs (Jacoby, 1997), and have been most commonly identified in *K. pneumoniae* (Tzouvelekis and Bonomo, 1999). The SHV β-lactamases share 68% amino acids similarity with TEM (Tzouvelekis and Bonomo, 1999). SHV variant with point mutations at positions 238 and 240 are involved in ESBL activity (Paterson *et al.* 2003; Huletsky *et al.,* 1993). The serine amino acid at position 238 of the peptide is required for effective inhibition of ceftazidime, while the lysine amino acid at position 240 is required for inhibition of cefotaxime (Huletsky *et al*., 1993). Over 120 SHV variants have been identified worldwide (Jacoby and Bush, 2009). In Europe the SHV-type ESBLs were the predominant in 1990s, and were found the dominant ESBL in the start of 21st century in the United States, with SHV-2, SHV-5 and SHV-12 variants the most prevalent (Paterson *et al.,* 2003).

2.6.1.3 CTX-M β-lactamases

CTX-M enzymes also represent an important group of class-A β-lactamases (ESBLs) that are rapidly spreading among *Enterobacteriaceae* worldwide. CTX-M was first described in *Enterobacteriaceae* strains 1986 in Europe (Matsumoto *et al.,* 1988). In 1989, more reports of CTX-M were found in other European countries like Argentina, France and Germany (Bauernfeind *et al.,* 1992). Since then over 80 different variants of CTX-M β-lactamase have been identified. CTX-M variants are

clustered in six groups based on their amino acids sequence homology (Rossolini *et al.,* 2008). These groups are CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTXM-25 and CTX-M-45. The CTX-M β-lactamases show significant affinity towards cefotaxime (and ceftriaxone) as compared to ceftazidime.

CTX-M β-lactamases have been reported worldwide in several species of *Enterobacteriaceae*. However, most commonly they are found in *E. coli, S. typhimurium, K. pneumoniae* and *P. mirabilis*. CTX-M-1 group (especially the *bla*CTX-M-15 gene), *bla*CTX-M-14 and *bla*CTX-M-9 are the most commonly reported CTX-M genes in Europe (Woodford *et al.,* 2004; Livermore *et al*., 2007). However, recent emergence of CTX-M-14 and CTX-M-15 has been reported in the United States (Castanheira *et al.,* 2008). This widespread prevalence of CTX-M genes across clinical bacterial communities of *Enterobacteriaceae* is attributed to horizontal gene transfer via plasmids (Bonnet, 2004).

2.6.2 Class B Beta-lactamases

2.6.2.1 Metallo β-lactamases

Class B β-lactamases are also known as metallo-β-lactamases. Metallo-β-lactamases are different from class A enzymes as they require Zn (2+) at their active site instead of Serine. Since few years, several novel zinc β-lactamases have been discovered in variety of pathogens. Metallo-β-lactamases belong to class B on the molecular classification (Ambler, 1980), and belong to group 3 on functional classification (Bush *et al.,* 1995). Metallo β-lactamases (MBLs) are subdivided on into three subclasses B1, B2 and B3 on the basis of sequence homology (Garau *et al*., 2005). However, recently an unusual metallo-β-lactamase was discovered in *Serratia proteamaculans* (SPR-1) which suggests the presence of another subgroup, *i.e.* B4

(Hou *et al.,* 2014). Metallo-β-lactamases are broad-spectrum in activity and inhibit several penicillins and cephalosporins (Poirel *et al.,* 2000), including carbapenems, but are inhibited by aztreonam (Walsh *et al.,* 2005). They can also be inhibited by EDTA but are resistant to clavulanic acid (Walsh *et al.,* 2005). Metallo-β-lactamases are generally found in nosocomial pathogenic strains of *K. pneumonia* (Queenan and Bush, 2007; Walsh *et al.,* 2005), *Pseudomonas* spp. and *Serratia* spp. (Bebrone, 2007).

Various types of metallo-β-lactamases have been described. They can be chromosomally encoded or plasmid mediated. Chromosomally encoded metallo-β-lactamases are reported from various bacterial strains such as BcII from *Bacillus cereus* (Meini *et al.,* 2014; Lim *et al.,* 1988), GOB1 from *Elizabethkingia meningoseptica* (Meini *et al.,* 2014), and L1 from *Stenotrophomonas maltophilia* (Walsh *et al.,* 1994). However, the clinically important metallo-β-lactamases are coded in mobile genetic elements and include VIMs (Verona Integron-encoded Metallo-β-lactamase) (Meini *et al.,* 2014; Lauretti *et al.,* 1999), IMPs (Imipenemase) (Laraki *et al.,* 1999), and the more recently emerged NDMs (New Delhi metallo-β-lactamase) (Meini *et al.,* 2014; Yong *et al.,* 2009).

2.6.3 Class C β-lactamases

Class C β-lactamases (AmpC) constitute a very important and second most widely distributed group of β-lactamases worldwide (Bush *et al.,* 1995).

AmpC β-lactamases are chromosomally encoded β-lactamases. They are present mainly in *Enterobacteriaceae* and pose resistance against cephalosporins, penicillins, and β-lactamases inhibitors (Jacoby, 2009). Mostly AmpC inhibit cephalosporins but they are also capable of inactivating all β-lactams antibiotics

(Hanson *et al.,* 2003; Bush *et al.,* 1995; Sanders, 1987). AmpC genes are present usually in the chromosomes of *Enterobacteriaceae*; however, they have also been reported from plasmids (Jacoby and Munoz-Price, 2005). FOX, CMY and MOX are are plasmid-mediated AmpC β-lactamases (Adler *et al.,* 2008; Doi and Paterson 2007; Bauernfeind *et al.,* 1996a). Around hundred different AmpC types commonly isolated from *Enterobacteriaceae* have been described (Jacoby and Bush, 2009).

2.6.4 Class D β-lactamases

Class D β-lactamases were first described as enzymes degrading oxacillin and cloxacillin much faster than benzylpenicillin (Bush *et al.,* 2005). Several class D β-lactamases are reported that have poor inhibitition of cloxacillin and oxacillin. However, all of these β-lactamases significantly inhibit both aminopenicillins and carboxypenicillins (Poirel *et al.,* 2010). Class D β-lactamases are normally not resistant to all clinically important β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam. However an interesting characteristic of Class D enzymes is that they are inhibited by NaCl. NaCl at 100 mM concentration inhibits class D β-lactamases (Girlich *et al.,* 2004).

Oxacillinases or OXAs were the first of class D enzymes described. Generally class D enzymes confer intrinsic resistance to several Gram-negative bacteria such as *A. baumannii* and *P. aeruginosa* (Poirel *et al.,* 2010). The chromosomally encoded OXAs generally do not show ESBL activities (Afzal-Shah *et al.,* 2001). Point substitutions are required at position 73, or position for the ESBL phenotype (Heritier *et al.,* 2005). Along with the chromosomally encoded OXAs, plasmid mediated OXAs have also been described (Naas and Nordmann, 1999). About 140 OXA-type β-lactamases have been identified (Jacoby and Bush, 2009). Most of them show variable

spectrum of activity. OXA have also been found inactivating carbapenems in several instances (Poirel *et al.,* 2010).

2.7 CARBAPENEMASES

Carbapenemases are β-lactamases with a wide range of hydrolytic capabilities for all generations of β-lactam enzymes. They can hydrolyze penicillins, various generations of cephalosporins, monobactams and carbapenems. Carbapenemases may belong to any of the three molecular classes A, B, and D. Class A and D β-lactamases have a serine while class B enzymes possess a zinc residue in the active sites. Carbapenemases that are inhibited by EDTA are zinc β-lactamase while those inhibited by clavulanic acid and tazobatam are serine β-lactamase (Rasmussen *et al.,* 1996).

2.7.1 Class A Carbapenemases

The class A carbapenemases can inhibit a range of β-lactam antibiotics, including carbapenems, cephalosporins and aztreonam. However, they are hydrolysed by β-lactamase inhibitors (Queenan and Bush, 2007). The class A carbapenemase group contain both chromosomal and plasmid encoded carbapenemases. Among the chromosomal carbapenemases SME and IMI are important. SME-1 (for "*Serratia marcescens* enzyme") was isolated in England from *S. marcescens* strains in 1982 (Naas *et al.,* 1994; Yang *et al.,* 1990). The SME-1 β-lactamase, along with other identical β-lactamases SME-2 and SME-3, has been identified in various sites throughout the United States (Queenaan and Bush, 2007). The IMI (for "imipenem-hydrolyzing β-lactamase") and NMC-A (for "not metalloenzyme carbapenemase") enzymes have been very rarely reported in *E. cloacae* in the United States, France,

and Argentina (Radice *et al.,* 2004; Pottumarthy *et al.,* 2003; Rasmussen *et al.,* 1996; Nordmann *et al.,* 1993).

Among the plasmid borne class A carbapenemases, KPC (for "*klebsiella pneumoniae carbapenemase*") is the most common. KPC was identified in North Carolina, United States in 1996 (Yigit et al, 2001).

Soon after the discovery of KPC-1 several investigators reported a single-amino-acid variant, KPC-2, from other places in United States (Miriagou *et al.,* 2003; Moland *et al.,* 2003; Yigit *et al.,* 2003). Today KPC is very common in United States, China and Europe (Woodford *et al.,* 2008; Bradford *et al.,* 2004). The GES/IBC family of Class A β-lactamases was described in 2000, but they are very rare. IBC-1 (for "integron-borne cephalosporinase") was isolated from *E. cloacae* in Greece (Giakkoupi et al., 2000) and GES-1 (for "Guiana extended spectrum") from *K. pneumoniae* isolate in French Guiana (Poirel *et al.,* 2000).

2.7.2 Class D Carbapenemase

The class D carbapenemases consist of OXA-type β-lactamases generally found in the *Acinetobacter baumannii* (Queenan and Bush, 2007). Paton *et al.,* (1993) identified the first OXA β-lactamase that has carbapenemase activity. These β-lactamases were isolated from a MDR *A. baumannii* strain in 1985. Several OXA type β-lactamases have been discovered in *Acinetobacter baumannii*. OXA-23 with carbapenemase activity were reported from outbreaks of *Acinetobacter* in Brazil, the United Kingdom, Korea, and Tahiti, Spian, Portagal (Lolans *et al.,* 2006; Jeon *et al.,* 2005; Naas *et al.,* 2005; Turton *et al.,* 2005; Da Silva *et al.,* 2004; Dalla-Costa *et al.,* 2003; Bou *et al.,* 2000) and also from military persons in Iraq and Afghanistan (Hujer *et al.,* 2006).

2.7.3 Class B Carbapenemase

Class B β-lactamase, also known as metallo-β-lactamases, are group of β-lactamases that require zinc for their hydrolysing activity. The first metallo-β-lactamases identified and studied in detail were chromosomal BCII, which was found in environmental bacteria *Bacillus cereus* (Kuwabara and Abraham, 1967). BC11 was the first metallo-β-lactamase whose entire amino acid sequence was determined (Hussain *et al.,* 1985).

In contrast to chromosomal metallo-β-lactamases, there are also many transferrable metallo β-lactamases. Transferrable metallo-β-lactamases include the VIM, IMP, GIM, and SIM enzymes, which are mediated through integrons (Queenan and Bush, 2007). IMP (for "imipenemase") was first identified in *P. aeruginosa* in 1990 in Japan (Watanabe *et al.,* 1991). VIM is another family of integron mediated metallo-β-lactamases. VIM-1 (for "Verona integron-encoded metallo-β-lactamase") was first reported in 1997 from Verona, Italy (Lauretti *et al.,* 1999). VIM was present in class 1 integrons in *P. aeruginosa* (Queenan and Bush, 2007). SPM is another family of uncommon metallo-β-lactamase. SPM-1 (for "Sao Paulo metallo-β-lactamase") was also reported first in *P. aeruginosa* strain in the city of Sao Paolo in Brazil. SPM-1 *P. aeruginosa* has caused several nosocomial outbreaks in Brazil (Poirel *et al.,* 2004). Similalry, another type of metallo-β-lactamase, GIM-1 (for "German imipenemase"), was identified in Germany in 2002 (Castanheira *et al.,* 2004). SIM (for "Seoul imipenemase") is another metallo-β-lactamase identified in Korea. The SIM-1 has genetic similarity of about 65% to IMP family.

IMP type metallo-β-lactamases is the most common carbapenemase explored in Japan in a country wide study (Kimura *et al.,* 2005; Nishio *et al.,* 2004). The first

class B carbapenemase reported from China was IMP-4 (Hawkey *et al.*, 2001). The VIM family carbapenemase, VIM-2 was first identified in China in 2006 (Wang *et al.*, 2006).

Several outbreaks of carbapenem resistant metallo-β-lactamases have been reported from European countries. VIM enzymes have been reported from Germany (Henrichfreise *et al.*, 2005), Turkey (Bahar *et al.*, 2004), Croatia (Sardelic *et al.*, 2003), Hungary (Libisch *et al.*, 2004), and Poland (Fiett *et al.*, 2006). Metallo-β-lactamases have also been reported from Australia. The first class B carbapenemase identified in Australia was IMP-4, reported in *P. aeruginosa* in 2004 (Peleg *et al.*, 2004).

A novel metallo-β-lactamases of sub-continental origin NDM-1 was first identified in a Swedish tourist to India when he was hospitalized in New Delhi and diagnosed with an untreatable infection, which later was named NDM-1. NDM-1 was identified in 2008 (Yong *et al.*, 2009), and was reported as predominant carbapnemase in Pakistan, India and Bangladesh (Kumarasamy *et al.*, 2010). NDM-1 harbouring bacteria have been found resistant to all available antibiotics which are in clinical use. NDM-1 was also detected in UK (Kumarasamy *et al.*, 2010) and Japan (Yuasa, 2010). Four variants of NDM have been reported uptill now (Coppo *et al.*, 2014).

Despite the tremendous discoveries made so far for controlling bacterial diseases, several enzymes such as class A (ESBL, KPC), class D (OXA) and class B (NDM) β-lactamases remains a global challenge for clinicians (Qin *et al.*, 2014). Bacteria harboring these enzymes are super resistant to the advanced generations of

antibiotics and significant level of morbidity is associated with them. The term "superbugs" refer to bacteria with enhanced virulence and resistance to drugs resulting in huge mortality and morbidity (Davies and Davies, 2010).

2.8 GENOMICS AND RESISTANCE MECHANISM

The increasing incidence of resistance to the latest drugs by bacteria poses a huge threat to human race today. It is extremely important to understand the very mechanisms behind these emerging super resistant bugs. Characterization of antibiotic resistance determinants at the genomic level can help us understand and control the spread of multidrug-resistant (MDR) pathogens (Kumar *et al.,* 2011). Identification of resistance determinants in microbial genomes is a critical step in combating infectious diseases (Gibson *et al.,* 2014). The most prevalent and easy method used to characterize microbial resistome from sequencing data is pairwise sequence alignment against ARG databases (Gibson *et al.,* 2014; Forslund *et al.,* 2013; Hu *et al.,* 2013). Identification of epidemiologically important resistant bacteria can no longer be traced on simple phenotypic tests (Rossolini and Thaller**,** 2010). Their genomic analysis is very important to understand their life style and evolution for survival against the antibiotics stress (Harris *et al.,* 2010). Genomic knowledge of the microbes will also be helpful in designing novel identification tools for pathogens of epidemiological importance to contain their infections (Rossolini and Thaller**,** 2010). Genome wide investigations of pathogens in this regard are of utmost importance to predict novel ARG and their evolution mechanisms (Rossolini and Thaller**,** 2010).

2.9 WHOLE GENOME SEQUENCING TO STUDY THE EMERGENCE OF ANTIBIOTIC RESISTANCE

Whole Genome Sequencing (WGS) are being used to develop novel diagnostic tests, and has been very promising in studying the emergence of antibiotic resistance. Rapid WGS methods have the potential to be used for ARG infection detection and control in the clinics (Köser *et al.,* 2010). WGS has also been used to study the evolution of resistance in real time (Palmer ad Kishony, 2013). For example, if carbapenem resistance is detected during screening of multiple isolates, WGS could be used as an efficient tool to trace carbapenemase producers (Reuter *et al.,* 2013). Although WGS methods could be expensive as compared to alternatives like multiplex PCR (Livermore and Wain, 2013), if used solely for identification. However, WGS data could be much promising tools in molecular epidemiology in tracing resistance outbreaks and containing them (Reuter *et al.*, 2013).

The advancement in sequencing technologies has made WGS a promising tool for comparing multiple bacterial genomes simultaneously (Wu *et al.,* 2009). The next-generation sequencing technology of Illumina, ABI and 454 Life Sciences (Roche), has made possible to generate millions of sequence data in a short time (Margulies *et al.,* 2005). This can be used to assemble the genomes using various softwares and identify the genes of interest in real time (Worley and Gibbs, 2010; Reinhardt *et al.,* 2009; Hogg *et al.,* 2007; McCutcheon *et al.,* 2007).

2.10 THE ANTIBIOTICS RESISTOME

The rapid dissemination and evolution of ARG in human pathogens is a huge clinical challenge (Arias and Murray, 2009). Several antibiotics are naturally produced in environmental bacteria and those bacteria have developed intrinsic resistance to them. In this regard, resistance is thought to be an inherited trait in certain bacteria, and which is shared among diverse bacterial communities through

HGT (Villegas, 2012). Although, the origin of resistance is not fully explored, several researchers believe that vast majority of bacterial communities in the environment possess collections of ARG, the resistome (Davies and Davies, 2010; Martínez, 2008; Wright, 2007; D'Costa *et al.*, 2006). Several researchers have studied the resistome in different environments but the exact size and diversity has not been fully explored yet (Hugenholtz and Tyson, 2008).

Environmental bacterial reservoirs have long been attributed as a source of antibiotic resistance in human pathogens (Benveniste and Davies, 1973). Examples of exact similar ARG between environmental bacteria and human pathogens are rare (Poirel *et al.*, 2005; Poirel *et al.*, 2002). Kluyvera and Shewanella are free-living bacteria in the environment, have resistance genes (CTX-M β-lactamase and qnrA genes) with high identity (100% identity in clinical Kluyvera isolates) to those of human pathogens (Poirel *et al.*, 2005; Poirel *et al.*, 2002). This significant ARG sharing between environmental and clinical bacteria poses concerns regarding the impact of environmental resistance on human pathogens (Forsberg *et al.*, 2012).

2.11 THE ANTIBIOTICS SUBSISTOME

Dantas *et al.* (2008) screened soil bacteria that degrade antibiotics. They collected hundreds of isolates from diverse soil samples and checked for their capability to grow on 18 different antibiotics, they tested, using them as a source of carbon and nitrogen. They identified many isolates that grew on several commonly used antibiotics including aminoglycosides and fluoroquinolones. Most of the strains that grew on antibiotics were proteobacteria, and more than 40% were *Burkholderia* spp. Antibiotic digestion reveals that environmental bacteria possess a

rich source of resistant determinant. Dantas *et al.* (2008) beautifully demonstrated the presence of antibiotics catabolizing genes in the environment.

Today, the Waksman drug discovery strategy is coupled with "omics" technologies, using genomics and metagenomics approaches to mine for novel antibiotics genes (Iqbal *et al.*, 2012; Knrerr and van der Donk, 2012; Sello, 2012), which in turn will help in coping with increasing threats from super resistant pathogens.

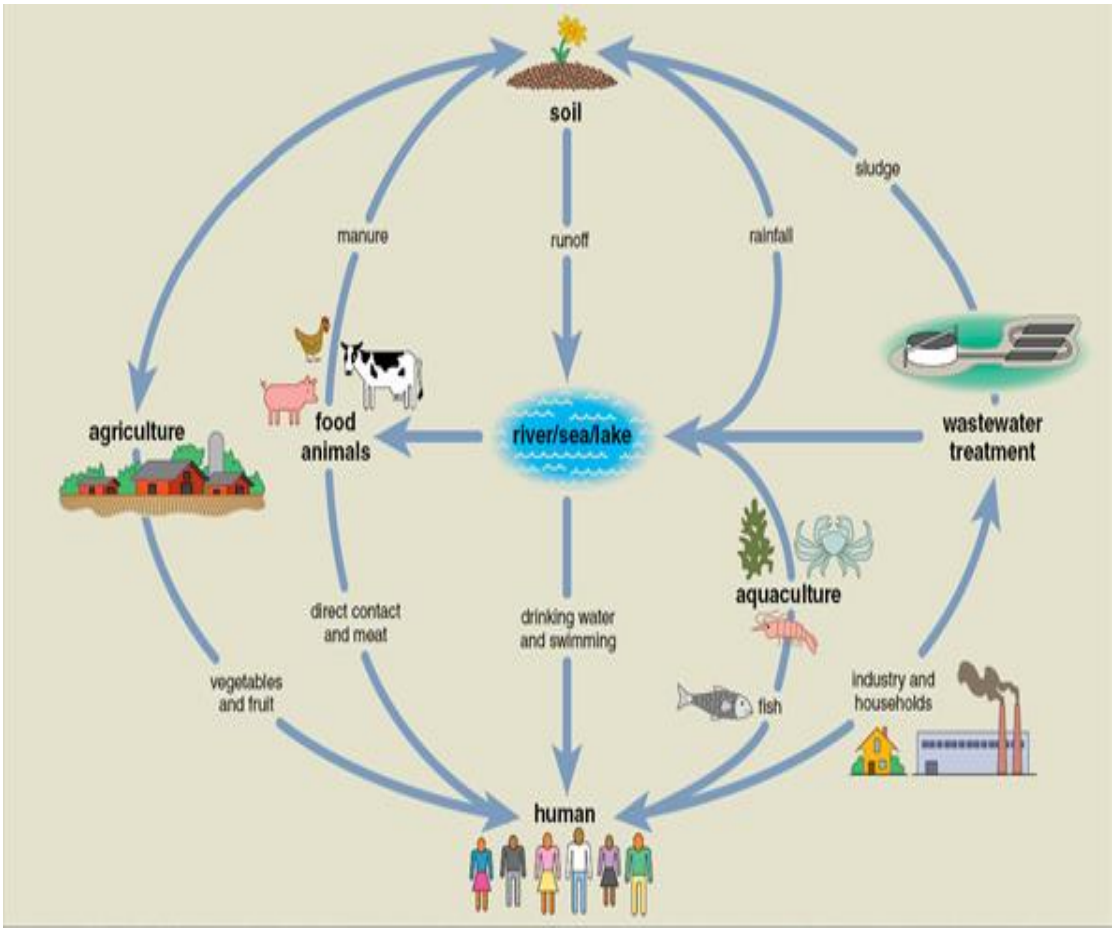

Figure 2.3: The cyclic dissemination of antibiotic resistance genes in human life

Antibiotic resistance genes come into humans contact through variety of natural and anthropogenic sources. Agriculture, aquaculture, dairy farming, industrial wastes, and natural sources like soil and water bodies, all contribute to antibiotic resistance accumulation. Adapted from (Dantas and Sommer, 2014)

Chapter 3

MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

Four hundred and fifty bacterial isolates were collected from microbiology laboratory, Pakistan Railway General Hospital, Rawalpindi, Pakistan and Pakistan Institute of Medical Sciences, Islamabad, Pakistan during January 2012 and June 2013. These bacterial specimens were isolated from various infections of blood, pus, urine, sputum, stools and high vaginal swab. Isolates were identified through Gram staining and standard biochemical techniques as described previously (Cheesbrough, 2006). Banked clinical isolates were also selected from frozen stocks at Barnes Jewish Hospital/Washington University School of Medicine in Saint Louis, Missouri, U.S.A. to represent both susceptible and MDR isolates recovered from U.S. specimens. U.S. strains were isolated from urine, respiratory specimens, bone, and bile specimens between January 2010 and June 2013. The research project was duly approved by the ethics review board at the Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and technology, Islamabad, Pakistan.

3.2 ANTIBIOTIC RESISTANCE PHENOTYPES

To establish the susceptibility profiles of each of the isolate, phenotypic tests were performed using Kirby Bauer Disk diffusion methods in accordance with CLSI guidelines (CLSI, 2013), against 12 antibiotics including 7 β-lactams (Table S, given in appendix).

3.3 DNA EXTRACTION FROM BACTERIA

Bacteria were grown in 5 mL liquid media (LB) for 24 hours. CTAB method with little modification was used for DNA extraction. About 1.5 mL of culture was centrifuged in a sterile microfuge tube for 2 minutes at 13,000 rpm. Supernatant was discarded and pellets were re-suspended in 576 µL TE buffer. A total of 30 µL of 10 % SDS and 3 µL of 20 mg/mL proteinase K was added and mixed thoroughly and incubated for 1 hour at 37 ºC. One hundred µL of 5 M NaCl was added to it. Eighty µL CTAB/NaCl solutions was added and incubated for 10 minutes at 65ºC. About 700 µL of 24:1 chloroform/isoamyl alcohol was added to it and centrifuged for 5 minutes at 13,000 rpm. The supernatant was collected and 1 volume of 25:24:1 phenol/chloroform/isoamyl alcohol was added to it and centrifiged. Supernatant was collected in a fresh tube and 0.6 volume of isopropanol was added and mixed gently until a stringy white DNA precipitate was formed. After centrifugation, 70 % ethanol was added to the pellet. Ethanol was evaporated and pellet was re-suspended in 50 µL TE buffer and stored at -20°C.

3.3.1 PCR Amplification of the *bla*SHV, *bla*TEM and *bla*CTX-M Genes

Reported primers (Monstein *et al.*, 2007) were used for the amplification of *bla*SHV, *bla*TEM and *bla*CTX-M group. The sequences of primers and amplicon size are given in Table 3.1.

Table 3.1. Primers used in PCR amplification

	Primers	Sequence	Amplicon size (base pairs)
SHV	bla-SHV.SE	5'-ATGCGTTATATTCGCCTGTG-3'	747
	bla-SHV.AS	5'-TGCTTTGTTATTCGGGCCAA-3'	
TEM	TEM-164.SE	5'-TCGCCGCATACACTATTCTCAGAATGA-3'	445
	TEM-165.AS	5'-ACGCTCACCGGCTCCAGATTTAT-3'	
CTX-M	CTX-M-U1	5'-ATGTGCAGTACCAGTAAAGTTATGGC-3'	593
	CTX-M-U2	5'-TGGGTGAAGTAAGTGACCAGAATCAGCGG-3'	

All DNA samples were subjected to PCR amplification by using the primers set given (Table 3.1). Different profiles were optimized respectively. Same reaction mixture was used for preparing master mix in PCR tubes (Axygen^{R,} California, USA). Total volume of recation mixture prepared was 25 µl, containing 2 mM DNTPs, 10x PCR buffer, 50 mM $MgCL_{2,}$ 50 pmol of each primer, 3 µl DNA sample, 1 unit of thermostable Taq DNA polymerase (Fermentas, USA). The reaction mixture was centrifuged for thorough mixing using microcentrifuge (Edison, New Jersey, USA), then PCR was performed in Swift^{TM} MaxPro thermal cycler (Applied Biosystem, Foster city, USA).

3.3.2 PCR profile for *bla*SHV, *bla*TEM and *bla*CTX-M

Initial denaturation of PCR reaction was given at 94 ºC for 4 minutes followed by 35 cycles of denaturation at 94 ºC for 45 seconds, annealing at 56 ºC for 45 seconds and extension at 72 ºC for 2 minutes. The reaction was ended with a final

extension of 10 minutes at 72 ºC and kept on hold at 4 ºC until tubes were taken out of the cycler.

3.3.3 Agarose gel electrophoresis

To analyze the PCR products, 2% agarose gel was prepared in 1X TAE (In 990 mL distilled water, 1M Tris HCl (10mL): pH 8.0 and 400 µL of 0.5 EDTA was added) and was run in the same buffer composition. For making gel, 0.8g 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; 5µL 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) at constant current 60mA 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 trans-illuminator (Biometra, Goettingen, Germany). The resulting gel was photographed by Gel Documentation System (Wealtec Dolphin Doc Sparks, USA).

3.4 ILLUMINA LIBRARY PREPARATION AND GENOME SEQUENCING

Bacterial isolates were re-identified before DNA extraction for Illumina genomic library preparation on MALDI-TOF-MS Vitek v2.0 knowledgebase (bioMerieux) as previously described (Manji *et al.,* 2014; Richter *et al.,* 2013).

3.4.1 Genomic and Plasmid DNA Extraction

Single colonies of each isolate were grown in LB broth liquid culture for DNA extraction. Total DNA was then extracted using the Invitrogen Charge Switch gDNA

Mini Bacteria kit per the manufacturer's protocol. Plasmid DNA was then extracted from all KPC and NDM-1 and CTX-M-15 using the Qiagen Large Construct kit per the manufacturer's protocol.

3.4.2 Shearing of DNA Samples

For each bacterial isolate, 500 ng of total DNA was sheared to 300 bp fragments in nine rounds of shearing of ten minutes each on the BioRupter XL. In each round the power setting was 'H' and samples were treated for 30s and allowed to rest for 30s.

3.4.3 End Repair and Adapters Ligation to DNA Samples

Each sample was concentrated using the Qiagen MinElute PCR purification kit per the manufacturer's protocol. End Repair of the sheared DNA fragments was initiated with the addition of 2.5 µl of T4 DNA ligase buffer with 10mM ATP (NEB, B0202S), 1 µl of 1 mM dNTPs (NEB), 0.5 µl T4 Polymerase (NEB, M0203S), 0.5 µl T4 PNK (NEB M0201S), and 0.5 µl Taq Polymerase (NEB, M0267S). This mixture was incubated at 25°C for 30 min, then at 75°C for 20 min. Barcoded adapters were then added to the solution along with 0.8µl of T4 DNA ligase (NEB, M0202M), for the purpose of ligating the adapters to the DNA fragments. This solution was then incubated at 16ºC for 40min, then 65°C for 10min. At this point the adapter-ligated DNA was purified using the Qiagen MinElute PCR purification kit per the manufacturer's protocol.

3.4.4 Size Selection of Barcoded DNA Fragments

The DNA fragments were then size selected on a 2% agarose gel in 1X TBE buffer stained with Biotium GelGreen dye (Biotium). DNA fragments were combined with 2.5uL 6X Orange loading dye before loading on to the gel. Adaptor-ligated DNA was extracted from gel slices corresponding to DNA of 250-300bp using a QIAGEN MinElute Gel Extraction kit per the manufacturer's protocol. The purified DNA was enriched by PCR using 12.5 µL 2X Phusion HF Master Mix and 1µL of 10µM Illumina PCR Primer Mix in a 25 µL reaction using 1µL of purified DNA as template. DNA was amplified at 98°C for 30 seconds followed by 18 cycles of 98°C for 10 seconds, 65°C for 30 seconds, 72°C for 30 seconds with a final extension of 5min. at 72°C.

3.4.5 Size Range Verification and Library Quantification

Finally a quality control check was done before submitting the samples for Illumina Sequencing. Size of the fragments was checked by bioanalyser and concentration of pooled samples was determined Qubit Fluorometer and 10nmol of each sample (upto 106 per lane of sequencing) were pooled. Pool tubes were submitted for Illumina HiSeq-2500 Pair-End (PE) 101 bp sequencing at GTAC (Genome Technology Access Center, Washington University in St. Louis) at concentration of 9 pmol per lane.

3.5 GENOMIC AND PLASMID SEQUENCE ASSEMBLY

3.5.1 Genomes Assembly

All sequence reads were de-multiplexed by barcodes into separate genome bins. Reads were quality trimmed to remove adapter sequence and bases with a quality score below 19. Any reads shorter than 31 bp after quality trimming were not used in further analysis. The best reference sequence was chosen for each isolate or plasmid by mapping 10000 reads using Bowtie 2 (Langmead and Salzberg, 2012).

The genome or plasmid against which the highest percentage of reads mapped was used as the reference sequence for that assembly. It was empirically determined that if this first mapping included fewer than 60% of the reads, then the assembly would be best done completely *de novo*. For isolates with >60% of reads matching a reference sequence, all reads were mapped to that sequence. SAMtools (Handsaker *et al.,* 2009), was used to call sequence variants for each position. The variant call format file was then filtered to remove SNPs with a quality score lower than 70 or coverage greater than twice the average coverage expected per base. Custom scripts were then used to extract DNA sequences from the reference genome with more than three independent reads, to create a fragment file of regions in the sample genome matching the reference genome modified with high-quality variant information.

3.5.2 *De-novo* Assembly of Draft Genomes

For isolates having fewer than 60 percent reads *de novo* assembly was performed. *De novo* assembly of the reads from each isolate was combined with the highly covered regions from the reference alignment to create the final assemblies using Velvet (Zerbino and Birney, 2008). Kmer coverage was calculated as: total read coverage*0.50 (because the kmer length was approximately half the read length), and the coverage cutoff was calculated as the kmer coverage divided by eight. Whenever

complete reference mapping was performed, then contigs from the *de novo* assembly and reference mapping were put in an additional Velvet assembly step as long reads with the original reads files. Finally all fragments were collapsed on nucleotide identity using CD-Hit (Fu *et al.,* 2012). All fragments smaller than 500bp were partitioned to a separate file by a custom script. Plasmid sequences were assembled by this same method, with the sequences of all complete plasmids encoding, NDM-1, KPC, or CTX-M used as references.

3.5.3 ORF Prediction and Annotation

ORF prediction for each genome was performed separately using GeneMark (Borodovsky and Lomsadze, 2011), models based on the closest reference genomes. Each ORF was compared to three databases of profile hidden Markov models using HMMR (Eddy, 2009), Pfam (Finn *et al.,* 2014), TIGRFAMs (Haft *et al.,* 2001), and Resfams (Gibson and Dantas, 2014). All functional annotations were concatenated into a single file by a custom script. 756 *E. coli* and 54 *K. pneumoniae* completed and draft genomes were downloaded from the National Center for Biotechnology Information on April 15th 2014, for the purpose of comparing to the isolate set.

GeneMark models from the completed genomes were used to predict ORFs for those genomes, while for draft genomes models created from *E. coli* K12 MG1655 (for *E. coli* draft genomes) or *K. pneumoniae* KCTC 2242 (for *K. pneumoniae* draft genomes) were used for ORF prediction. All annotated genome and plasmid assemblies were deposited into NCBI (BioProject accession number: PRJNA261540).

3.5.4 *In Silico* MLST and Phylogenetic Analysis

Multi-Locus Sequence Typing (MLST) profiles and sequences for *E. coli* and *K. pneumoniae* downloaded from PubMLST (pubmlst.org, accessed July 26th, 2013) were used against appropriate loci from each genome. When an absolute MLST profile could not be identified for an organism (because of ambiguous bases or incomplete assembly of one or more loci) the remaining possible sequence types (ST) based on the incomplete information were identified. In all cases a strain could be identified as one of at most 19 ST using this methodology.

To assess within species relatedness at the highest possible resolution, whole genome alignment was performed using mugsy (Angiuoli *et al.,* 2011). For *E. coli*, *K. pneumoniae*, and *E. cloacae* a single reference genome was included in the alignment to provide context (*E. coli* K12 MG1655, *K. pneumonaie* KCTC 2242, and *E. cloacae* ATCC 13047 respectively). Poorly aligned regions (which could create noise in the phylogenetic signal) were removed using Gblocks (Talavera and Castresana, 2007), leaving only the core genome alignment. From these alignments maximum likelihood phylogenetic trees were made using RaxML (Stamatakis, 2014) and FastTree (Price *et al.,* 2010). In all cases trees made by both methods were in agreement and the output from FastTree was used for visualization.

3.5.5 Antibiotic Resistance Profile Determination from Genomes

During development of resistance prediction model, expected resistance profiles were assigned against the β-lactam antibiotics to each isolate based on their β-lactamase composition using a custom BLAST database built from the Bush and Jacoby database at www.lahey.org/Studies/ (accessed March 25th, 2014) (Bush and

Jacoby, 2010). For genes lacking a specific functional classification, the resistance profile of the most resistant members of the same molecular class (i.e. TEM, SHV) was used. Resistance was then predicted for each isolate as the union of all resistance profiles for individual β-lactamases. Hierarchical clustering of β-lactamase profiles was computed using the R command hclust with the binary Euclidean distance metric. The resulting dendrogram and presence-absence data were visualized with R heatmap.

Resistance to ciprofloxacin was compared by comparison of the QRDR regions (amino acids 68-106) of *gyrA* and *parC* for each isolate against the wild type. Wild type for all *E. coli, K. pneumoniae,* and *E. cloacae* complex isolates was defined as the *E. coli* K12 MG1655 *gyrA* and *parC* sequences (Blattner *et al.,* 1997). Wild type for *E. aerogenes* isolates was the same except for a conserved S83T transition in *gyrA.*

3.5.6 NDM-1, KPC and CTX-M Plasmid Comparisons

Plasmid sequences were compared by an all-against-all pairwise nucleotide BLAST. For each pair of plasmids, the percent of each plasmid was calculated that aligned at >99% identity. The percentages were then binned from each pairwise alignment into collections based on the defining β-lactamase of their query and subject plasmids. Network diagrams were also generated from the pairwise BLASTs using custom Python scripts, only including regions above 99% identity and over 500 bp.

3.5.7 Statistical Analysis

For comparisons of total resistant gene content between Pakistani *E. coli* (n=24), U.S. *E. coli* (n=9), NCBI *E. coli* (n=744), Pakistani *K. pneumoniae* (n=19),

U.S. *K. pneumoniae* (n=11), and NCBI *K. pneumoniae* (n=52) the Student's t-test (two-sided) was used against the null hypothesis of no difference between groups.

For comparing sequence diversity of plasmids containing NDM-1 (n=28), KPC (n=24), and CTX-M (n=24) β-lactamases, two independent statistical methods were used. First, the Mann-Whitney U test (two-sided) was used on each pair of collections of pairwise alignments with the null hypothesis that they came from the same population. Second, 10,000 Monte Carlo experiments were performed randomly exchanging subject plasmid defining β-lactamase labels and finding the median sequence length conserved between two collections of alignments. Results from the Mann-Whitney U test were reported as a p-value, while results from the Monte Carlo simulations were reported as the percent of simulated data with an equal or greater difference in medians.

Chapter 4

RESULTS

4.1 IDENTIFICATION OF BACTERIA

Among the 450 samples collected from different Pakistani hospitals, *Enterobacteriaceae* represented the most frequently encountered pathogens and was selected for the study. The *Enterobacteriacae* isolates included *Escherichia coli* (n = 157), *Klebsiella pneumoniae* (n = 97), *Proteus vulagaris* (n = 34), *Citrobacter freundii* (n = 28), and *Enterobacter* spp. (n = 25). The U.S. *Enterobacteriacae* samples comprised of *Escherichia coli* (n=33), *Klebsiella pneumoniae* (n = 30) and *Enterobacter* spp. (n = 15).

4.2 ANTIBIOTIC RESISTANCE PATTERN IN PAKISTANI AND U.S. *ENTEROBACTERIACEAE* ISOLATES

Clinical *Enterobacteriaceae* isolates collected in Pakistani and U.S. hospitals were assayed for susceptibility to 12 antibiotics, including 7 β-lactams. 71% of Pakistani isolates were found resistant to ciprofloxacin, a fluoroquinolone commonly used to treat urinary tract infections. Resistance to trimethoprim-sulfamethoxazole was found to be 76%. Gentamicin, doxycycline, and chloramphenicol were resisted by 55% of isolates each. In the β-lactams, a universal resistance to ampicillin (96% of isolates) was observed. Variable resistance to the cephalosporins, and a high rate of resistance to meropenem (24 % of isolates were resistant) was observed in Pakistani isolates.

Looking at this high prevalence of carbapenem resistance, a set of U.S. isolates was compared, specifically selecting for a mixture of carbapenem resistant and susceptible isolates. About 50 % of the U.S. isolates were resistant to ciprofloxacin, and 45 % were resistant to trimethoprim-sulfamethoxazole. About 21 % of the isolates were found resistant to gentamycin, 28 % to doxycycline, and 31 % to chloramphenicol. This resistance is very low as compared to the resistance observed in Pakistani isolates. Similarly resistant to various cephalosporin antibiotics were also quite less compared to the resistance seen in Pakistani set of isolates (Figure 4.1).

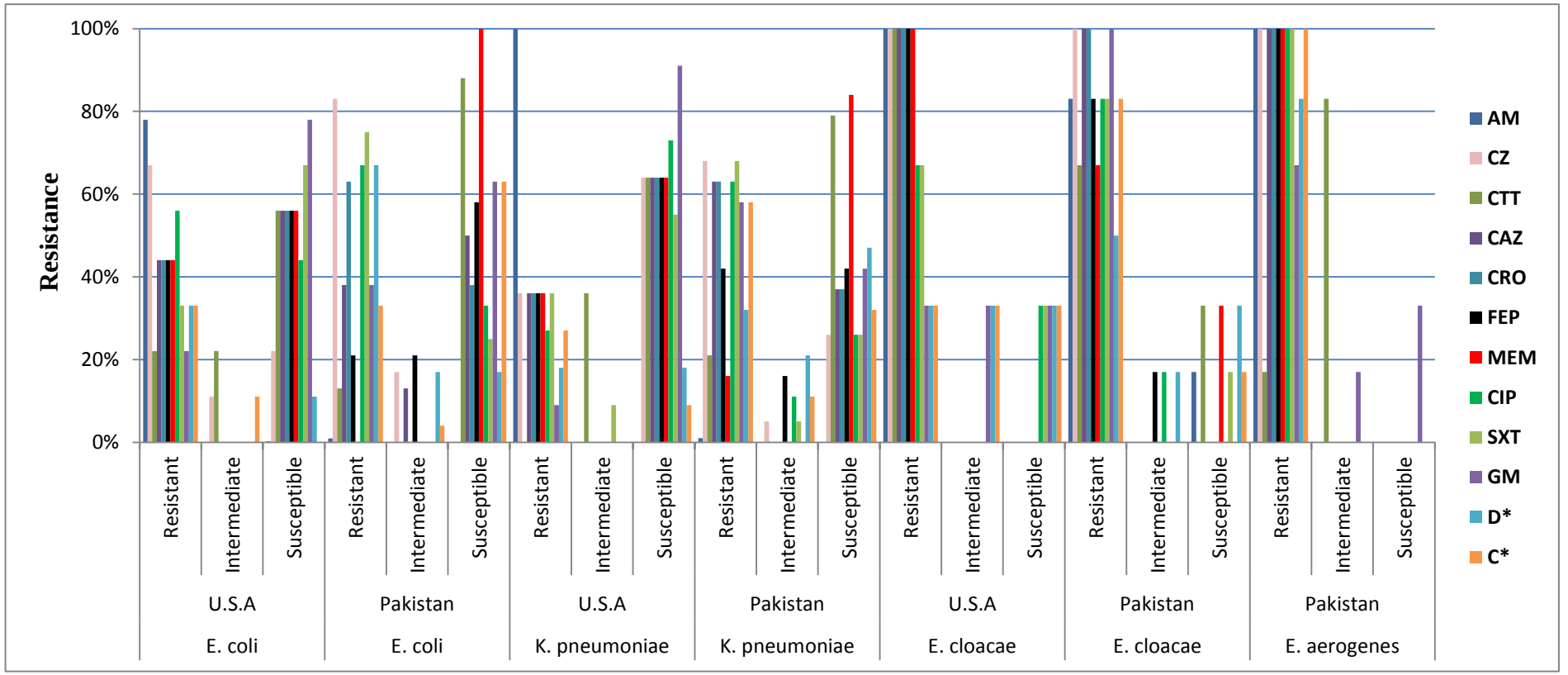

Figure 4.1: Antibiotic susceptibility profiles of Pakistani and U.S. clinical isolates.
AM = ampicillin, CZ = cefazolin, CTT = cefotetan, CAZ = ceftazidime, CRO = ceftriaxone, FEP = cefepime, MEM = meropenem, CIP = ciprofloxacin, SXT = trimethoprim-sulfamethoxazole, GM = gentamicin, D = doxycycline and C = chloramphenicol.

4.3 PREVALENCE OF SHV, TEM AND CTX-M BETA-LACTAMASES IN CLINICAL *ENTEROBACTERIACAE*

Three β-lactamases (CTX-M, TEM and SHV) that are involved generally in outbreaks and exhibit resistance to penicillin and cephalosporin antibiotics were detected in Pakistani *Enterobacteriaceae* isolates. PCR assay amplified unique, characteristic bands corresponding to the genes for each of these β-lactamases. The overall prevalence of *bla*CTX-M (593 bp), *bla*TEM (445 bp) and *bla*SHV (747 bp) in the screened isolates of *E. coli* (n = 50), *K. pneumoniae* (n = 27), *Enterobacter* spp. (n = 27), *P. vulgaris* (n = 19), and *C. freundii* (n = 12), is given below (Figures 4.2- 4.7).

Prevalence of *bla*CTX-M was highest in *E. coli* (68 %), followed by *P. vulgaris* (47 %). About 35 % of *K. pneumoniae* and 40 % of *C. freundii* were positive for *bla*CTX-M. The prevalence of genes encoding TEM β-lactamases was observed in all bacterial species, including *K. pneumoniae* (62 %), *E. coli* (40 %), *E. cloacae.* (33 %), *C. freundii* (24 %) and *P. vulgaris* (22%). Prevalence of *bla*SHV was relatively low as compared to *bla*TEM and *bla*CTX-M among the *Enterobacteriacae* isolates. About 38 % of *K. pneumoniae*, 24 % *C. freundii*, 20 % *E. cloacae*, 18 % of *P. vulgaris* and *E. coli* were positive for *bla*SHV.

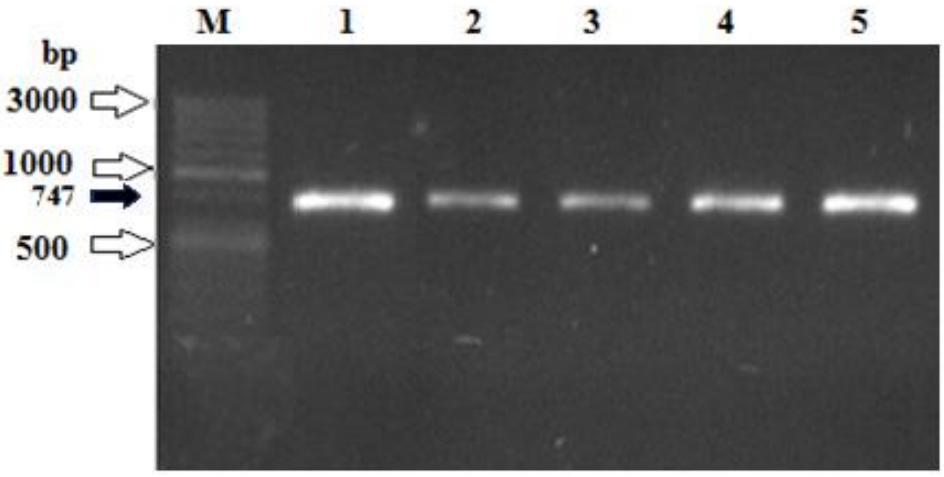

Figure 4.2: Identification of *bla*SHV by PCR. Amplification of *bla*SHV gene sequence in *Enteobacteriacae* isolates by polymerase chain reaction (PCR). Lane 1, 2, 3, 4 and 5 show amplified DNA fragment (approximately 747 bp) in *E. coli*, *K. pneumoniae*, *P. vulgaris*, *C. freundii* and *E. cloacae*, respectively. Lane M shows a DNA Ladder, 100bp.

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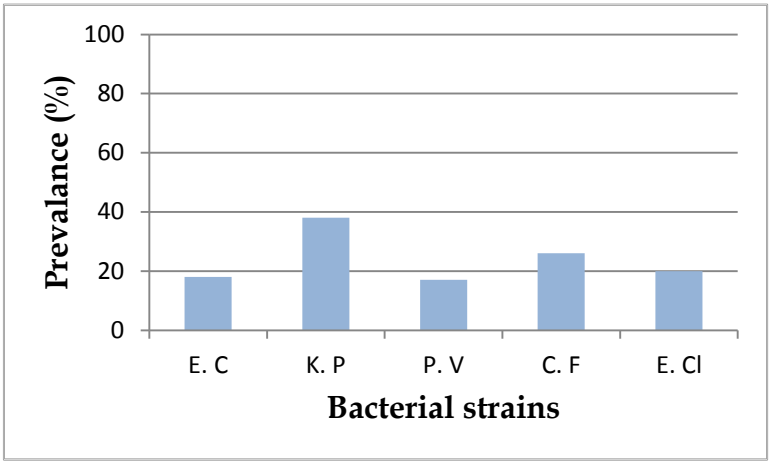

Figure 4.3: Prevalence (%) of SHV β-lactamases in clinical *Enterobacteriaceae* isolates.
E.C: *E. coli*, K.P: *K. pneumoniae*, P.V: *P. vulgaris*, C.F: *C. freundii*, E.Cl: *E.cloacae.*

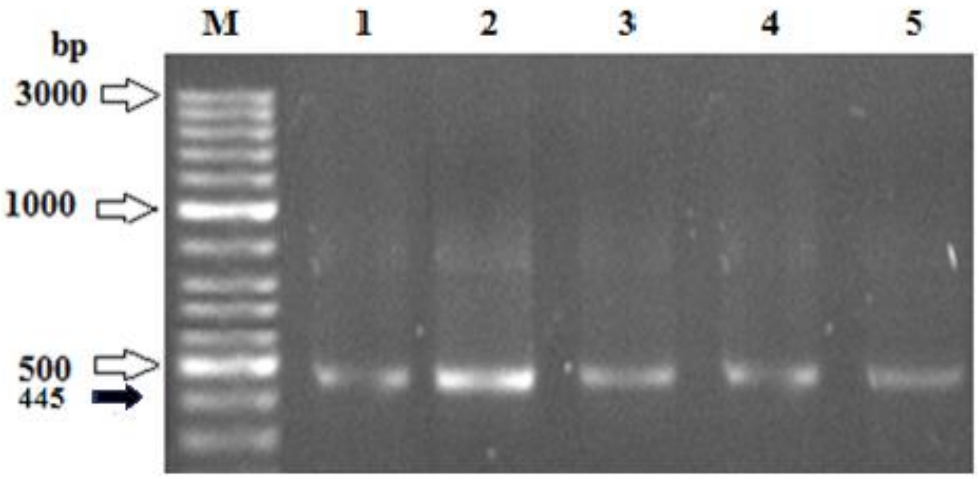

Figure 4.4: Identification of *bla*TEM by PCR. Amplification of *bla*TEM gene sequence in *Enteobacteriacae* isolates by polymerase chain reaction (PCR). Lane 1, 2, 3, 4 and 5 show amplified DNA fragment (approximately 445 bp) in *E. coli*, *K. pneumoniae*, *P. vulgaris*, *C. freundii* and *E .cloacae*, respectively. Lane M shows a DNA Ladder, 100bp.

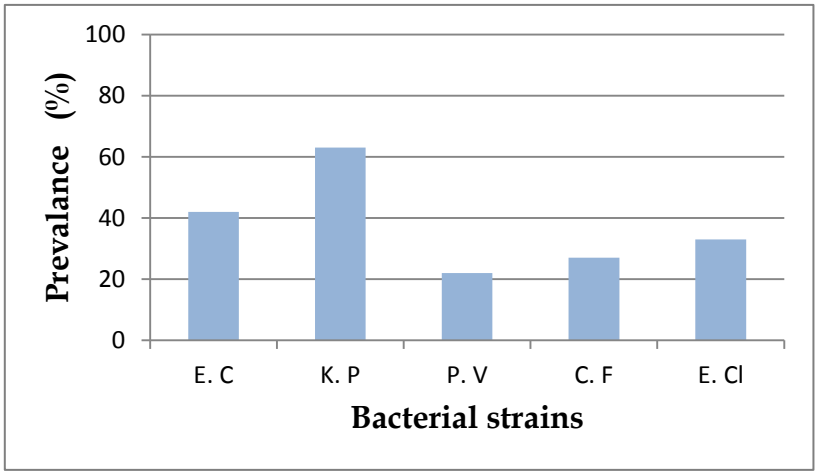

Figure 4.5: Prevalence (%) of TEM β-lactamases in clinical *Enterobacteriaceae* isolates.
E.C: *E. coli*, K.P: *K. pneumoniae*, P.V: *P. vulgaris*, C.F: *C. freundii*, E.Cl: *E.cloacae.*

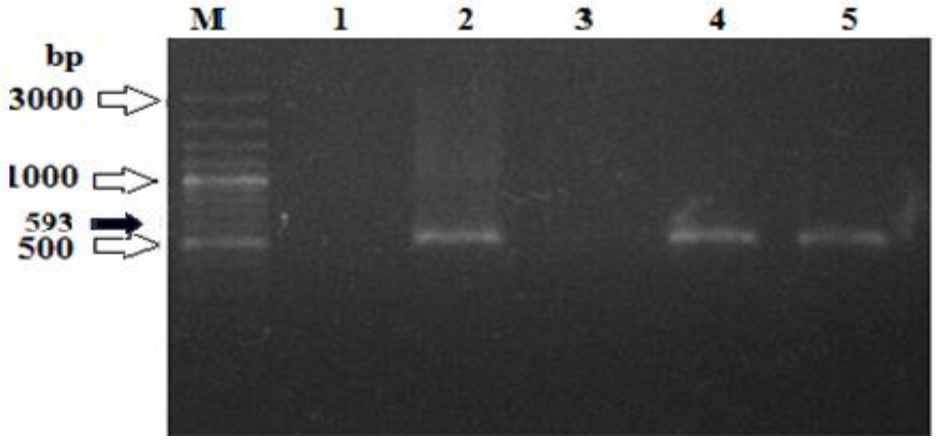

Figure 4.6: Identification of *bla*CTX-M by PCR. Amplification of *bla*CTX-M gene sequence in *Enteobacteriacae* isolates by polymerase chain reaction (PCR). Lane 1, 2, 3, 4 and 5 show amplified DNA fragment (approximately 593 bp) in *E. coli*, *K. pneumoniae*, *P. vulgaris*, *C. freundii* and *E. cloacae*, respectively. Lane M shows a DNA Ladder, 100bp.

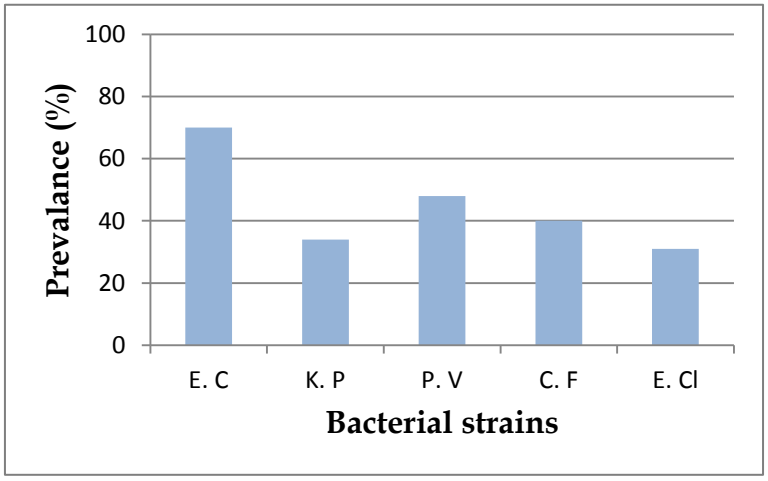

Figure 4.7: Prevalence (%) of CTX-M β-lactamases in clinical *Enterobacteriaceae* isolates.
E.C: *E. coli*, K.P: *K. pneumoniae*, P.V: *P. vulgaris*, C.F: *C. freundii*, E.Cl: *E.cloacae.*

4.4 PHYLOGENETIC DISTRIBUTION OF PAKISTANI AND U.S. ISOLATES

Whole genome sequencing was performed on each of the Pakistani and U.S. isolate, including on 33 *E. coli* isolates, 30 *K. pneumoniae* isolates, 9 *E. cloacae* complex isolates, and 6 *E. aerogenes* isolates. The draft genomes were assembled using a hybrid assembly method that utilizes closely related reference genomes, while preserving sequences of novel mobile elements. Next, whole genome alignment was performed to reconstruct the phylogenetic relationships of each isolate at high resolution (Figure 4.8). The specie trees demonstrate that genomes were captured from a variety of evolutionary clades as well as from multiple members of specific clades. *In silico* MLST was also performed for each strain to compare the phylogenetic analysis to previously identified sequence types. It was found that the clades on the tree represent globally-disseminated pathogen sequence types, such as ST131 in *E. coli* and ST11 (single locus variant of ST258) in *K. pneumoniae*. The Pakistani and U.S. isolates did not cluster separately on the species trees.

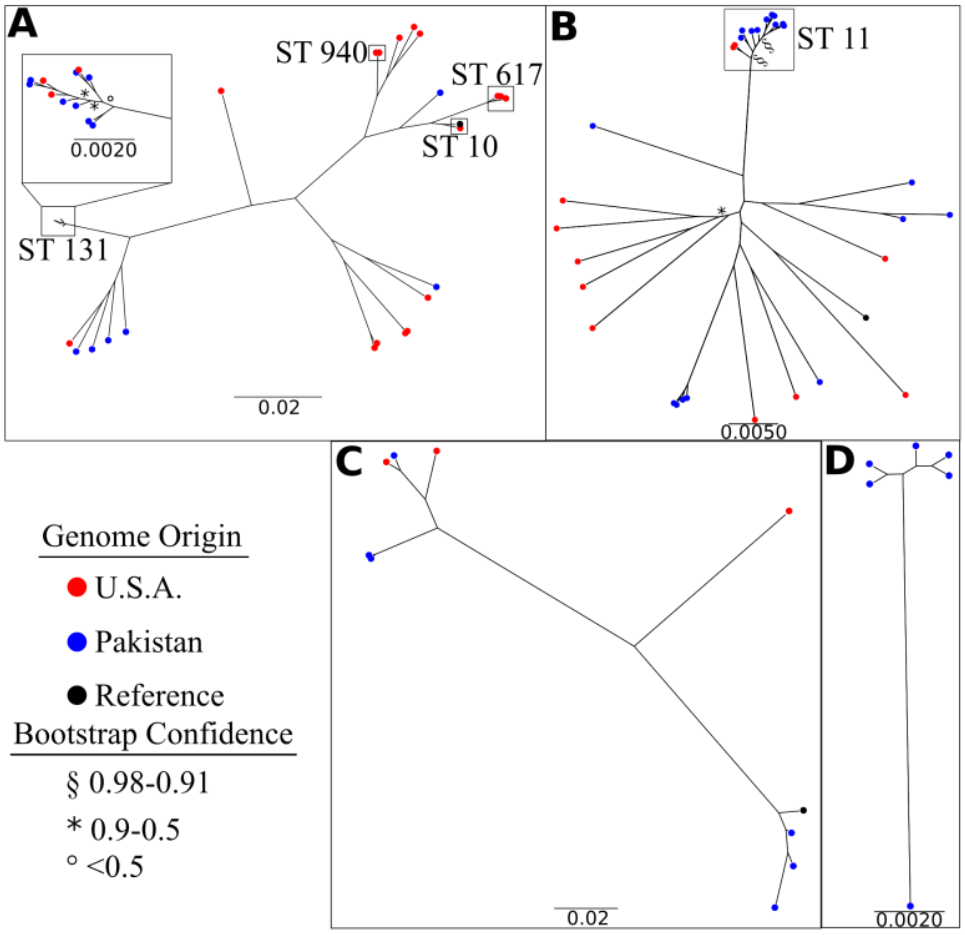

Figure 4.8: Phylogenetic trees for Pakistani and U.S. isolates.

Trees are separated by species, A) *E. coli*, B) *K. pneumoniae*, C) *E. cloacae*, and D) *E. aerogenes*, but not rooted. For each species approximately 50% of the genome aligned well and was used for phylogenetic inference. Scale bars indicate the nucleotide substitutions per site. In a) and b) clades that correspond to a known ST (as determined by *in silico* MLST) and contain more than one isolate are indicated. Reference genomes included for *E. coli* (K12 MG1655), *K. pneumoniae* (KCTC 2242), and *E. cloacae* (ATCC 13047) on their respective trees.

4.5 COMPARISON OF BETA-LACTAMASE CARRIAGE BETWEEN PAKISTANI, U.S. AND REFERENCE GENOMES

From the draft genome sequences, resistance genes were identified that were carried by each isolate. Those genes were separated into four broad gene mechanism categories: efflux (including TetA and RND efflux pumps), β-lactamases, aminoglycoside modification enzymes, and other mechanisms (including ribosome protection proteins and stress response regulators). β-lactamase genes in each category were compared between the two geographies and against all complete or draft *E. coli* and *K. pneumoniae* genomes from the National Center for Biotechnology Information (NCBI, *Enterobacter* spp. were excluded from this analysis due to low number of genomes, both in our set and in NCBI). The *E. coli* genomes from Pakistani set of isolates encoded similar numbers of AR efflux pumps to those of U.S. and NCBI genomes, but encoded significantly higher numbers of β-lactamases ($p<0.0001$) and aminoglycoside AR genes ($p<0.0001$) than reference genomes in NCBI. In contrast, the Pakistani and U.S. *K. pneumoniae* genomes encoded significantly fewer antibiotic efflux pumps than NCBI genomes ($p=0.026$ and $p=0.0076$ respectively, Figure 4.9 A & B).

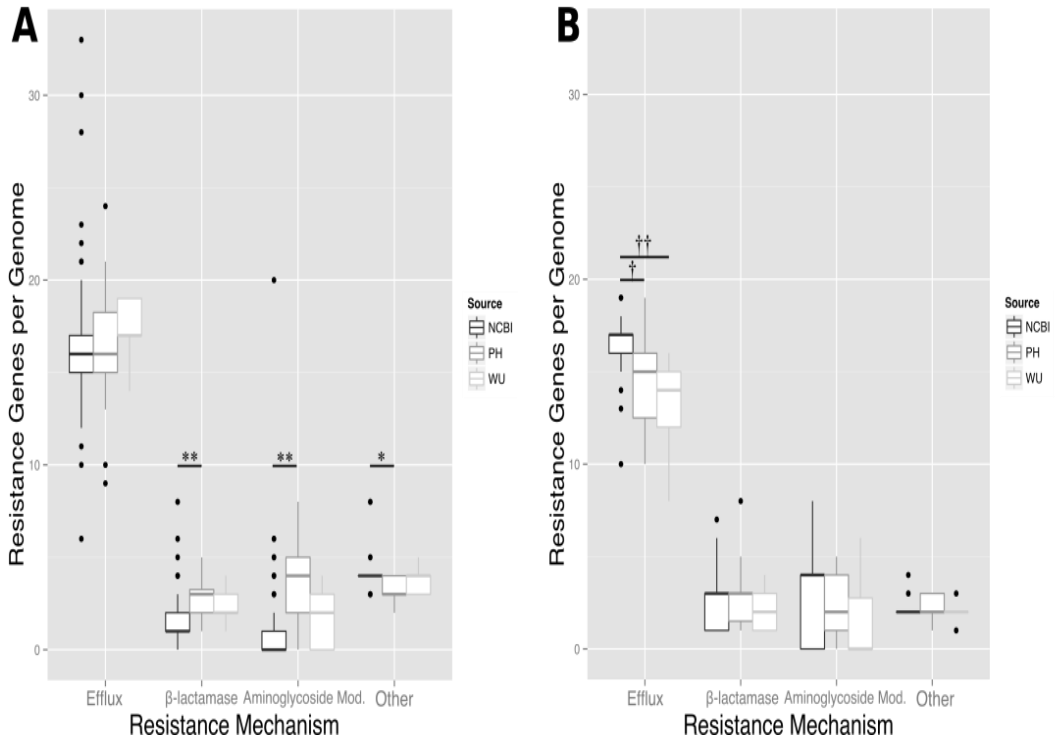

Figure 4.9: Prevalence of resistance genes per genomes.

A) *E. coli* and B) *K. pneumoniae*. NCBI genomes include all finished or draft genomes available for *E. coli* (n=744) and *K. pneumoniae* (n=52) as of 4/16/2014. Difference between all groups within a resistance mechanism was tested using student's t-test against the null hypothesis of no difference between groups. P-values were corrected for testing 24 hypotheses using the Bonferroni correction. Only significant differences are indicated. †p=0.026. ††p=0.0076 *p=0.00083. **p<0.0001

4.6 BETA-LACTAMASE CARRIAGE AND PATHOGENS EVOLUTIONARY HISTORY

From draft genome sequences the specific β-lactamase carriage of each isolate was identified (Figure 4.10A-D), suspected chromosomal *ampC* from *E. coli* and *Enterobacter* spp. were excluded). β-lactamases from each Ambler class are present in the isolates, including several novel variants. The most abundant β-lactamases, CTX-M-15, TEM-1, SHV-11, SHV-12, and OXA-1 were found in both U.S. and Pakistani isolates, but the KPC and NDM-1 carbapenemases showed strict geographic separation within this set. To determine if β-lactamase resistance profiles cluster by geography, hierarchical clustering was used to generate a dendrogram of β-lactamase carriage (Figure 4.11). This dendrogram did not cluster Pakistani isolates separately from U.S. isolates, though its primary separation was driven by the presence or absence of CTX-M-15, which was enriched in Pakistani isolates.

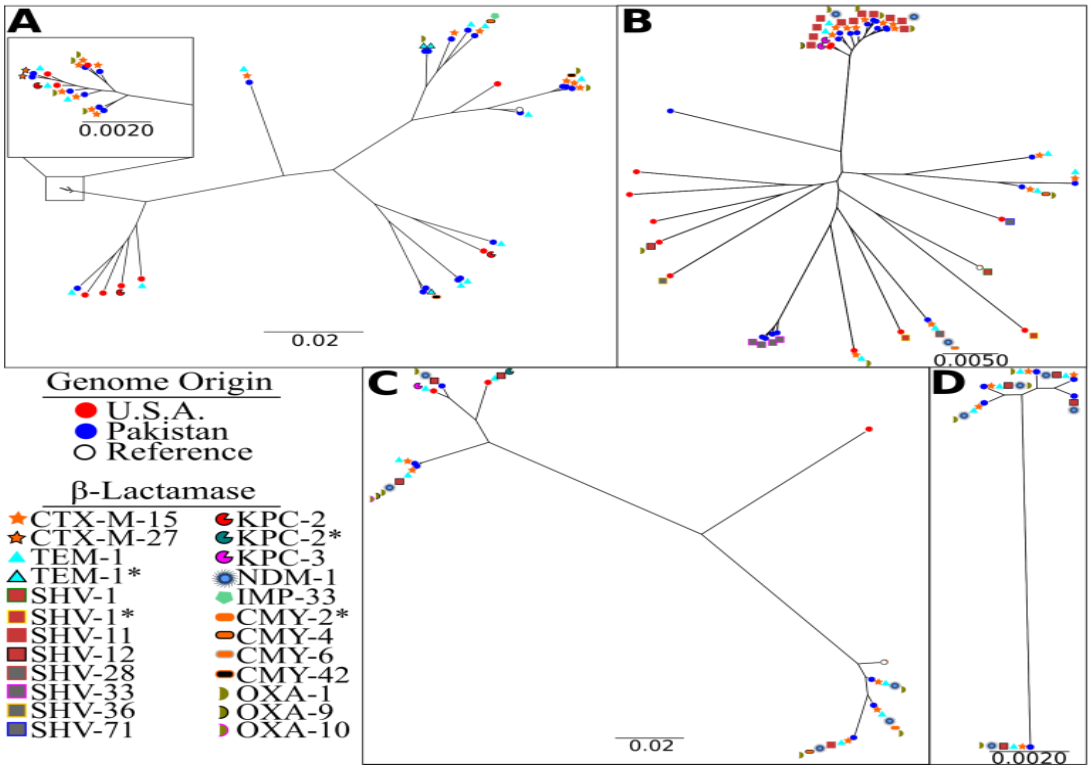

Figure 4.10: Distribution of antibiotic resistance and virulence genotypes across isolate phylogenies.

A-D) phylogenetic trees from Figure 4.9 have been annotated with the specific β-lactamases encoded by those isolates in lines extending from the isolate leaf. Circles at each leaf are colored to represent whether the leaf represents a Pakistani or USA isolate or a reference strain.

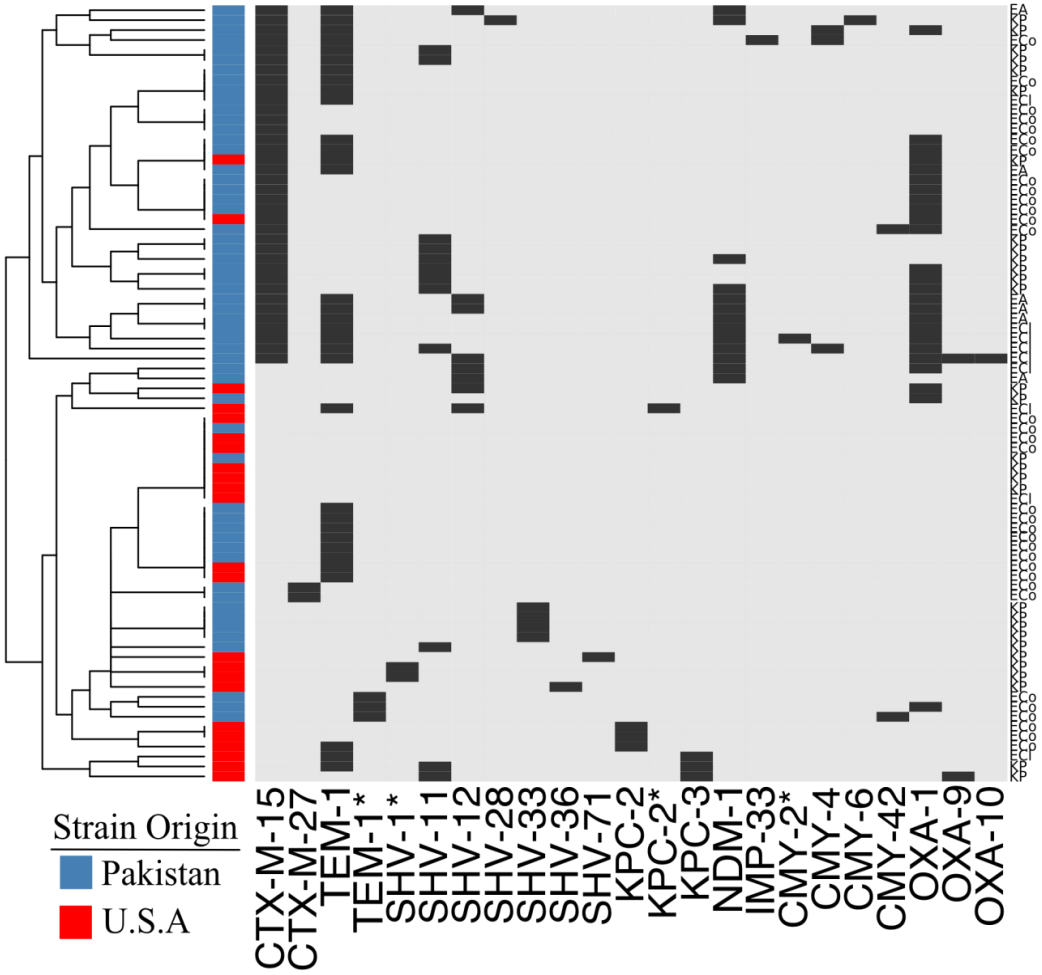

Figure 4.11: β-lactamase gene profiles of Pakistani and U.S. isolates.

Dark rectangles indicate presence of a β-lactamase, while light rectangles indicate its absence from the genome. An asterisk next to a gene name indicates a novel variant of that gene at least 1 amino acid different. The dendrogram on the left was generated by hierarchical clustering using the binary Euclidean distance between β-lactamase profiles. Asterisk denotes novel variant of gene (at least 1 amino acid different). EA = *Enterobacter aerogenes*. ECl = *Enterobacter cloacae* complex. ECo = *Escherichia coli*. KP = *Klebisella pneumoniae*.

4.7 IDENTIFICATION OF *E. COLI* PATHOTYPES BASED ON CLOSEST REFERENCE GENOME ALIGNMENT

E. coli genomes were evaluated from Pakistani and U.S. isolates for the presence of a previously reported panel of extraintestinal pathogenic *E. coli* (ExPEC) virulence factors (Johnson and Russo, 2002) as well as the toxins *sat*, *senB*, and *shET2* (Figure 4.12). In comparison with the distribution of β-lactamases, (Figure 4.10A) virulence factors show higher conservation within clades. Seven of the 11 isolates in ST131 encode the same virulence profile, compared to four out of 11 isolates showing the most common β-lactamase profile in ST131. To estimate the pathogenicity of each isolate, a literature search was performed on the closest reference genomes for each isolate. It was found that the distribution of virulence factors tracks closely with pathogenicity of the nearest reference genomes: isolates closely related to known ExPEC (including uropathogenic *E. coli*, UPEC) strains encode many ExPEC virulence factors, while genomes closely related to intestinal pathogens encode fewer ExPEC virulence factors, but are more likely to encode the *Shigella* enterotoxin, *shET2*. No major differences were seen between Pakistani and U.S. isolates within the same pathogenicity classes.

Table 4.1: Closest reference pathogenicity determination of *E. coli* to the annotated genomes available on NCBI

Closest Reference Genome	Isolates	Pathogenecity	Reference (PMID)
Escherichia coli NA114	PK101-2, PK129, PK151-2, PK156-1,PK18, PK31, PK5, PK90, US31, US34, US44, US45	UPEC	21685291
Escherichia coli UMN026	PK105, PK108, PK141, PK92-1, PK93, US32	ExPEC	19165319
Escherichia coli O104:H4 str. 2011C-3493	PK100, PK135, PK20, PK98	EAEC	23133618
Escherichia coli str. K12 MG1655star	PK39, PK94	Non-Pathogenic	21422176
Escherichia coli UMNK88	PK118, PK143	ETEC	22081385
Escherichia coli O7 K1 CE10	PK114	ExPEC	22123760
Escherichia coli SE11	PK51	Non-Pathogenic	18931093
Escherichia coli IHE3034	PK85	ExPEC	20439758
Escherichia coli 536	US33	UPEC	12379716
Escherichia coli ATCC 8739	US35	Non-Pathogenic	
Escherichia coli ABU 83972	US40	Non-Pathogenic	20865122
Escherichia coli UM146	US43	AIEC	21075930

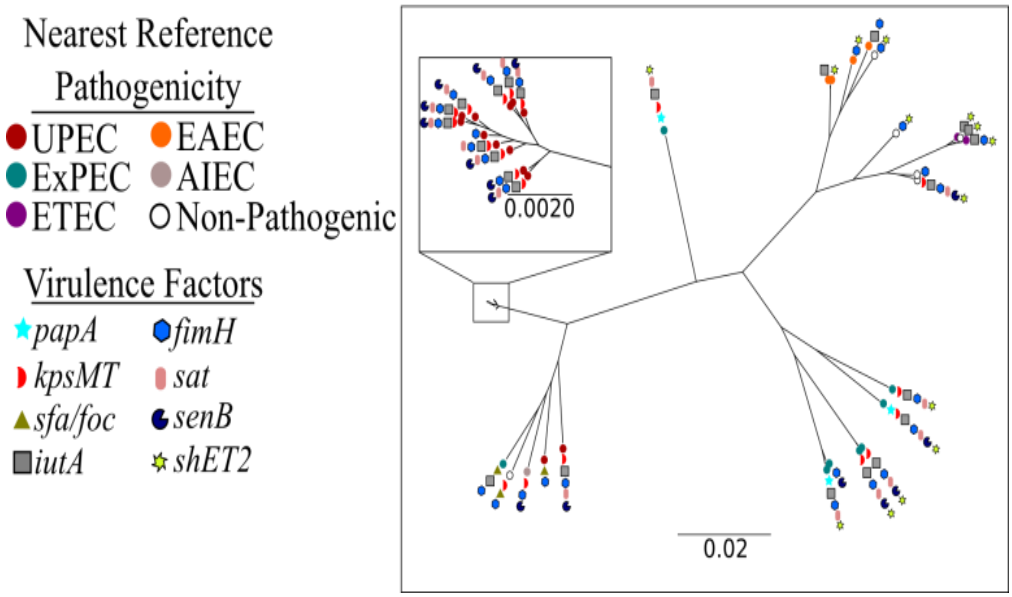

Figure 4.12: The *Escherichia coli* tree with ExPEC and non-ExPEC virulence factors

The *Escherichia coli* tree annotated with selected ExPEC virulence factors and non-ExPEC toxins extending from each leaf. The circles on each leaf represent the published pathogen type of the reference genome used in assembly of the draft genome of the isolate represented by that leaf. UPEC = uropathogenic*E. coli*, ExPEC = extraintestinal pathogenic *E. coli*, ETEC = enterotoxigenic *E. coli*, EAEC = enteroaggregative *E. coli*, and AIEC = adherent-invasive *E. coli*

4.8 ANALYSIS OF KPC, NDM-1 AND CTX-M PLASMIDS

KPC and NDM-1 have previously been predominantly observed in plasmids (Dortet*et al.*, 2014; Cuzon*et al.*, 2010). To determine whether the clonal distribution of NDM-1 and KPC in our isolate set was due to the spread of a single or a small number of multidrug resistance plasmids, plasmid DNA was isolated and sequenced from all the isolates encoding NDM-1 or KPC, and from three isolates encoding CTX-M-15, as non-carbapenemase controls. This represents the first sequences of NDM-1-encoding plasmids that were isolated directly in the Indian subcontinent rather from patients hospitalized elsewhere. Within the plasmid sequences many transposons and other repetitive mobile elements were observed that contribute to plasmid diversity. The targeted β-lactamase were identified from nine isolates for NDM-1, eleven isolates for KPC, and three isolates for CTX-M-15 (with one additional CTX-M-15 identified from a plasmid preparation that also included NDM-1). Using reciprocal BLAST between each pair from this group of 23 plasmid preparations, it was determined that how often the same DNA segments occurred in different plasmids. Regions with above 99% identity were considered to be shared between the plasmids, and the percentage of each plasmid shared was calculated with each other plasmid. Repeat regions in a plasmid were counted towards its total length, and towards the percentage shared if that region was found one or more times in the comparison plasmid.

This same analysis was performed for all sequenced plasmids containing NDM-1, KPC, or CTX-M available in NCBI and from our set separately (Figure 4.14), and together (Figure 4.13A). Median BLAST identity for this pairwise comparison was below 12%, even when only considering plasmids with the same β-

lactamase. This trend holds true for globally disseminated resistance genes, such as CTX-M and KPC, as well as NDM-1. Similar values for a pairwise BLAST were seen only for the NCBI plasmids, while pairwise BLAST within the Pakistani and U.S. plasmid sequences had median values up to 25% (Figure 4.14). Not surprisingly, plasmids consistently shared more of their sequences with other plasmids that have the same β-lactamase, but NDM-1 plasmids did not share more with other NDM-1 plasmids than KPC plasmids shared with other KPC plasmids. This suggests that the diversity of plasmids carrying NDM-1 is as high as the diversity of plasmids carrying KPC.

Outliers were also observed with high pair-wise identity, representing the same plasmid configuration in multiple isolates. A few of these outliers with very high sequence sharing were also observed between plasmids containing different β-lactamases. Since KPC genes were not found in the same plasmid as NDM-1 for any of the plasmids in this comparison, this finding highlights that the KPC and NDM-1 transposons both have access to extremely similar plasmid backbones. Though only a small number of plasmids share large portions of their sequences with other plasmids in this set, there is some sharing between almost every pair of plasmids compared for this study (Figure 4.13B).

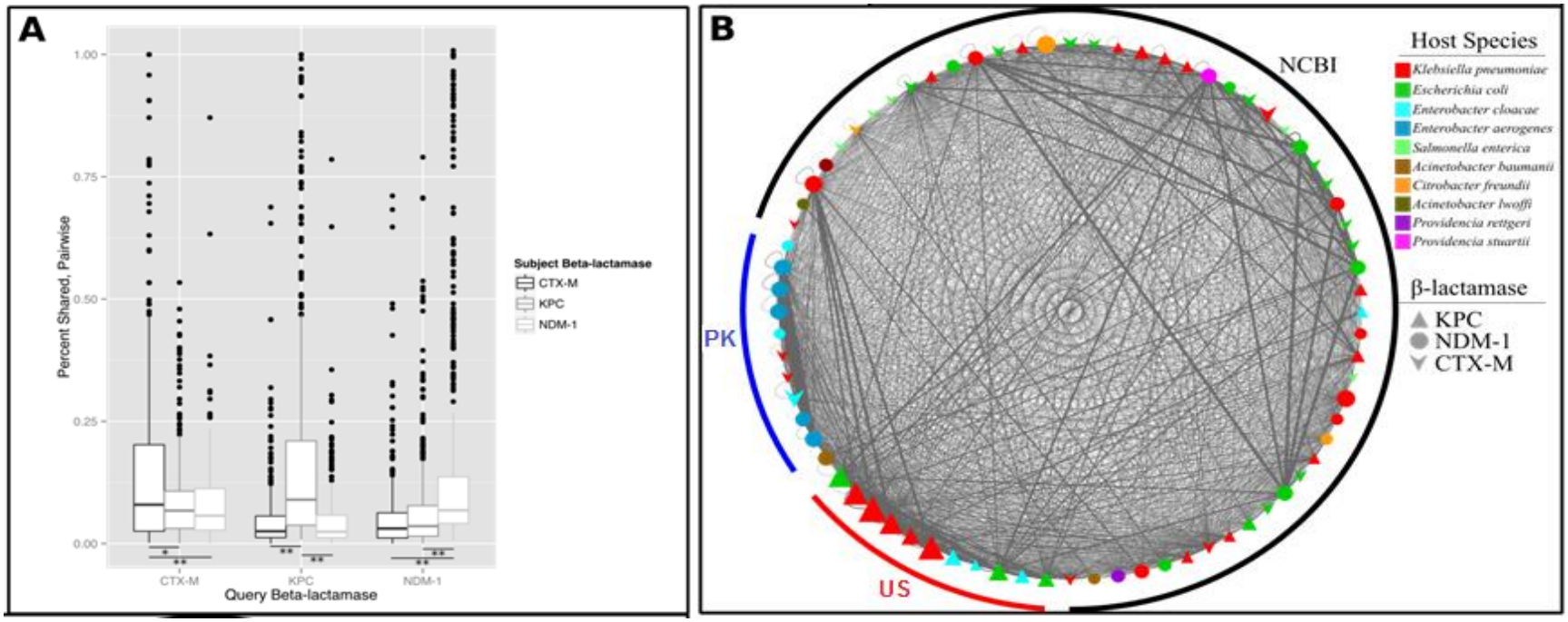

Figure 4.13:Pairwise BLAST identity of all CTX-M, KPC, and NDM-1 plasmids from Pakistani and U.S. and NCBI plasmids

An all-against-all plasmid BLAST was performed and plasmid interactions were defined by the percentage of the query plasmid conserved (at ≥ 99% identity) in the subject plasmid.A) plasmid interactions were collected based on the defining β-lactamase of their query and subject plasmids. The null hypothesis that two plasmid interaction collections were part of the same population was tested by the Mann-Whitney U test and by 10,000 Monte Carlo experiments. All collections with the same query β-lactamase were compared by this method, as were the three collections whose query and subject β-lactamases were the same; p-values are only given for significantly different collections. P-values were corrected for testing 3 hypotheses using the Bonferroni correction. *p= 0.00059, percentile = 0.002%. **p<0.00001, percentile > 0.0001%.B) a network map in which nodes represent individual plasmids and edges represent conserved regions between plasmids, with edge width proportional to the number of nucleotides contained in fragments > 500bp in length at >99% sequence identity. Plasmid sequence origin is indicated in arcs around the network.

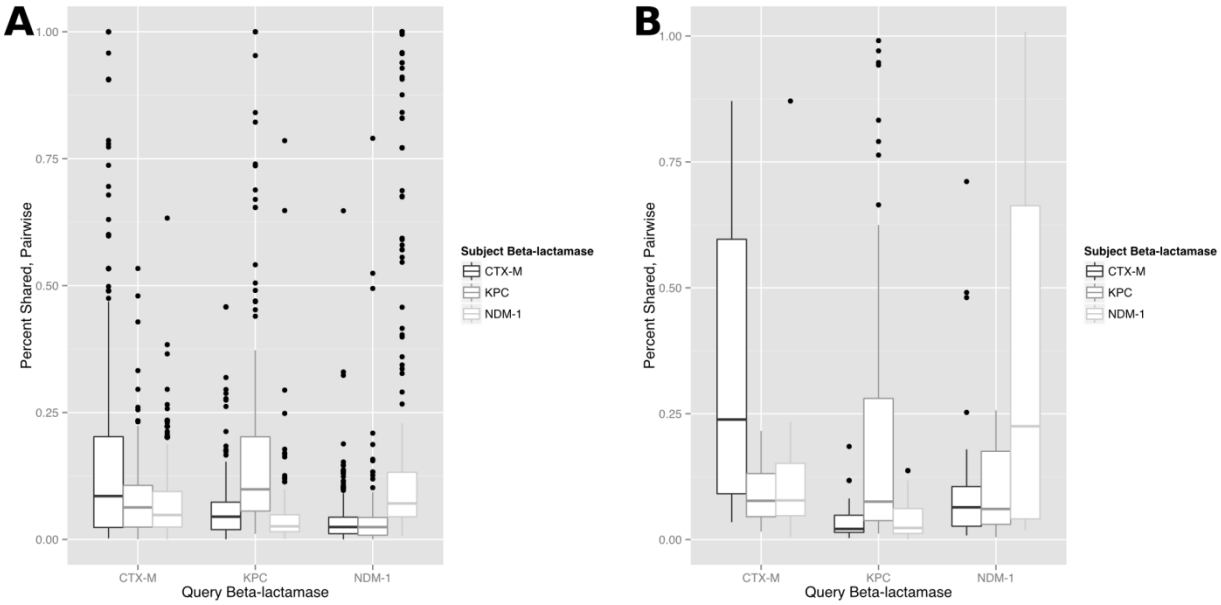

Figure 4.14: Sequence conservation between plasmids containing NDM-1, KPC, or CTX-M β-lactamases

All plasmids from A) NCBI and B) this study that contained a NDM-1, KPC, or CTX-M β-lactamase were analyzed by and all-against-all BLAST. Plasmid interactions were defined by the percentage of the query plasmid conserved (at > 99% identity) in the subject plasmid. Plasmid interactions were plotted based on the defining β-lactamase of their query and subject plasmids.

4.9 GENOMIC SEQUENCE BASED PREDICTIONS OF BETA-LACTAM AND FLUOROQUINOLONE SUSCEPTIBILITY

To evaluate the accuracy of β-lactam phenotypic resistance predictions based on the organism genotype, an expected β-lactam resistance profile was constructed for each named gene observed in our set based on the functional and molecular characterization work of Bush and Jacoby (Bush and Jacoby, 2010) (www.lahey.org/Studies/, accessed March 25th, 2014, Table 4.2). When a β-lactamase could not be matched to a specific named variant, either because the gene was not completely assembled or because it represents a novel β-lactamase sequence within a class, then the conservative assumption was made that the gene provided the highest level resistance provided by any gene within that subclass (e.g; KPC or CTX-M). The predictions were generally accurate across each genus (Figure 4.15A) and antibiotic (Figure 4.15b). The very major error rate was 3.34%, and the major error rate was 7.42%. The predictions were most accurate for ampicillin and meropenem.

Table 4.2: Phenotype predictions from presence of specific β-lactamases

Name	Database phenotype	AM	CZ	CTT	CAZ	CRO	FEP	MEM
CTX-M-15	2be	R	R	S	R	R	R	S
TEM-1	2b	R	R	S	S	S	S	S
OXA-1	2d	R	R	S	S	S	S	S
SHV-1	2b	R	R	S	S	S	S	S
SHV-2	2be	R	R	S	R	R	S	S
SHV-3	2be	R	R	S	R	R	S	S
SHV-4	2be	R	R	S	R	R	S	S
SHV-5	2be	R	R	S	R	R	S	S
SHV-6	2be	R	R	S	R	S	S	S
SHV-7	2be	R	R	S	R	R	S	S
SHV-8	2be	R	R	S	R	R	S	S
SHV-9	2be	R	R	S	R	R	S	S
SHV-10	2br	R	R	S	R	R	S	S
SHV-11	2b	R	R	S	S	S	S	S
KPC-2	2f	R	R	V	R	R	R	R
KPC-3	2f	R	R	V	R	R	R	R
NDM-1	3a	R	R	R	R	R	R	R
CTX-M-27	2be	R	R	S	R	R	S	S
TEM-12	2be	R	R	S	R	R	S	S
CMY-4	1e	R	R	R	R	R	S	S
CMY-42	1e	R	R	R	R	R	S	S
IMP-33	3a	R	R	R	R	R	R	R
SHV-33	2b	R	R	S	S	S	S	S
SHV-28	2be	R	R	S	R	R	S	S
SHV-71	2b	R	R	S	S	S	S	S
SHV-36	2be	R	R	S	R	R	S	S
SHV-12	2be	R	R	S	R	R	S	S
SHV-2A	2be	R	R	S	R	R	S	S
MIR-5	1e	R	R	R	R	R	S	S
OXA-9	2d	R	R	S	S	S	S	S
OXA-10	2d	R	R	S	S	S	S	S

CTXM	2be	R	R	S	R	R	S	S
TEM	2be	R	R	S	R	R	S	S
OXA	2de	R	R	S	S	V	S	S
SHV-LEN	2be	R	R	S	R	R	S	S
KPC	2f	R	R	V	R	R	R	R
NDM-CcrA	3a	R	R	R	R	R	R	R
ClassA	2be	R	R	S	R	R	S	S
ClassC-AmpC	1e	R	V	S	S	S	S	S
ClassD	2de	R	R	S	S	V	S	S

Antibiotic susceptibility profiles of Pakistani and U.S. clinical isolates.
AM = ampicillin, CZ = cefazolin, CTT = cefotetan, CAZ = ceftazidime, CRO = ceftriaxone, FEP = cefepime and MEM = meropenem. Database phenotype represents Bush and Jacoby functional classification groups of the given β-lactamases.

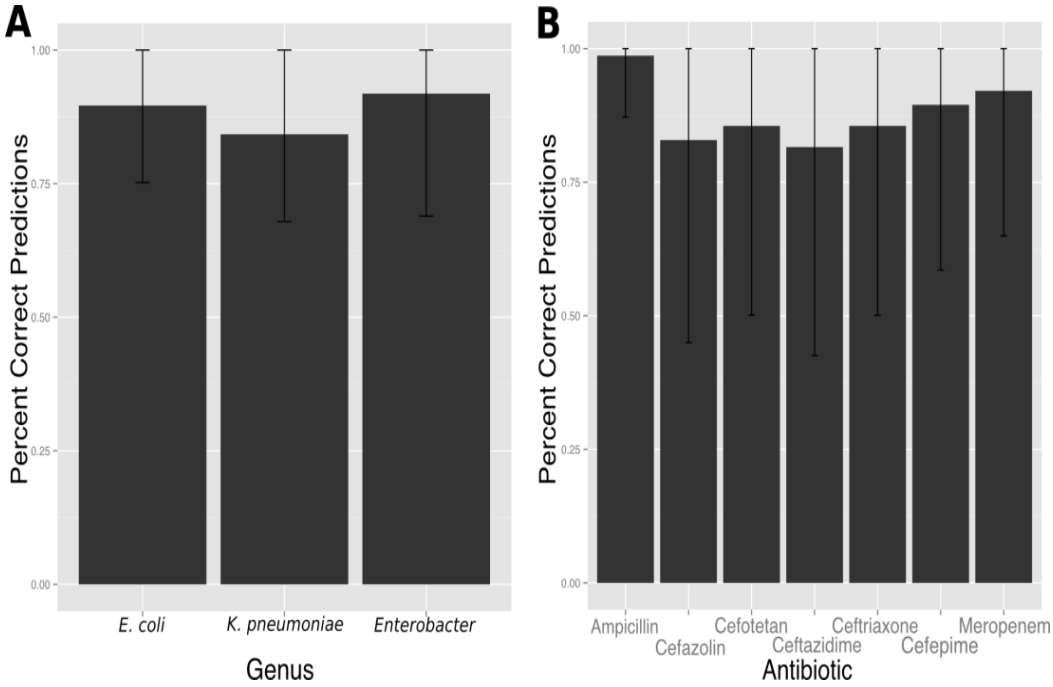

Figure 4.15: Agreement of genotype based antibiotic susceptibility predictions with phenotypic tests

The results were divided by genus of testing organism in part a), and by antibiotic predicted in part b). Error bars indicate one standard deviation above and below the mean for the test group.

4.10 QRDR MUTATIONS AND RESISTANCE PREDICTIONS FOR PAKISTANI AND U.S. ISOLATES

The fluoroquinolones are an important treatment for urinary tract infections with *Enterobacteriaceae* and because the Pakistani isolates also show high levels of ciprofloxacin resistance, ciprofloxacin susceptibility was predicted from WGS data. Resistance to the fluoroquinolones occurs almost exclusively because of mutations in the quinolone resistance-determining regions (QRDR) of the DNA gyrase gene "*gyrA"* and topoisomerase gene "*parC"* of the *Enterobacteriaceae*. Using a simple model where any mutation in the QRDR of *gyrA* or *parC* relative to wild-type gave a prediction of resistance, resistance was predicted correctly for 87.5% of the *E. coli* and *K. pneumoniae* isolates (Table 4.3). Of the eight incorrect predictions in those two species, four (6.25%) were cases where confident prediction could not be made because full-length genes for *gyrA* or *parC* were not assembled. The remaining four (6.25%) incorrect predictions were major errors resulting from the *gyrA* mutation S83L being insufficient to provide ciprofloxacin resistance on its own. Interestingly, for both *E. aerogenes* and *E. cloacae* complex isolates with no mutations in the QRDR of *gyrA* or *parC*, resistance was still observed to ciprofloxacin, which resulted in seven very major errors out of 15 strains tested. It is possible that this phenotype could be explained by the presence of gyrase protection (*qnr*) or quinolone efflux (*oqxAB*) genes in each of these resistant strains. However, previous studies have found *qnr* to be insufficient to provide ciprofloxacin resistance on its own (Rodriguez-Martinez *et al.*, 2007). In this study close homologues of *oqxAB* were observed in susceptible *K. pneumoniae* as well as resistant *Enterobacter* spp.

Table 4.3: QRDR mutations and resistance predictions

Isolate	gyrA Mutations	parC Mutations	Prediction	Phenotype	Error	*qnr*	*oqxAB*
***E. coli* PK100**	-	-	S	S	-	-	-
***E. coli* PK101-2**	S83L,D87N	S80I,E84V	R	R	-	-	-
***E. coli* PK105**	S83L	-	R	S	M	-	-
***E. coli* PK108**	S83L,D87N	S80I	R	R	-	-	-
***E. coli* PK114**	S83L,D87N	S80I	R	R	-	-	-
***E. coli* PK118**	S83L,D87N	S80I	R	R	-	Y	-
***E. coli* PK129**	S83L,D87N	S80I,E84V	R	R	-	-	-
***E. coli* PK135**	-	-	S	S	-	Y	-
***E. coli* PK141**	S83L,D87N	S80I	R	R	-	-	-
***E. coli* PK143**	S83L,D87N	S80I	R	R	-	-	-
***E. coli* PK151-2**	S83L,D87N	S80I,E84V	R	R	-	-	-
***E. coli* PK156-1**	S83L,D87N	S80I,E84V	R	R	-	-	-
***E. coli* PK18**	S83L,D87N	S80I,E84V	R	R	-	-	-

***E. coli* PK20**	**S83L,D87N**	**S80I**	**R**	**R**	**-**	**-**	**-**
***E. coli* PK31**	**S83L,D87N**	**S80I,E84V**	**R**	**R**	**-**	**-**	**-**
***E. coli* PK39**	**S83L**	**-**	**R**	**S**	**M**	**-**	**-**
***E. coli* PK51**	**S83L,D87N**	**INC**	**R**	**R**	**-**	**-**	**-**
***E. coli* PK5**	**S83L,D87N**	**S80I,E84V**	**R**	**R**	**-**	**-**	**-**
***E. coli* PK85**	**S83L**	**-**	**R**	**S**	**M**	**-**	**-**
***E. coli* PK90**	**S83L,D87N**	**S80I,E84V**	**R**	**R**	**-**	**-**	**-**
***E. coli* PK92-1**	**-**	**-**	**S**	**S**	**-**	**-**	**-**
***E. coli* PK93**	**S83L**	**-**	**R**	**S**	**M**	**-**	**-**
***E. coli* PK94**	**S83L,D87N**	**S80I**	**R**	**R**	**-**	**-**	**-**
***E. coli* PK98**	**-**	**-**	**S**	**S**	**-**	**-**	**-**
***E. coli* US31**	**S83L,D87N**	**S80I,E84V**	**R**	**R**	**-**	**Y**	**-**
***E. coli* US32**	**S83L,D87N**	**S80I**	**R**	**R**	**-**	**-**	**-**
***E. coli* US33**	**-**	**-**	**S**	**S**	**-**	**-**	**-**
***E. coli* US34**	**S83L,D87N**	**S80I,E84V**	**R**	**R**	**-**	**-**	**-**

***E. coli* US35**	**-**	**-**	**S**	**S**	**-**	**-**	**-**
***E. coli* US40**	**-**	**-**	**S**	**S**	**-**	**-**	**-**
***E. coli* US43**	**-**	**-**	**S**	**S**	**-**	**-**	**-**
***E. coli* US44**	**S83L,D87N**	**INC**	**R**	**R**	**-**	**-**	**-**
***E. coli* US45**	**S83L,D87N**	**S80I**	**R**	**R**	**-**	**-**	**-**
***K. pneumoniae* PK102**	**INC**	**-**	**-**	**R**	**-**	**-**	**-**
***K. pneumoniae* PK10**	**S83I**	**S80I**	**R**	**R**	**-**	**Y**	**Y**
***K. pneumoniae* PK11**	**S83I**	**S80I**	**R**	**R**	**-**	**-**	**Y**
***K. pneumoniae* PK124**	**S83F**	**-**	**R**	**R**	**-**	**Y**	**Y**
***K. pneumoniae* PK12**	**S83I**	**S80I**	**R**	**R**	**-**	**Y**	**Y**
***K. pneumoniae* PK139**	**S83I**	**S80I**	**R**	**R**	**-**	**-**	**Y**
K. pneumoniae	**S83I**	**S80I**	**R**	**R**	**-**	**Y**	**-**

PK150-2							
***K. pneumoniae* PK152**	**S83I**	**S80I**	**R**	**R**	**-**	**Y**	**Y**
***K. pneumoniae* PK24-1**	**S83I**	**S80I**	**R**	**R**	**-**	**Y**	**Y**
***K. pneumoniae* PK25**	**S83I**	**S80I**	**R**	**R**	**-**	**-**	**-**
***K. pneumoniae* PK28-1**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
***K. pneumoniae* PK38-1**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
***K. pneumoniae* PK40**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
***K. pneumoniae* PK44**	**S83F,D87A**	**S80I**	**R**	**R**	**-**	**-**	**Y**
***K. pneumoniae* PK49-2**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
K. pneumoniae	**INC**	**INC**	**-**	**R**	**-**	**Y**	**-**

PK72							
***K. pneumoniae* PK73**	**INC**	**-**	**-**	**S**	**-**	**-**	**-**
***K. pneumoniae* PK88**	**-**	**S80I**	**R**	**R**	**-**	**-**	**-**
***K. pneumoniae* PK9**	**S83I**	**S80I**	**R**	**R**	**-**	**-**	**-**
***K. pneumoniae* US10**			**S**	**S**	**-**	**-**	**Y**
***K. pneumoniae* US12**	**S83I**	**S80I**	**R**	**R**	**-**	**-**	**-**
***K. pneumoniae* US18**	**S83I**	**S80I**	**R**	**R**	**-**	**-**	**-**
***K. pneumonia* US21**	**S83F,D87A**	**-**	**R**	**R**	**-**	**Y**	**Y**
***K. pneumoniae* US23**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
K. pneumonia	**-**	**-**	**S**	**S**	**-**	**-**	**-**

US2							
***K. pneumoniae* US3**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
***K. penumoniae* US6**	**-**	**INC**	**-**	**S**	**-**	**-**	**-**
***K. penumoniae* US7**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
***K. penumoniae* US8**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
***K. penumoniae* US9**	**-**	**-**	**S**	**S**	**-**	**-**	**Y**
***E. aerogenes* PK112-2**	**-**	**-**	**S**	**R**	**VM**	**Y**	**-**
***E. aerogenes* PK113**	**INC**	**-**	**-**	**R**	**-**	**-**	**-**
***E. aerogenes* PK134**	**-**	**-**	**S**	**R**	**VM**	**Y**	**-**
***E. aerogenes* PK138**	**T83S**	**-**	**R**	**R**	**-**	**Y**	**Y**

***E. aerogenes* PK63**	**-**	**-**	**S**	**R**	**VM**	**Y**	**-**
***E. cloacae* PK3**	**-**	**-**	**S**	**R**	**VM**	**Y**	**Y**
***E. cloacae* PK242**	**-**	**-**	**S**	**R**	**VM**	**Y**	**Y**
***E. cloacae* PK125**	**S83I**	**S80I**	**R**	**R**	**-**	**Y**	**Y**
***E. cloacae* PK158**	**-**	**-**	**S**	**R**	**VM**	**Y**	**Y**
***E. cloacae* PK82**	**S83I**	**S80I**	**R**	**R**	**-**	**Y**	**Y**
***E. cloacae* US26**	**S83F,D87A**	**S80I**	**R**	**R**	**-**	**-**	**Y**
***E. cloacae* US27**	**S83I**	**-**	**R**	**R**	**-**	**Y**	**-**
***E. cloacae* US29**	**S83T**	**-**	**R**	**S**	**M**	**-**	**-**

QRDR mutations and resistance predictions for Pakistani (PK), and U.S. (US) isolates. INC =Incomplete Assembly. M = Major Error. VM = Very Major Error.

Chapter 5

DISCUSSION

Antibiotic resistance is an extremely threatening public health concern all over the world. The rapid increase in resistance to antimicrobials drugs and the decreasing interests of pharmaceutical companies into novel drugs development is a huge concern. Pharmaceutical companies find it less profitable since it takes about 10-15 years and approximately 1-2 billion dollars to make a drug available in the market and by the time the drug is available, the bacteria might have already developed resistance or may become very quickly resistant as there is no check on antibiotic usage. The inappropriate prescription and then the overuse and underuse of antibiotics in medications have challenged their efficacy. *Enterobacteriaceae* isolates screened in this study against eight different classes of antimicrobials viz; penicillin, cephalosporin, tetracycline, sulfonamides, aminoglycosides, monobactam, β-lactamase inhibitors and carbapenem showed varying levels of resistance. Most of the isolates were found resistant to more than six antimicrobials belonging to different groups.

Susceptibility profile of MDR *Enterobacteriaceae* isolates show that resistance is getting severe with time. Comparing the results obtained in this study (Figure 4.1) to previous studies from Pakistan (Tanvir *et al.,* 2012; Abdullah *et al.,* 2012; Hassan *et al.,* 2011; Qamar *et al.,* 2010; Khan *et al.,* 2010; Amin *et al.,* 2009; Mahmood, 2001; Iqbal *et al.,* 2001; Farooqui *et al.,* 1989), there is a gradual increase in resistance, and this increase is either because of the gene enrichment in bacterial population or clonal expansion of resistant bacteria.

Enterobacteriaceae isolates showed maximum resistance to ampicillin. This significant resistance to ampicillin is seen because of its continuous and widespread use since decades. It is administered to treat a variety of bacterial infections, including pneumonia, bronchitis, ear, lung, skin, and urinary tract infections.

Trimethoprim-sulfamethoxazole is a drug frequently prescribed for urinary tract infections. Overwhelming resistance was observed to trimethoprim-sulfamethoxazole. Resistance to trimethoprim-sulfamethoxazole was reported as early as in 1968 (Bushby *et al.,* 1968), but due to its easy availability and low cost in developing countries, it is still considered a standard drug for treating urinary tract infections (Stuck *et al.,* 2012). In a study reported from the United Kingdom (Enne *et al.,* 2001), even when the prescription of trimethoprim-sulfamethoxazole was controlled, the frequency of resistance in *E. coli* was still high. The reason for this replicating resistance could be mobile genetic elements like integrons or plasmids (Huovinen *et al.,* 1995), which can transfer resistance very quickly not only to closely related bacteria but also to bacteria from different families. Moreover, Resistance to sulfonamides has been attributed to integrons, which also cause resistance to multiple antibiotics (Jones, 1997)

Similarly, a high level of resistance was observed to cephalosporins in Pakistani isolates as compared to the U.S. isolates. Resistance to the quinolones occurs almost exclusively because of mutations in the quinolone resistance-determining regions (QRDR) of the DNA gyrase and topoisomerase genes (*gyrA* and *parC*, respectively) of the *Enterobacteriaceae*. Hence resistance to ciprofloxacin is directly linked to its overuse. The Overuse of ciprofloxacin not only make bacteria

resistant to these antibiotics, but induces resistance to several other classes of antibiotics as well (Borgmann *et al.,* 2010).

Carbapenem were most effective in inhibiting bacterial isolates. Carbapenems are thought to be the most effective antibiotics and are administered against multidrug resistant bacteria as a last option drug. The results obtained in this study showed that resistance to carbapenem is emerging among bacterial communities. Pakistani isolates showed substantial level of resistance to carbapenems.

Looking at this high prevalence of carbapenem resistance in Pakistani set of isolates, a comparison set of 50 isolates was assembled from Barnes Jewish Hospital, Washington University, United States, specifically selecting for a mixture of carbapenem resistant and susceptible isolates.

Various classes of carbapenem hydrolysing enzymes have been described but NDM-1 and KPC are most frequently encountered in clinical settings. KPC is prevalent globally while NDM-1 has been found endemic in Pakistan, India and Bangladesh. Resistance to cephalosporins is because of Class A β-lactamases, also known as Extended Spectrum B-Lactamases (ESBLs), which are frequently encountered β-lactamases in hospitals and community infections. CTX-M, SHV and TEM are common ESBLs. ESBLs are globally prevalent and cause resistant to several generations of β-lactam antibiotics such as penicillin and cephalosporin.

The results in this study showed that prevalence of all these three β-lactamases is very high in all the clinical *Enterobacteriaceae* isolates. As they are encoded by plasmids, and gene enrichment or clonal expansion both are very possible in Pakistani hospitals and community because of substandard hygienic and treatment practices.

The results suggest that these β-lactamases are spreading very rapidly and causing the failure of treatments. Together, the susceptibility and molecular study suggests that antibiotic resistance is rapidly spreading among the *Enterobacteriaceae* clinical isolates in Pakistan, and all the popular ESBL types that is CTX-M, SHV, and TEM are highly prevalent in clinical isolates. And since genes for ESBLs are present on plasmids which also co-carry the genes for other antibiotics such as aminoglycosides, quinolones, chloramphenicol and tetracycline (Al-Zahrani *et al.,* 2005), so resistance to multiple drugs is on the rise. Consistent surveillance programs for antibiotic resistance both at phenotypic and molecular level are very essential to identify any emerging threats.

Carbapenem resistant *Enterobacteriaceae* (CRE), encoding carbapenemases such as New Delhi Metallo-β -lactamase-1 (NDM-1) or *Klebsiella pneumoniae* Carbapenemase (KPC), are an urgent global health threat with limited treatment options. CREs harboring NDM are highly prevalent in the Indian subcontinent, while KPC isolates are globally distributed. Accurate genomic-based diagnostics of these and other multidrug resistant (MDR) pathogens could enable more rapid and efficacious treatment of bacterial infections.

The results with WGS (whole genome sequencing) of multidrug resistant pathogens collected from Pakistani and US hospitals provides interesting findings regarding the AR genes carriage. Various studies have reported a high level of AR and metallo-β-lactamase carriage in pathogenic strains from the Indian sub-continent (Walsh *et al.,* 2011; Mushtaq *et al.,* 2011; Nahid *et al.,* 2013), but this study is the first to characterize many clinical isolates from multiple species of *Enterobacteriaceae* from the Indian sub-continent at a genomic level. This deep analysis allowed us to

compare the phylogeny, antibiotic susceptibility phenotype, AR genotype, and plasmid sequence of isolates from Pakistan to isolates from the United States as well as to the sequenced bacteria from around the world.

The data presented herein confirm that bacterial isolates with the ability to harbor broad spectrum AR are globally dispersed; not only do Pakistani and U. S. isolates belong to the same clades (Figure 4.8), but in some cases they belong to the same recognized globally disseminated lineages, such as *E. coli* ST131 (Rogers *et al.,* 2011) and *K. pneumoniae* ST11 (Pena *et al.,* 2014).*E. coli* ST131 has been identified as a causative agent of human infections in all six inhabited continents, and frequently carries a large complement of AR and virulence genes (Rogers *et al.,* 2011). A recent study found that the rapid global expansion of ST131 has been driven by the success of a specific subclone of ST131 that encodes fluoroquinolone resistant *gyrA* and *parC* alleles and CTX-M-15 (Price *et al.,* 2013), a characterization which fits seven of the total 11 of ST131 isolates studied. A single ST131 isolate was also identified carrying KPC-2, which was resistant to all β-lactams tested. Similarly, *K. pneumoniae* ST258, and its single locus variant ST11, have been found to be highly common worldwide and frequently encode carbapenemases (Cuzon *et al.,* 2010; Pena *et al.,* 2014), similar to the ST11 isolates in this study.

No significant difference was found in the number of AR genes of various mechanisms encoded by Pakistani versus U.S. isolates, though it was found that Pakistani *E. coli* encoded significantly more β-lactamases than *E. coli* strains sequenced in the past (Figure 4.9). Exact same AR genes for several β-lactamase types were also observed in both sets of isolates (Figure 4.10); however, strains clustered on β-lactamase profile did not cluster by phylogeny (Figure 4.11). These

observations together demonstrate that β-lactamases have transferred between Pakistan and the U.S., either directly or through shared intermediates, and between species within each location. Furthermore, it demonstrates that even high-resolution phylogenetic profiling is insufficient for inferring β-lactam susceptibility phenotypes without specific β-lactamase detection. The β-lactamases in the Pakistani and U.S. isolates (with the exception of chromosomal *ampC*) are associated with mobility elements such as plasmids, promoting AR sharing. In contrast, a set of virulence factors (Figure 4.12), which would also be selected for during human infection, shows much more conservation within phylogenetic clades. This is possibly because the β-lactamaseshave overlapping functions, and are perhaps more interchangeable than virulence factors. The major exception to the apparently free exchange of β-lactamasesbetween Pakistani and U.S. isolates is the carbapenemases, with NDM-1 found only in Pakistani isolates and KPC genes found only in the U.S. isolates.

The deep characterization of plasmids that carry NDM-1 and KPC demonstrated that NDM-1 carrying plasmids are at least as diverse as KPC carrying plasmids (Figure 4.13). Thus, even though NDM-1 had not been observed outside of the Indian subcontinent until 2008, it appears the variety of mobile elements available to plasmids carrying NDM-1 is not restricted. Despite the fact that NDM-1 carrying plasmids were more likely to share DNA fragments with other NDM-1 carrying plasmids than with KPC carrying plasmids, plasmids in this set shared at least 500bp with an average of 58 of the other plasmids analyzed (Figure 4.13b). In many cases the conserved sequence consisted of just a transposase, but in some cases an NDM-1 carrying plasmid shared a large portion (maximum: 79.0%) of its sequence with a KPC carrying plasmid, indicating that KPC and NDM-1 have existed in extremely

similar plasmid backbones. Together, this data suggests that strains and plasmids nearly identical to those carrying NDM-1 have already been found around the world, and it is likely only a matter of time before NDM-1 becomes as globally disseminated as other β-lactamases. It is possible that effective surveillance and containment procedures at hospitals worldwide have thus far prevented more rapid NDM-1 dissemination.

β-lactam and ciprofloxacin susceptibility was accurately predicted in MDR pathogens with high accuracy based on genotype information alone. Current epidemiological trends suggest that MDR strains could represent a growing proportion of infections in coming years. As the options available in treating each specific pathogen become more limited, faster diagnostic tools will become increasingly necessary to combat these strains. The results of successful prediction of AR, and success in other measures such as virulence gene and high-resolution phylogeny detection, support the development of WGS as a clinical diagnostic tool in the future. The instances where the predictions were not accurate can be used as a guide for refinement of WGS based diagnostics. Majority of the major errors was attributed to detection of novel β-lactamase variants, which were conservatively assumed that would show an extended spectrum phenotype. For four of the seven isolates whose β-lactamase susceptibility profiles contained very major errors, retrospectively porin deletions were identified with relative to near neighbours on their species phylogenetic tree. A complete deletion or deleterious mutation in the outer membrane porins that β-lactam antibiotics must pass through in Gram negative bacteria can extend the activity of β-lactamases were also found. Though these porin deletions were retroactively identified, it required comparison to closely related

isolates, and thus it was conservatively chosen not to include these predictions in the model.

In the case of fluoroquinolone resistance, the results suggest that more basic research is needed on the mechanisms of fluoroquinolone resistance in *Enterobacter* spp. before accurate predictions can be made in those organisms. For these antibiotic classes, a small number of well-defined studies could improve the base knowledge enough to bring our model within FDA parameters. Susceptibility predictions for only two important classes of antibiotics were attempted in this study, but it is anticipated that the high accuracy of WGS-based diagnostics would extend to other classes of antibiotics as well.

In conclusion, β-lactmases in *Enterobactreiacae* with the ability to dessiminate through horizontal gene transfer is an increasingly growing threat to the human race in diverse geographic regions. The whole genomes analysis of MDR clinical pathogens suggests that antibiotic resistance could substantially be increased in coming years. The deep analysis of NDM-1 and KPC plasmids, irrespective of their geographic exclusivity, suggests that NDM-1 has the genetic characteristics for global dissemination and is likely to gain prevalence outside of the Indian subcontinent in the near future. It was also found that WGS-based diagnostics show great promise for accurate susceptibility prediction of MDR infections and efforts should continue to increase the speed of such techniques to optimize them for clinical practice.

Chapter 6

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Appendix:

TABLE. S. Antibiotics discs used for phenotypic susceptibility tests of bacterial isolates

Ampicillin (30µg)	Cefazolin (30µg)	Cefotetan (5µg)	Ceftriaxone (30µg)	Ceftazidime (30µg)
Cefepime (30µg)	Meropenem (10µg)	Doxycycline 30µg	Gentamycin (30µg)	Ciprofloxacin (5µg)
Chloramphenicol (30µg)	Trimethoprim/ Sulfamethoxazol (25µg)			