Determining risk factors for the spread of XDR bacteria in Pakistan, and examining interventions to prevent their dissemination

By

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Summary

Most epidemiological studies concerning multidrug resistance (MDR) or extensively drug resistance (XDR) emphasise the prevalence of resistance in clinical settings whereas environmental contamination is often ignored a topic of increasingly great concern, especially in low middle-income countries (LMICs). This thesis reports on the prevalence and associations of carbapenemases and extended spectrum β-lactamases (ESBLs) in clinical and environmental settings in and the effects of climate factors and local hospital cleaning regimes on the XDR rate in post-surgical infections. Furthermore, I attempted to restore sensitivity to carbapenem antibiotics by introducing peptide nucleic acid (PNA) as an alternative therapy.

A preliminary study of environmental samples isolated from Karachi, Pakistan demonstrated the presence of New Deli Metalo-β-lactamase (bla_{NDM}) and cefotaxime β-lactamase ($bla_{CTX\text{-}M\text{-}15}$) in animal's faeces, insects, hospital surface and drinking water. Similarly, the environmental (insect and hospital surface) and clinical (surgical wound) samples from Peshawar, Pakistan revealed very high rates of bla_{NDM}, $bla_{CTX\text{-}M\text{-}15}$ and Oxacillin β-lactamase ($bla_{OXA\text{-}48}$)- like. Additionally, strains with similar restriction length polymorphism (REP) profiles and STs (sequence types) were recovered from environmental and clinical samples indicating their possible spread across clinical and environmental sectors.

In the Peshawar study, the collected in winter were shown to carry more resistance compared to summer. However, the cleaning regime had no significant association with the carriage of resistance.

Finally, it was attempted to use PNA to inhibit the expression of carbapenemases. However, despite exhibiting antimicrobial properties when targeted to the acyl carrier protein gene (*acpP*), PNA was unable to restore the sensitivity of resistant strains to carbapenem antibiotics.

The current work herein contributes to the understanding that contaminated touch surfaces may play an essential role in the transfer of MDR bacteria, whereas insects may be a key vector in their dissemination across distant niches. Such holistic studies are crucial in determining the role of atypical environmental co-factors in contributing to the increased resistance dissemination in LMICs.

Posters and Presentations from data presented in this thesis

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Declaration

I declare that this thesis represents my own work, except where otherwise acknowledged. The opinions given are my own and not those of Cardiff University.

No portion of this thesis has been submitted for any other degree or award at this or any other university or place of learning, nor is it being submitted concurrently in candidature for any degree or other award.

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List of abbreviations

Amino acid(s)	AA
Aminoglycosides	AG
Aminoglycoside modifying enzyme(s)	AMEs
Antimicrobial resistance	AMR
α-cyano-4-hydroxycinnamic acid	HCCA
β-lactamase gene	*bla*
Base pair	bp
Carbapenem-resistant Enterobacteriaceae	CRE
Centre for disease control and prevention	CDC
Clinical and Laboratory Standards Institute	CLSI
Conserved segment	CS
Cycle threshold value(s)	Ct
Cefotaxime	CTX-M
Deoxyribonucleic acid	DNA
Ribonucleic acid	RNA
Extensively-drug resistant	XDR
European Committee on Antimicrobial Susceptibility Testing	EUCAST
Genomic island(s)	GEI(s)
Extended spectrum β-lactamase(s)	ESBL
High income country	HIC
Horizontal gene transfer	HGT
Insertion sequence(s)	IS
Integrative and conjugative element(s)	ICE(s)
Integrative and mobilizable element(s)	IME(s)
Klebsiella pneumoniaee carbapenemase	KPC

Luria Bertani (agar/ broth)	LB
Low middle-income county	LMIC
Metalo-β-lactamase	MBL
Messenger Ribonucleic acid	mRNA
Minimal inhibitory concentration	MIC
Mobile genetic element(s)	MGE
Mueller Hinton (agar)	MH
Multi-drug resistant	MDR
Multilocus sequence typing	MLST
NDM-producing Enterobacteriaceae	NPE
NDM-producing GNB	NPGNB
New Delhi metallo-β-lactamase	NDM
Open reading frame	ORF
Oxacillinases	OXA
Penicillin binding protein(s)	PBP(s)
Polymerase chain reaction	PCR
Pulsed Field Gel Electrophoresis	PFGE
Quantitative PCR	qPCR
Restriction length polymorphism	REP
Single nucleotide polymorphism(s)	SNP(s)
Strain type	ST(s)
Transposon(s)	Tn(s)
Tris-borate-EDTA (buffer)	TBE
Tris-EDTA (buffer)	TE
Whole genome sequencing	WGS
Verona integron-encoded metallo-β-lactamase	VIM

Extreme drug resistace	XDR

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1. Introduction

1.1. Antibiotics and the Emergence of Resistance

1.1.1. The Discovery of Antibiotics

The discoveries of Salvarsan, in 1910, and Prontosi, in 1935, are documented as the earliest events in the commencement of antimicrobial chemotherapy for bacterial infectious diseases. However, the mass production of penicillin and streptomycin in the 1940s can be regarded as a real breakthrough in establishing the golden era of antibiotics discovery (Jayaraman 2009; Aminov 2010). Antibiotic use dramatically reduced the rate of infectious diseases and the associated mortality and morbidity. It is perceived that penicillin alone is responsible for saving approximately 200 million people (Fernandes 2006). However, it was soon realized that exposing bacteria to antimicrobials results in the development of resistance which could compromise the drug efficiency. Eventually, multiple resistance mechanisms started to emerge and spread rapidly. To overcome the resistance, modification to the existing antibiotics and some synthetic antibiotics such as fluoroquinolones were introduced. New bacterial cell targets were also identified and multiple metabolic pathways were targeted (Figure 1). However, none of the synthetic derivatives, as well as the natural compounds, were able to sustain its antimicrobial properties effectively and as the number of antibiotics increased, the resistance mechanisms also increased in parallel (Fernandes 2006; Aminov 2010). Furthermore, the emergence MDR (a bacterial strain resistant to more than one antimicrobial agent), and more recently XDR (resistant to multiple antimicrobial agents and likelihood of becoming resistant to almost all approved antimicrobial agents), complicated an already alarming situation (Walsh and Toleman 2012; Lewis 2013).

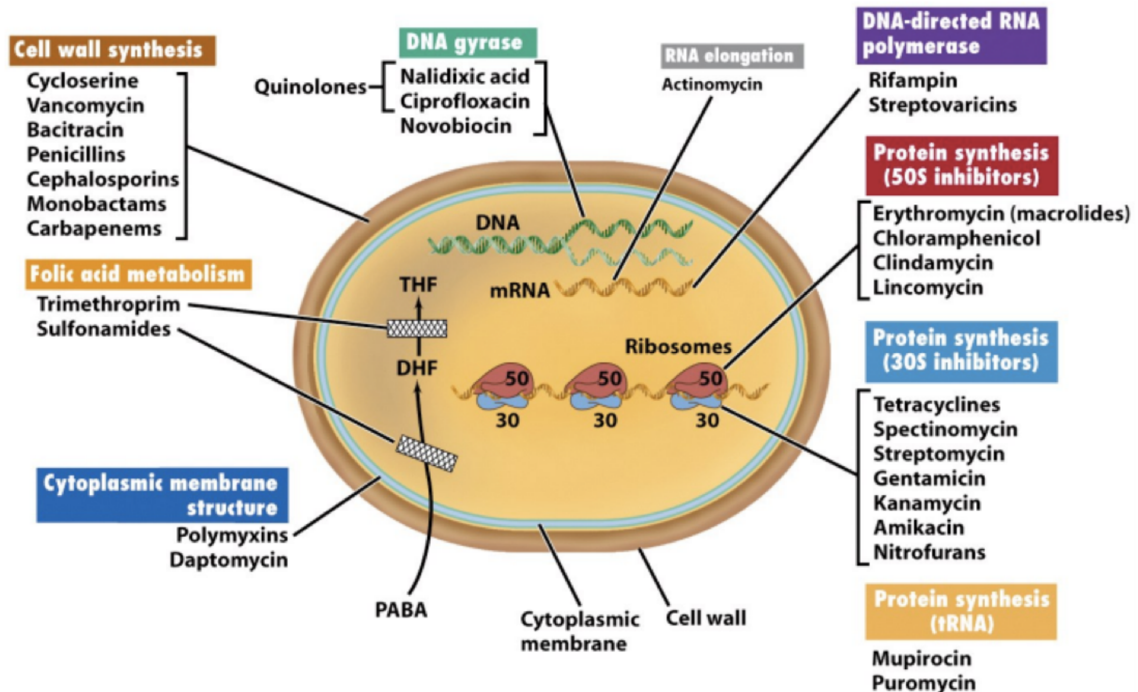


Figure 1. The target and mechanism of antibiotics. The image above shows the different targets of antimicrobials within a bacterial cell and its derivatives. Reproduced with permission (Rasheed 2006).

1.1.2. Anthropology of Antibiotic Resistance

Owing to the benefits seen with antibiotics, they have been used widely all over the world (Orzech and Nichter 2008; Aminov 2010). Several developed nations quickly introduced prescription policies, especially the UK and Sweden. However, this is not true for all developed nations, for example Greece, and there is no or little control on the consumption of antibiotics in the third world countries. The extensive use of antibiotics has contributed to the pharmaceuticalisation of the global health (Orzech

and Nichter 2008). This is detrimental for the efficiency of antibiotics as it encourages greater unnecessary use and, as a consequence, aids in the development of antibiotic resistance (WHO 2015a). Lack of knowledge and understanding is also a contributing factor. Antibiotics are often deemed as an instant remedy to improve symptoms of other unrelated conditions with no need for antibiotic therapy. Again, this is especially true for developing countries where a general lack of knowledge, in addition to the availability of unprescribed "over the counter" antibiotics, is exerting an unnecessary selection pressure on the bacteria. Furthermore, the environment has also been contaminated extensively by using antibiotics in veterinary, agriculture, aquaculture and farming (Quintela-Baluja et al. 2015). There has been no restriction on limiting or reserving some antibiotics exclusively for human use and, as a result, resistance could arise due to extensive use in other veterinary, agriculture and food industry which may circulate to distinct environments and eventually colonize humans (Wang et al. 2017). Over the years several resistance mechanisms have emerged, and spread globally, leading the world into a post-antibiotic era, where once treatable common infections and minor injuries could now lead to fatal consequences (Clatworthy et al. 2007; WHO 2013a). The continuous increase in drug resistant pathogens and decreased production of new antibacterial agents are key contributory factors. The pharmaceutical companies have somewhat lost their interest in the discovery and development of new antimicrobials. It is estimated that the development of a new antibiotic takes about seven to ten years and it takes the same time for bacteria to generate a considerable resistant population which decreases the efficiency of the drug (Clatworthy et al. 2007; Lewis 2013). Most antibiotics in use today have the resistance mechanisms already distributed around the world. In addition, conventional drug discovery

methods are proving to be ineffectual and, over the last 30 years, no major new class of antibiotics have been developed (Figure 2) (WHO 2013a). Recently a new class of antibiotic, (teixobactin) has been developed (not shown in the figure below) which is active against Gram-positive bacteria only and inhibits cell wall synthesis by binding to a highly conserved region of peptidoglycan teichoic acids in the cell wall. However, teixobactin is only active against Gram-positive bacteria and is not effective against Gram-negative bacteria (Ling et al. 2015).

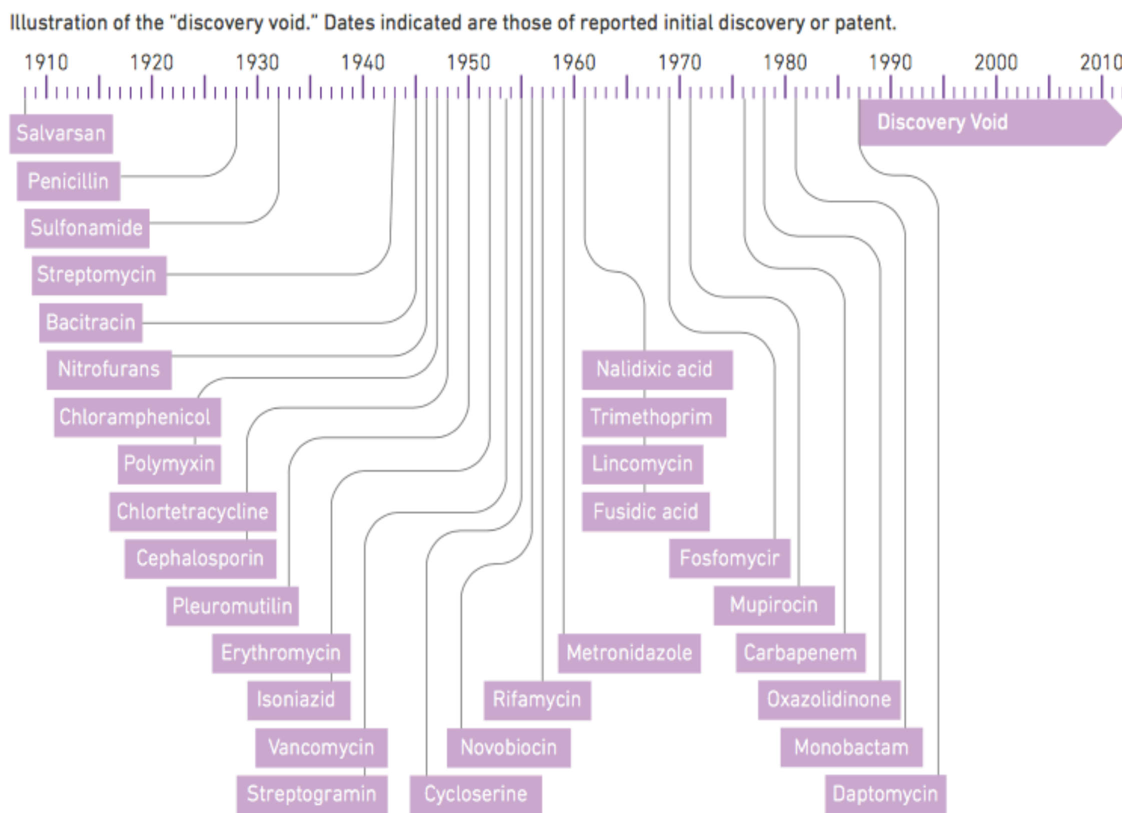


Figure 2. Timeline of antibiotic development and the emerging resistance. The image shows the timeline for the clinical antibiotics' introduction. Since 1987 a discovery void has been observed and no new class of antibiotics have been introduced in the market. Image copied from (WHO 2013a).

1.1.3. Resistance Mechanisms and Transfer

Several mechanisms may produce a resistance phenotype such as changes in the permeability of the bacterial cell wall to restrict the access of the active compound in to the cell, efflux pumps to discharge the antibiotic out of the cell, enzymatic modification of the antibiotic, degradation of the antimicrobial agent, acquisition of alternative metabolic pathways, modification of antibiotic targets and overproduction of the target enzyme (Van Hoek et al. 2011). Resistance can also be inherited or intrinsic. Intrinsic resistance is the inherited innate ability of bacterial species to withstand the activity of a drug. It is independent of selective pressure and present among pathogens as well as environmental bacteria (Blair et al. 2014).

Gram-negative bacteria are intrinsically more resistant to certain antibiotics than Gram-positive bacteria due to the presence of an additional outer membrane providing an extra layer of protection and limiting the entry of some compounds (Denyer and Maillard 2002; Holmes et al. 2016a). Smaller hydrophilic molecules can pass through by diffusion with a size limit of < 600 Da whereas the entry of large or hydrophobic molecules is either restricted completely or 50-100 times slower than for Gram-positive bacteria; for example, vancomycin, a 1500 Da hydrophilic glycol-peptide antibiotic inhibiting peptidoglycan crosslinking in the periplasm of Gram-positive bacteria, is ineffective against Gram-negative bacteria due to its large size (Denyer and Maillard 2002; Blair et al. 2014).

Intrinsic mechanisms enable bacteria to proliferate in a competitive environment with no link to previous antibiotic exposure. In contrast, acquired resistance arises through mutations in the genes or by gene acquisition which render bacteria resistant to a previously susceptible antibiotic (Holmes et al. 2016a). Mutations are

rare events in nature occurring at approximately 1×10^{-3} per genome per generation. However, once established successfully, they can persist in the organism and passed on by direct descent (Lee et al. 2012). Furthermore, they may transfer from one species to another by horizontal gene transfer (HGT) via mobile genetic elements (MGE) such as plasmids, transposons and integrons (Bennett 2008; Van Hoek et al. 2011).

1.1.4. Horizontal Gene Transfer of Plasmids, Integrons and Other Mobile Genetic Elements

HGT is the transfer of foreign DNA between different bacterial species. It is responsible for much of the plasticity observed between strains and has been linked to the evolution of species. Large sections of DNA can be acquired over a short period of time creating remarkable diversity. Comparing whole genomes of *Escherichia coli* revealed that the genome consists of approximately 5000 genes, 3100 of which constitute the core genome (genes found in all *E. coli* genomes) whereas the others are accessory genes (genes found in some strains). However, for one *E. coli* only 10% of the genes are present in the pan-genome (all genes found in the species tested) (Land et al. 2015).

Although challenging the neo-Darwinian paradigm, HGT has an important role in the evolution of different species-specific features and characteristics such as antimicrobial resistance and virulence traits. Yet, once acquired, “natural selection” can select the genes providing survival advantage and spread across to the other organisms (Boto 2010). The movement of antimicrobial resistance (AMR) via HGT in the population provides a genetic pool with easy access for the acquisition and propagation of the preferred genes. Nevertheless, AMR has caused a paradigm

shift since the discovery of HGT of plasmids and other mobile genetic elements (MGEs) (Darmon and Leach 2014).

1.1.4.1. Conjugation, Transformation and Transduction

Bacteria use three main mechanisms for the exchange of DNA between cells: namely conjugation, transformation and transduction. All these mechanisms are not restricted to one MGE and some may use more than one mechanism to successfully transfer DNA. In addition, all of them have contributed to the acquisition and spread of AMR. Plasmid transfer by conjugation is, by far, the most superior mechanism found in bacteria. However, integrative and conjugative element (ICE) transfer is the most common mechanism of HGT and has extensively contributed to the development of MDR acquisition and transfer (Holmes et al. 2016a). Plasmids are extra-chromosomal genetic material with the ability to replicate autonomously and control their copy number to ensure constant inheritance. They have been classified according to their replicon (Carattoli 2009). Plasmids depends on their host for the regulation of replication and maintenance. They usually carry other non-essential genes that code for certain characteristics and features that may be useful in exploiting specific environmental conditions; for example resistance to antibiotic and toxic heavy metals (Gullberg et al. 2014). The classification scheme for plasmids is based on their replicon and their ability to maintain stable conjugation. Plasmids with the same, or closely related, replicons cannot be maintained together in a bacterial cell and are therefore termed as incompatible. However, the above statement is not entirely true and a few base pair mutations in the replicon region can result in the maintenance of same or closely related Inc group plasmids in the same cell (Toleman and Walsh 2011).

Plasmids can have a narrow host range or can be promiscuous, with a broad-host-range, with the ability of HGT between species from different genera. Although the role of plasmids in AMR spread is undisputable, promiscuous plasmids are regarded as very important tools in the HGT of AMR among different bacterial kingdoms (Toukdarian 2004; Carattoli et al. 2012). Some plasmids, termed as conjugative, are capable of self-transfer and carry genes required for conjugation, whereas mobilisable plasmids lack genes required for self-transfer but can use conjugative plasmids mate-forming machinery for horizontal transfer (Ramsay et al. 2016). Mate-pairing is initiated by the transfer gene which encodes the 'coupling protein' through a characteristic secretion type IV pilus formation. A single stranded nucleoprotein complex, termed a relaxosome, is formed by nicking the plasmid DNA at the origin of transfer by a relaxase enzyme (Holmes et al. 2016a). The relaxosome is transferred via pilus across the host cell membrane into the recipient and the complementary strands are synthesised in both cells (Figure 3). (Frost et al. 2005; Darmon and Leach 2014). Plasmids are often seen to reduce the fitness of the host and costly to maintain in the cell in the absence of selection pressure. Therefore, they are usually maintained in the cells in the presence of selection pressure. However, once a favourable environment (without selection pressure) is achieved, the plasmid may be lost from the new cells (Gullberg et al. 2014).

Other MGE such as ICE and conjugative Transposons (Tns) also use conjugation for DNA transfer (Holmes et al. 2016a). Tns and ICE are normally integrated into the host chromosome but can also initiate conjugation upon excision whereby the DNA is nicked and a single strand is transferred. Integrative and mobilisable elements (IMEs) lack the conjugation machinery but, like conjugative deficient plasmids, IMEs

also depend on the conjugation machinery of other MGEs for transfer (Blair et al. 2014).

Transformation is the uptake of free DNA from the environment which is then integrated into the new host (Blair et al. 2014). Some cells can be naturally capable of transformation, termed as “naturally competent”, whereas others only become competent under certain physiological conditions. The DNA acquired by transformation is incorporated into the host chromosome or plasmid by recombination and, therefore, it can only incorporate a slightly different form of the same gene and is an insignificant vehicle for HGT. Unlike transformation, transduction is virus, bacteriophages or phage mediated transfer of host DNA from one cell to another (Figure 3). The life cycle of a bacteriophage involves a lytic and lysogenic cycle (Lin et al. 2017). The lytic cycle uses the host machinery to replicate and releases new phages until triggering a lysogenic cycle, in which the phage DNA is integrated into the host chromosome as a prophage. The phage DNA is replicated along the host DNA and eventually excised to undergo lytic cycle. Random host DNA packaging during lytic cycle is termed as generalised transduction whereas specific nearby DNA packing during lysogenic cycle is termed specialised transduction. Generally, bacteriophages are very specific and usually can only invade a single or a few strains of a given species. Therefore they are not very important for HGT as can only transfer a gene from one strain of *E. coli* to exactly the same strain (Frost et al. 2005; Seed 2015).

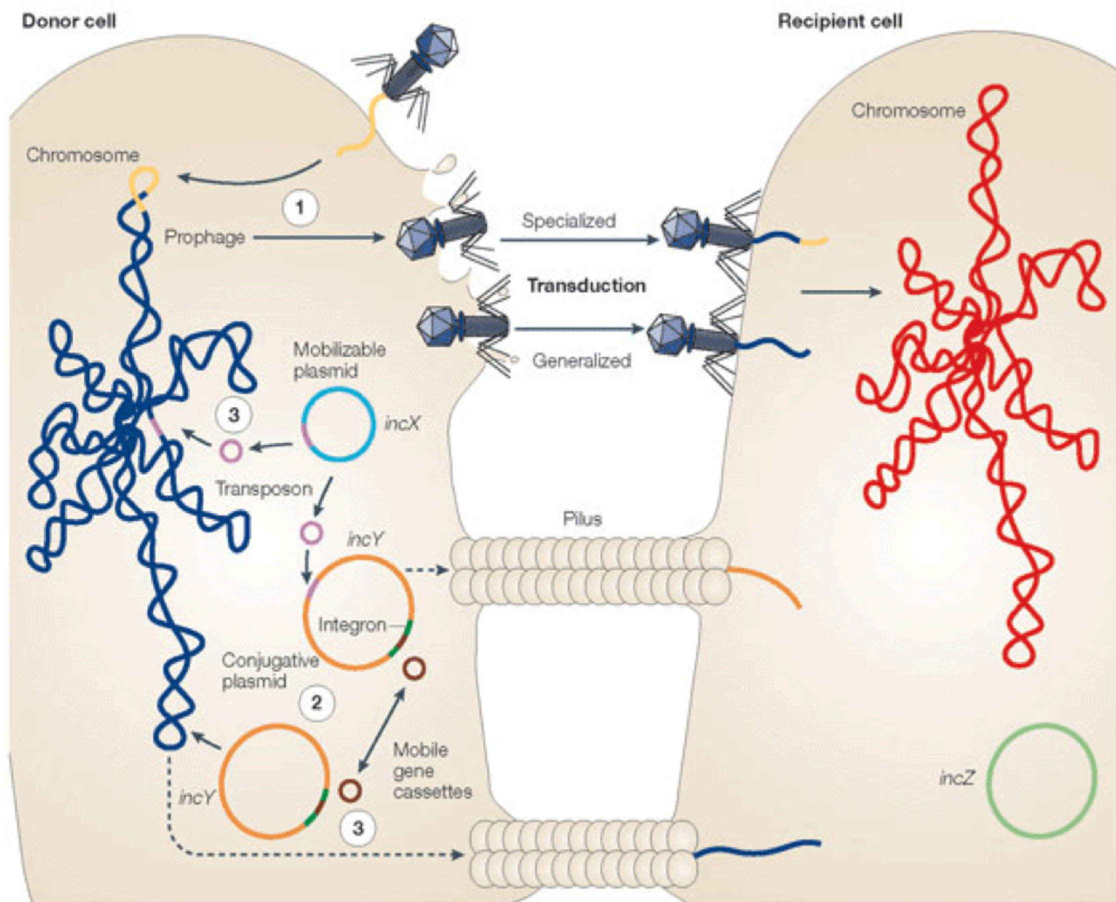


Figure 3. Mechanisms involved in HGT between bacterial cells. The image represents examples of HGT mechanisms; Transduction of phages (1), Conjugation of plasmid (2) and Transposition of integron gene cassettes (3). Reproduced with permission from (Frost et al. 2005).

1.1.4.2. Integrons

One of the important events in the HGT of Gram-negative AMR is regarded as the construction of class 1 integrons. It is believed to be a “three-step”, one-off evolutionary event that gave rise to the basic structure of the class 1 integron of today. Initially, the class 1 integrase, and its attachment site, were captured by Tn*5090*/Tn*402* transposon which attached to a fused *qacE* (quaternary ammonium

disinfectant resistance gene cassette) and *sul1* (sulphonamide resistance) to form the 3' conserved segment (3'CS). This was subsequently followed by the fusion of insertion sequence common regions-1 (IS*CR1)* to the 3' CS of *qacEΔsul1* providing a structure equipped with sophisticated machinery to capture and carry gene cassettes by site-specific recombination and play a central role in the carriage and dissemination of AMR (Toleman and Walsh 2011). Integrons are composed of a gene encoding for integrase (IntI), a recombination site (attI) and a promoter (Pc) (Deng et al. 2015). Gene cassettes are independent mobile units which contain an open reading frame (ORF) and a recombination site (attC) and are integrated into integrons by a site-specific recombination event between the attI site of the integron and the attC sites of the cassette. Excision of gene cassettes occur through a site-specific recombination event between two attC sites (Figure 4) (Vinué et al. 2011). Different types of integrons have been identified; however, class 1 integrons have received the most attention due to their increased association with AMR (Diene and Rolain 2014).

An integron may carry multiple resistance genes to various antibiotics. (Deng et al. 2015). Since the gene cassettes are generally promoter-less, they are transcribed from the Pc promoter. Different variants of Pc promoters have been identified and the location or distance from the genes has been shown to influence the strength of transcription (Jové et al. 2010; Wozniak and Waldor 2010). Although class 1 integrons are deficient in initiating their own transfer, they can be acquired by other MGEs for subsequent transfer (Diene and Rolain 2014). Other type of integrons such as superintegrons are described as chromosomal integrons and are very large, non-mobile elements accounting for approximately 3% of the host genome. Superintegrons usually carry genes (minimum array of 20) encoding for proteins

involved in a cell's environmental interaction. However, not all genes are expressed and the Pc promotor can only induce the expression of a few genes based on the proximity from the promotor and most of the genes remain silent (Darmon and Leach 2014).

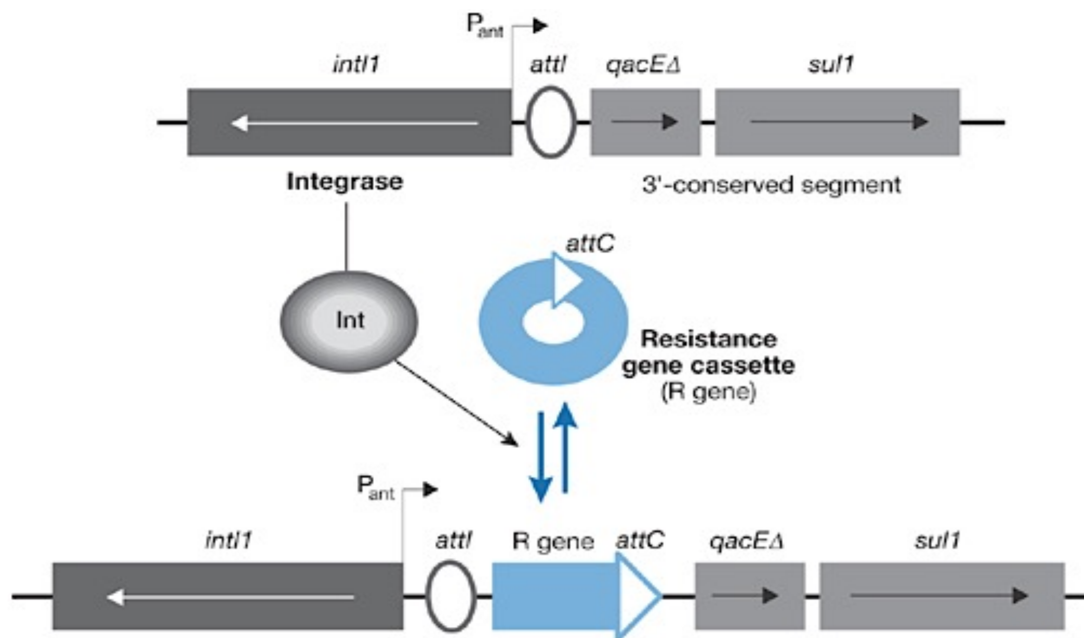


Figure 4. Class 1 integron gene. The figure shows schematic representation of the class 1 integron structure and the process involved in the integration and excision of gene cassette (Davies 2007).

1.1.4.3. Transposons, ISs and IS*CR*s

Transposons are termed as the jumping gene systems that can carry a variety of genes including AMR. They can move between plasmids and bacterial chromosomes and generally do not require DNA homology with the insertion sites (Darmon and Leach 2014). Transposons can be divided into composite and unit transposons. Unit transposons carry genes involved in transposition in addition to

other genes such as antibiotic resistance. Composite transposons are formed by insertion sequences (IS) flanking a section of DNA or gene (Dortet et al. 2014). IS are short DNA sequences with one or two ORFs usually coding for proteins required for transposition. Both IS and transposons are flanked by inverted repeats which, upon insertion into the host cell, can generate direct repeats (Dortet et al. 2014).They can have profound effects by inactivating, interrupting or altering the expression of adjacent genes upon insertion into a sequence. Furthermore, the presence of multiple transposons in high number in one cell could lead to genetic information loss by recombination events and increase the mutation. Therefore, some transposons use transposition immunity to limit the number of transposons to two copies per cell; for example, Tn*3*. However, increasing chances of mutation can be beneficial in certain circumstances, especially if the mutation provides a growth advantage in the presence of selection pressure; for example, IS*26* with no transposon immunity. The early distribution of bla_{SHV} gene from the chromosome of *K. pneumoniae* was attributed to the resistance gene acquisition by IS*26*. It is believed that *IS26* inserted on both sides of the resistance gene mobilising it on a plasmid to distribute among different strains and species (Toleman and Walsh 2011).

In some cases, IS may contribute to altering the expression of a gene by inserting partial or full -35 promoter sequence (Darmon and Leach 2014). For example, IS*Aba125* is commonly seen to enhance the expression of $bla_{NDM\text{-}1}$ by providing a -35 sequence motif of the promoter for $bla_{NDM\text{-}1}$ gene (Dortet et al. 2014). A different kind of transposition, termed as one-ended transposition, is also shown to be a powerful gene movement tool as it only requires one IS moving adjacent to the gene of interest to initiate transposition (Toleman and Walsh 2011).

Other transposable elements such as ICEs and IS*CR* are also associated with the acquisition and spread of AMR genes. ICEs are capable of transposition as well as conjugation (Garneau-Tsodikova and Labby 2015; Holmes et al. 2016a). IS*CR*, on the other hand, shares similarities with IS*91* and has gained a lot of attention due to the movement of gene clusters on plasmids and chromosomes. However, unlike IS and other Tns, IS*CR* do not contain IR but rather they have definite sequences involved in initiation and termination of movement found at either end of the element ie ori and ter sequences and movement is mediated by rolling circle replication. Occasionally the ter sequence is not recognised and in these cases replication and movement events mobilise several genes adjacent to the ter sequence. In IS*CR1* the ter sequence is missing entirely and, in this case, the IS*CR* normally mobilises adjacent sequences and can produce variant forms of the class1 integron called extended class one integrons (Toleman et al. 2006; Toleman and Walsh 2010).

ICEs are vastly more common than plasmids and chimeric i.e. formed by the fusion of a bacteriophage and a conjugative plasmid. They contain genes that behave both like bacteriophages and insert in the host chromosome for replication, and like plasmids and transfer to other cells through conjugation (Toleman and Walsh 2011; Zakharova and Viktorov 2015). Multiple ICEs have been identified carrying a vast array of AMR genes and, among them, one of the most detailed is the SXT/R391 family. SXT carries genes encoding resistance to sulphamethoxazole and trimethoprim and was initially identified in isolates of *Vibrio cholera* that were causing cholera endemic in India in 1992. Since then, SXT elements are commonly found in all clinical *V. cholera* serovars in Asia (Ryan et al. 2016). ICEs, typically, have three distinct modular structures which carry genes for integration and excision, conjugation, and regulation. The SXT/R391 family contains gene modules closely

related to that of IncA/C plasmids. This includes the shared similarity between the location and the amino acid sequence of the transfer genes and many other genes of unknown function, implying a common ancestor for both ICE and IncA/C plasmid. It is suggested that the insertion of a phage into the IncA/C backbone most probably gave rise to the early SXT/R391 ICE family (Toleman and Walsh 2011). SXT/R391 has 52 core genes which are highly conserved among the family. However, inter-ICE recombination events are frequent at the “hot-spot” regions. As a result, different variants are formed with various genes encoding for the antibiotic and heavy metal resistance among the many other functions (Zakharova and Viktorov 2015; Ryan et al. 2016).

1.2. Antibiotic Resistance

1.2.1. Antibiotic Inactivating Enzymes

Resistance mechanisms are usually defined as active or passive. Passive resistance mechanisms are independent of the selective pressure, for example the outer membrane of Gram-negatives, whereas active resistance can arise as a response to a specific evolutionary pressure to antibiotics, for example efflux pumps membrane proteins pumping antibiotic resistance out of the cell, modification of the antibiotic target and synthesis of antibiotic destroying enzymes. Antibiotic inactivation enzymes act on a specific molecule through hydrolysis or chemical group transfer (Blair et al. 2014).

Multiple variants of enzyme-catalysed drug inactivating agents have been identified producing resistance to different classes of antibiotics including β-lactams, aminoglycosides and macrolides. The enormous family of β-lactamases has thousands of variants, some of which can hydrolyse multiple different classes of β-lactam antibiotics such as penicillins, cephalosporins, carbapenems and monobactams (Nordmann 2014; Woodford et al. 2014). Other common clinical antibiotics such as aminoglycoside, chloramphenicol, Rifamycin and Fosfomycin are usually inactivated by enzymes through the addition of a chemical group to the susceptible sites of antibiotics (Spanogiannopoulos et al. 2012; Castañeda-García et al. 2013; Garneau-Tsodikova and Labby 2015).

Rifamycins are broad-spectrum antibiotics that target β-subunit of bacterial RNA polymerase. Resistance usually arises by amino acid mutation in the target.

However, enzymatic inactivation of the drug occurs through group transfer mechanisms of glycosylation, ADP ribosylation and phosphorylation, phosphotransferases and glycosylation (Spanogiannopoulos et al. 2012). Similarly, resistance to fosfomycin could occur through enzymatic inactivation by FosA, FosB or FosX which follows the same mechanism of adding a chemical group to inactivate the antibiotic but differ from each other by using different substrates to conduct the task (Castañeda-García et al. 2013). On the other hand, Chloramphenicol resistance is most often associated with the presence of chloramphenicol acetyltransferases. All these classes of enzymes are evolutionarily diverse and evolved by multiple genetic mutation events fuelled by the presence of selection pressure (Garneau-Tsodikova and Labby 2015).

1.2.2. Resistance to Aminoglycosides

Since 1943, aminoglycosides (AG) have been used widely as effective broad-spectrum antibiotics against life threatening conditions of Gram-negative and Gram-positive bacteria such as plague, tularemia, brucellosis and endocarditis (Ramirez and Tolmasky 2011). They can bind to the 30s subunits of RNA constraining the subunit assembly and inhibiting protein translation. Like most antibiotics, AG are derived from a natural source (*actinomycetes*) and hence the resistance exists in the environment. However, several other mechanisms have evolved, for example, inactivation of AGs by aminoglycoside modifying enzymes (AMEs), mutations of the ribosome by methyltransferase enzymes (RMTases), cell membrane impermeability by lipid modification and the formation of efflux pumps (Figure 5) (Doi et al. 2016).

AMEs are also commonly associated with β-lactam resistance and hence, limit the therapeutic options considerably (Garneau-Tsodikova and Labby 2015).

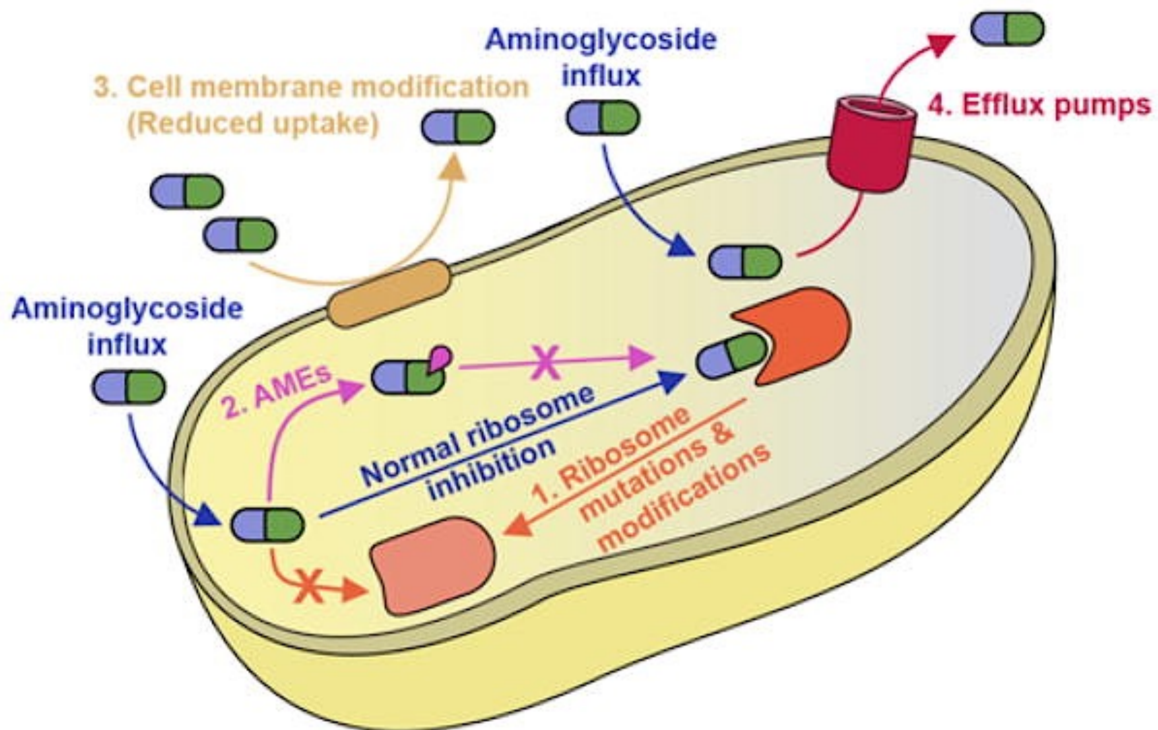


Figure 5. Mechanisms involved in AG resistance. The diagram is a schematic representation of the different intrinsic and acquired resistance mechanism to AG resistance. Reproduced from (Ramirez and Tolmasky 2011) with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry.

1.2.2.1. Ribosomal Mutation Ribosomal methylation

The first plasmid encoded RMTases gene, *RmtA,* was discovered in Japan from a *P. aeruginosa* strain in 1997 which was followed by subsequent identification of other

acquired genes displaying less than 30% sequence similarity to intrinsic naturally occurring RMTases in *actinomycetes* (Garneau-Tsodikova and Labby 2015). This suggests a possible evolution event occurring, not very recently, to give rise to RMTases enzymes. These enzymes can provide resistance by methylation of the 16S *rRNA* AG-binding site. They are further divided based on the position of the nucleotide they methylate: *ArmA, RmtA, RmtB, RmtC, RmtD1, RmtD2 RmtE, RmtF, RmtG* and *RmtH* methylate at the N7 position of nucleotide G1405 whereas *NpmA* methylate at the N1 position of A1408 (Galimand et al. 2012; Zhang et al. 2017). Currently, *RmtB* and *ArmA* are the most prevalent RMTases and have spread to various locations around the world. Interestingly, aminoglycosides are usually transferred with other ESBLs and carbapenmase genes and are commonly found on the same MGE (Doi et al. 2016).

1.2.2.2. AG Modifying Enzymes

AME are the most common type of resistance against AG. They are divided into three main families based on their mode of action, such as aminoglycoside phosphoryltransferase [APH], aminoglycoside acetyltransferase (AAC), and aminoglycoside nucleotidyltransferase (ANT) or also known as aminoglycoside adenytransferase (AAD) (Zhang et al. 2017). The nomenclature is somewhat confusing and two main systems exist. One of them uses a three-letter identifier based on the activity and amino acid position of enzyme modification. Additionally, for sub-classification, some may add further information such as a roman number to identify the resistant profile and lower-case letter as a unique identifier. For example, *aac(6')- ia* represents N-acetyltransferase with acetylation activity at position 6′ and

an identical resistance profile to aac(6′)-i enzymes (Doi et al. 2016; Zhang et al. 2017). Another system uses the gene name followed by a capital letter for modification site and a number as a unique identifier. For example, *aacB* is AME catalysing AG at position 6 and ANTs catalysing AME at position 6 is ANT(6) (Ramirez and Tolmasky 2011). For the purpose of this thesis the first nomenclature explained above will be used.

Among the AME, aac family of acetyltransferase enzymes consisting of *aac(1), aac(2'), aac(3) and aac(6')* is the most widely spread across the globe providing resistance against tobramycin, netilmicin, kanamycin, amikacin and gentamicin (Zhang et al. 2017). The genes are frequently isolated from Gram-negative species of *Acinetobacter, Enterobacteriaceae* and *Pseudomonas* and commonly associate with MGE such as plasmids and integrons. Among them, aac(6′) class of enzymes is the most common and clinically relevant type. In pseudomonas species *aac(6')-Ib* is frequently identified as a gene cassette within class 1 integrons (Ramirez and Tolmasky 2011; Garneau-Tsodikova and Labby 2015). Similarly, the genes responsible for adenylation of aminoglycosides by ANT or AAD enzymes, providing resistance against gentamycin and streptomycin, also exist as gene cassettes and are commonly carried on plasmids and transposons (Doi et al. 2016). For example, aadA type gene aadA15 is frequently found fused to the 3′ end of bla_{OXA-10}. On the other hand, aac(6′)-Ib is usually integrated within integrons carrying other ESBLs and more specifically CTX-M genes (Ramirez and Tolmasky 2011). Other AME enzymes, APH, possess inactivating phosphorylation activity against aminoglycosides such as kanamycin, neomycin and streptomycin. Although most of APHs are rarely found in clinical isolates, they are mostly associated with transposons and frequently manipulated as a molecular biological tool. For example, APH enzymes *aph (3')-II,*

aph(6)-Ic and *aph(3')-IIa* or *aphA-2* are associated with transposon Tn5 and *aph(3')-Ia* or *aphA-1* is part of Tn903 transposon (Zhang et al. 2017). Similarly, *aphA-1, aphA-2* and *aph(6)-Ic* are widely used as resistance marker genes in cloning vectors (Garneau-Tsodikova and Labby 2015).

1.2.3. β-Lactamases

β-Lactam antibiotics were the first antibiotics introduced in clinical settings with broad-spectrum activity and they are still the major component of modern anti-infective medicine. They act on bacterial cell walls which are composed of peptidoglycan strands with peptide side chains cross linked by penicillin binding proteins or trans peptidases. Trans-peptidases are also a substrate for β-lactam antibiotics and, upon binding with the antibiotics, they form inert enzyme intermediates, resulting in halting the cell wall synthesis and eventually cell lysis occurs (King et al. 2012). All β-lactam antibiotics have a common four membered β-lactam (four membered cyclic amide) ring attached to different functional groups which is regarded as fundamental for establishing the range of activity for a drug. Alteration in attached functional groups led to the production of different varieties of β-lactam antibiotics, many with broad-spectrum activity and valuable clinical use. Therefore, since their introduction, β-lactams antibiotics have been used widely and consequently resistance developed and spread rapidly (King et al. 2012; Zeng and Lin 2013). Resistance may arise by multiple mechanisms including the decreased expression of outer membrane proteins, efflux pumps and changes in the active site. However, the production of β-lactamases that bind to PBPs and hydrolyse the antibiotic is by far the most common mechanism of resistance found (Holmes et al. 2016b). Phylogenetic analysis, based on the sequence homology, revealed that β-

lactamases and the PBPs share a common ancestor. It has also been observed that β-lactamases can interact with both β-lactam antibiotics and trans-peptidases. However, the hydrolysed or inactivation of the antibiotic is much faster than the trans-peptidase enzymes (Zeng and Lin 2013; Meini et al. 2014).

Various β-lactamases have been identified and they are generally grouped using two classification systems. The Ambler system is based on the amino acid sequence homology whereas the Bush classification scheme uses biochemical and functional characteristics to group β-lactamases (Queenan and Bush 2007). The mechanism of hydrolysis is not completely understood but class A, C and D contain an active serine residue whereas class B metalo-β-lactamases (MBL) contain an active zinc residue which is believed to be involved in the hydrolysis of the β-lactam ring (Meini et al. 2014).

1.2.4. ESBLs: An Introduction

Antibiotic resistance to the first β-lactam antibiotic, penicillin, was identified in 1940 even before its introduction in clinical practice in 1945. Chain and Abraham noticed that an enzyme extract from *E. coli* was capable of destroying the properties of penicillin (Abraham and Chain 1940). In 1940, the mortality rate due to bacterial infections was huge with one clinical study from Boston reporting over 80% death due to bacteraemia associated with *S. aureus* (Skinner and Keefer 1941). However, the introduction of penicillin saw a reduction in bacterial infections and, as a result, penicillin use increased which led to the spread of plasmid mediated transferable resistance and ESBLs (Tumbarello et al. 2012). The most well-known first ESBLs

were derived from TEM-1 (named after the patient its isolated from, Temoniera) and SHV-1 (for sulphydryl variable). While SHV-1 was chromosomally encoded, TEM-1, being on a plasmid, was soon transferred to other species of bacteria (Datta and Kontomichalou 1965; Brun-Buisson et al. 1987). After the introduction of oxyimino-cephalosporins in the 1980s, SHV and TEM variants, expressing resistance to these expanded-spectrum β-lactam antibiotics, started to emerge and spread rapidly due to the presence of the resistance gene on the plasmids (Kliebe et al. 1985; Sirot et al. 1987; Paul et al. 1989). ESBL enzymes provide resistance against penicillin, first, second, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) and are inhibited by β-lactamase inhibitors such as clavulanic acid (Vasoo et al. 2015).

1.2.4.1. CTX-M General Characteristics and Properties

Among all the ESBLs, CTX-M is by far the most important public health concern because of its dramatic spread over a very short period. Although being carried by multiple species, the acquisition of *bla*$_{CTX\text{-}M}$ by pathogens such as *K. pneumoniae* and *E. coli* was the most important event. It's not only limited to nosocomial infections but spread to the community and frequently isolated from the environment and animals (Pitout and Laupland 2008). CTX-M ESBLs only share 40% identity with other ESBLs of TEM and SHV. Both SHV and TEM ESBLs arose from point mutations in their progenitor genes whereas CTX-M enzymes originated from several different *Kluyvera* species (Sirot et al. 1987; Bradford 2001). Some of the chromosomally encode cefotaximases from *Kluyvera spp*. such as KLUA-2, c-CTX-M-2, c-CTX-M-3 from *K. ascorbata*, KUUY-1, KLUG-1 and c-CTX-M-78 from *K.*

georgiana and c-CTX-M-37 and KLUC-2 from *K. cryocrescens* show 87.6-100% to plasmid encoded *bla*$_{CTX-M}$ genes and, hence, each cluster can be traced back to its progenitor gene of *Kluyvera spp*. (Zhao and Hu 2013). So far, CTX-M enzymes have been classified into 7 clusters based on amino acid sequence homology, namely CTX-M-2 (16 members), CTX-M-3 (42 members), CTX-M-8 (3 members), CTX-M-14 (38 members), CTX-M-25 (7 members), CTX-M-64 (2 members) and CTX-M-45 containing only one member (Zhao and Hu 2013; Lahlaoui et al. 2014). All members of the clusters are also very similar and share more than 95% of the sequences (Lahlaoui et al. 2014).

1.2.4.2. Epidemiology and Genetics

Shortly after their introduction in the mid 1980s, there was some sporadic incidence of CTX-M associated outbreaks in UK, Argentina, China and Europe, mainly associated with *K. pneumoniae* and *E. coli* (Radice et al. 2002; Brenwald et al. 2003; Livermore and Hawkey 2005). However, they are the most common type of ESBL isolated nowadays and spread across the whole world in many different species of Enterobacteriaceae. Yet, *E. coli* and *K. pneumoniae* remain the most prevalent *bla*$_{CTX-M}$ carriers. Surprisingly, different variants of *bla*$_{CTX-M}$ show an association with various geographical locations; however, *bla*$_{CTX-M-15}$ is the most dominant type worldwide (Figure 6) (Zhao and Hu 2013). The immense spread of blaCTX-M is mainly attributed to its genetic plasticity and acquisition by a pathogenic strain of *E. coli,* ST131. The gene is linked with an upstream IS*Ecp1* which, besides providing a promotor for its increased expression, also facilitates transposition to integrons and mobile plasmids predominantly IncF group (FIA, FIB and FII) plasmids. These

plasmids are exclusive to the family of Enterobacteriaceae and mostly associated with *E. coli* (Lahlaoui et al. 2014). Since F plasmid is thought to have low fitness cost, it may maintain the antibiotic resistance gene even in the absence of selection and, as a result, may persist even in the absence of selection pressure and contribute to the increased distribution (Woodford et al. 2011; Zhao and Hu 2013). The first IS*Ecp1* associated $bla_{CTX\text{-}M\text{-}15}$ was isolated from a hospital in New Delhi in 1999 and since then has been commonly detected in many other epidemiologically prevalent CTX-M enzyme variants such as 1, 3, 10, 13, 14, 15, 17, 19, 24, 27, 32, 54, 62 (Zhao and Hu 2013; Hawkey 2017)

CTX-M type enzymes are widely spread across the world and certain geographical locations are represented by a very large prevalence rate; for example,in countries of Asia and specifically South-East Asia, the Middle East and Africa. The immense spread of $bla_{CTX\text{-}M}$ in those regions is mainly attributed to inefficient sewage infrastructure, sanitation and public health facilities (Hawkey 2017). CTX-M enzymes have contaminated the environment and have also been isolated from animal and food sources (Zhao and Hu 2013; Hawkey 2017). The prevalence rate has increased considerably since the first identification and a large number of the human population carry $bla_{CTX\text{-}M}$ in their gut, serving as reservoirs. India alone represents approximately 60% of faecal carriage for $bla_{CTX\text{-}M\text{-}15}$ in the community (Woerther et al. 2013). Population dynamics are also believed to have a role in the worldwide distribution of CTX-M genes. A study by Tham et al. demonstrated that travelling to those regions can result in the acquisition of $bla_{CTX\text{-}M}$ compared to travel inside Europe. For example, 78% patients returning from India, 50% from Egypt and 22% from Thailand were carrying ESBLs compared to 3% of the patients who had travelled in Europe.

Among those ESBLs, 90% of the genes were of CTX-M type belonging to Group 1 and followed by Group 9 (Tham et al. 2010).

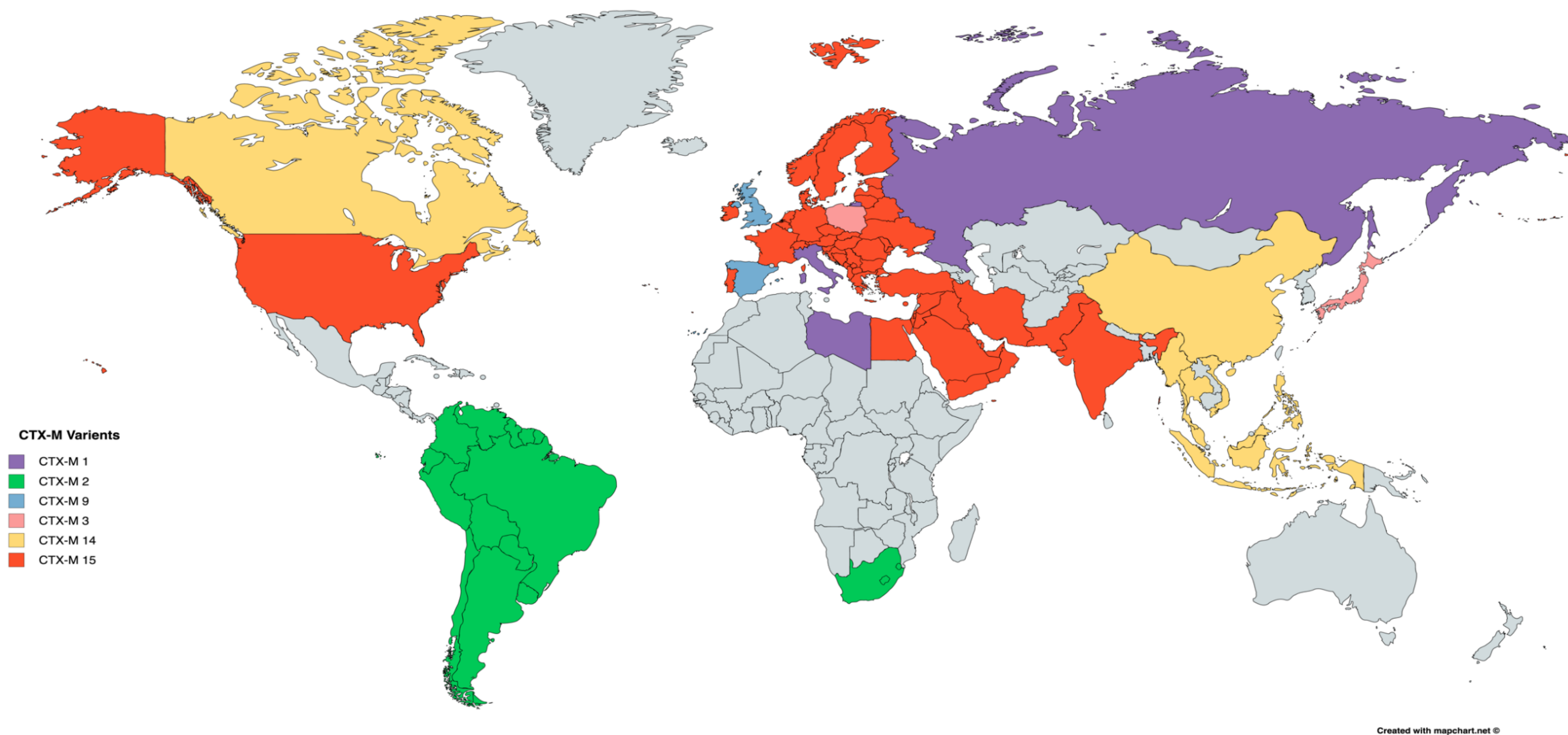


Figure 6. The spread of most common variants of CTX-M enzymes in different regions of the world. The map shows the distribution of the most prevalent CTX-M enzyme variants around the world. CTX-M-1 is most prevalent in Italy, Libya and Russia; CTX-M-2 in South America and South Africa; CTX-M-3 in Japan, South Africa and Poland; CTX-M9 in Spain and England; CTX-M-14 in Canada and South-East Asia; CTX-M-15 in Europe, USA, Middle-East and India. Map created with Mapchart.net and the data derived from (Zhao and Hu 2013; Lahlaoui et al. 2014).

1.2.4.3. Clinical Significance

CTX-M enzymes are presented as efficient hydrolytic agents of cefotaxime with MICs in the range of 64 μg/ml. Aztreonam MICs are usually variable and although ceftazidime MICs are generally in the susceptible range of 2-8 μg/ml, some might show higher MICs of up to 256 μg/ml (Zhao and Hu 2013). Furthermore, it is not uncommon that some organisms may exhibit an altered antibiotic profile due to the presence of other ESBLs producing enzymes such as SHV and TEM or AmpC-type β-lactamases or even insensitivity to inhibitor due to the loss of membrane porins (Livermore 2012; Vasoo et al. 2015). Theoretically, a combination of antibiotic and inhibitor or quinolones for the treatment would be recommended; however, they are not advised due to the increased risk of treatment failure. $bla_{CTX\text{-}M}$ containing plasmids may also carry multiple resistance genes against other antibiotics. For example, in the UK, an *E. coli* ST131 strain containing $bla_{CTX\text{-}M\text{-}15}$, also carries resistance to other antibiotics such as aminoglycosides (*aac6'-Ib-cr, aadA5*), macrolides (*mph(A)*), chloroamphenicol (*catB4*), tetracycline (*tet(A)*), trimethoprim (*dfrA7*) and sulfonamide (*sul1*) resistance and other β-lactamases such as $bla_{OXA\text{-}1}$ and $bla_{TEM\text{-}1}$ (Woodford et al. 2011; Zhao and Hu 2013). A study in Sweden on 198 *E. coli* showed that 68% percent of the $bla_{CTX\text{-}M}$ isolates were resistant to several other non-β-lactam antibiotics such as trimethoprim, trimethoprim–sulfamethoxazole, ciprofloxacin, gentamicin, and tobramycin (Balkhed et al. 2013). Therefore, due to the possibility of therapeutic failure, carbapenems, and more specifically meropenem, is regarded as the treatment of choice. As a result, an increase use of carbapenem antibiotics is observed which in turn is giving rise to the widespread dissemination of carbapenemases (Laxminarayan et al. 2013).

1.3. Carbapenemases

1.3.1. Carbapenamases: An Introduction

Carbapenem antibiotics retained good activity against all ESBLs for over 20 years and, even now, most ESBL-associated infections rely on carbapenem for their treatment. However, the extensive use and abuse of carbapenem is fuelling the spread of carbapenem resistance in the form of drug inactivating carbapenemases. Currently, carbapenemases are distributed worldwide and KPC, NDM MBL, OXA-48 and Verona integron-encoded (VIM) MBL remains to be the most common types (Kitchel et al. 2009; Kumarasamy et al. 2010). Although, all carbapenemases can hydrolyse penicillin, 1st and 2nd generation cephalosporin and carbapenem, their activity differs by the variable hydrolysis profile to the 3rd and 4th generation cephalosporin and inhibition by β-lactam inhibitors. For example, class B MBLs such as NDM and VIM type enzymes can generally hydrolyse all β-lactam antibiotics, except aztreonam; class A KPCs can hydrolyse all β-lactam antibiotics but are inhibited by clavulanic acid; class D oxacillinases of OXA-48 type can hydrolyse all β-lactam antibiotics except 3rd and 4th generation cephalosporin and show partial activity against carbapenem and no inhibition by β-lactamase inhibitors (Kumarasamy et al. 2010; Nordmann 2014). However, the differential antibiotic profiles may be represented by a minority of resistant strains in vivo as they are usually accompanied by other genes such as $bla_{CTX\text{-}M}$ and AMpc-type β-lactamases providing resistance to the otherwise sensitive strain. Additionally, like CTX-M, carbapenemases are also commonly associated with other non-β-lactams antibiotic

resistance such as quinolones and aminoglycosides and, hence, the treatment options are very limited (Livermore 2012; Nordmann 2014).

1.3.2. General Characteristics and Properties of OXA Type Carbapenemases

The first carbapenem-hydrolysing oxacillinase gene, *bla*$_{OXA-23}$, was reported from Edinburgh in the United Kingdom in 1985. This was soon followed by scattered reports of other carbapenem hydrolysing OXA enzymes from Europe (Scaife et al. 1995). Based on sequence homology alone, class D carbapenemase genes can be divided into different clusters with, *bla*$_{OXA-48}$-like genes being the most significant (Evans and Amyes 2014). The *bla*$_{OXA-48}$ gene was initially identified from a *K. pneumoniae* isolate recovered from a patient in Istanbul, Turkey (Poirel et al. 2004). The resistance gene was carried on a plasmid and showed greater amino acid and structural homology with other non-carbapenem hydrolysing oxacillinase genes (Laurent et al. 2004). Structural crystallography of the OXA-48 enzyme revealed that the carbapenemase activity is a result of minor conformational changes in the active site region causing changes in the tertiary structure and thus creating a favourable hydrophilic environment for the hydrolysis of carbapenems (Docquier et al. 2009). However, very little carbapenem resistance is observed when only *bla*$_{OXA-48}$ is responsible and a preferential greater activity against imipenem is recorded (Poirel et al. 2004). Over the years, other variants of *bla*$_{OXA-48}$ have been reported such as *bla*$_{OXA-162}$, *bla*$_{OXA-163}$, *bla*$_{OXA-181}$, *bla*$_{OXA-199}$, *bla*$_{OXA-204}$, *bla*$_{OXA-232}$, *bla*$_{OXA-245}$ and *bla*$_{OXA-247}$. Usually, the variations involve only a few amino acid substitutions and the enzymes have similar hydrolytic profiles which also includes *bla*$_{OXA-181}$ However, *bla*$_{OXA-163}$ shows very weak carbapenem hydrolysis (Docquier et al. 2009; Poirel et

al. 2011) and shows a more similar hydrolytic profile to ESBLs which is usually represented by greater activity against ceftazidime, aztreonam, cefotaxime and cefepime (Poirel et al. 2004). Among all variants of OXA-48 type carbapenemases, *bla*$_{OXA-48}$ and *bla*$_{OXA-181}$ have been distributed worldwide and commonly isolated from Acinetobacter and Enterobacteriaceae species, mainly *K. pneumoniae* and *E. coli* (Evans and Amyes 2014).

1.3.2.1. Epidemiology and Genetics

Like *bla*$_{CTX-M}$, *bla*$_{OXA-48}$ variants also show geographical associations,for example *bla*$_{OXA-48}$ is frequently isolated in European countries especially France, UK, Germany, Belgium and Turkey, the Middle East and North African countries whereas, *bla*$_{OXA-181}$ is more prevalent in Asia specifically India, China Pakistan and Bangladesh (Figure 7) (Lee et al. 2016). The acquisition of *bla*$_{OXA-48}$ and *bla*$_{OXA-181}$ by a single plasmid may have contributed to its worldwide spread, predominantly in Enterobacteriaceae (Scaife et al. 2012). Usually, *bla*$_{OXA-48}$ and *bla*$_{OXA-181}$ are carried by a 62.3 kb IncL/M-type plasmid, pOXA-48a. The gene itself sits on a composite transposon and can be flanked by various types and combinations of insertion sequences which might aid in its mobilization and may also provide a promoter for stronger gene expression (Poirel et al. 2004; Aubert et al. 2006). For *bla*$_{OXA-48}$, three different combinations are identified such as Tn*199* (flanking of *bla*$_{OXA-48}$ by IS*99* on both sides), Tn*199.2* (IS*1R* inserted upstream of IS*1999* or Tn*199.3* (IS*1R* flanking IS*1999* upstream and downstream of *bla*$_{OXA-48}$ (Laurent et al. 2004; Aubert et al. 2006; Giani et al. 2012). The chimeric IS*1R*/IS*1999* element provides a hybrid promoter believed to be responsible for a slightly stronger hydrolysis of imipenem

compared to the other variants (Carrër et al. 2008). On the other hand, other *bla*$_{OXA-48}$ variants have shown an association with additional insertion sequences, for example, *bla*$_{OXA-163}$ gene is commonly associated with IS*Ecl4* element whereas *bla*$_{OXA-181}$, *bla*$_{OXA-204}$ and *bla*$_{OXA-232}$ genes are associated with different arrangements of IS*Ecp1* elements (Potron et al. 2011; Potron et al. 2013).

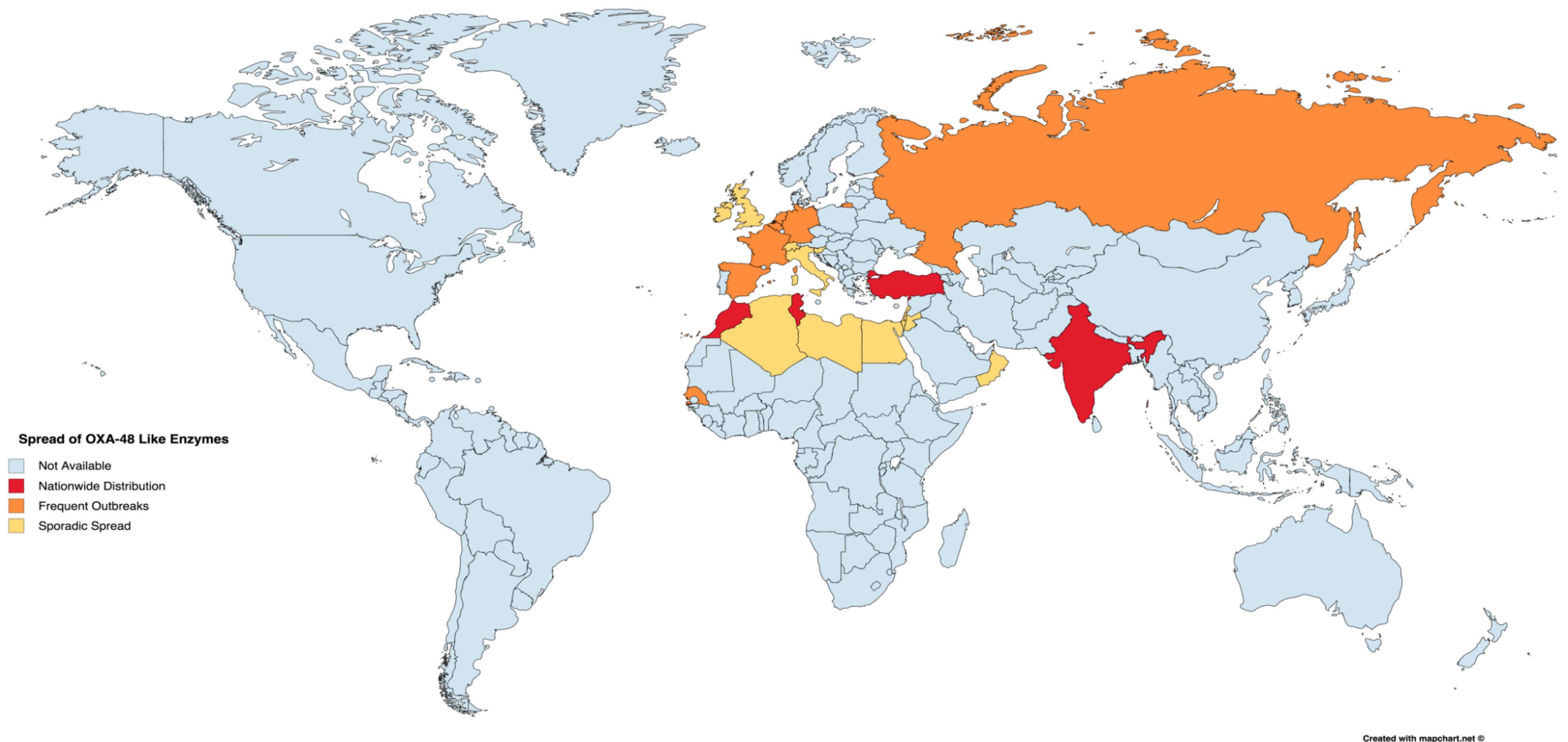


Figure 7. The prevalence of OXA-48 like carbapenemases around the world. The Map shows the countries where OXA-48 like enzymes are more prevalent. It is most commonly spread in India, Turkey, Morocco and Tunisia. Frequent outbreaks occur in Senegal, Spain, France, Netherland, Germany and Russia whereas sporadic appearances have been reported in Algeria, Libya, Switzerland, UK, Ireland, Oman, Greece, Israel, Jordan and Kuwait. Map created with Mapchart.net and the data derived from (Nordmann 2014).

1.3.2.2. Clinical Significance

The past few years have seen a gradual increase in the occurrence of OXA-48-like enzymes. There has been multiple outbreaks of $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-181}}$ mostly in European countries such as Turkey, United Kingdom, France Spain, Netherland, Belgium and Germany (Poirel, Potron, et al. 2012; Evans and Amyes 2014; Lee et al. 2016). Perhaps one of the reasons for this could be the difficulty in detection of these enzymes due to the reduced susceptibility to carbapenem and, in some cases, greater resistance to carbapenem and broad spectrum cephalosporin due to the co-existence of OXA-48 enzymes with other ESBLs (Evans and Amyes 2014). This phenomenon of $bla_{\text{OXA-48}}$ carrying bacteria presents a complicated scenario where its detection becomes difficult. It is also believed that the variable carbapenemase' activity could lead to underestimating the actual prevalence rate of these enzymes and the genes may be present at a higher frequency than anticipated. This also makes treatment very difficult and appropriate therapy would demand a much more thorough analysis on case-by-case basis. For treatment of $bla_{\text{OXA-48}}$ associated infections, carbapenem is not regarded as a suitable therapy but rather a combination therapy of non-carbapenem antibiotics and 3rd or 4th generation cephalosporin is advised. However, since bla_{OXA-48} is commonly co-expressed with other ESBLs, the treatment options are very limited and a different combination of antibiotics in addition to β-lactam inhibitors is advised (Dautzenberg et al. 2014; Bakthavatchalam et al. 2016).

1.3.3. General Characteristics and Properties of KPC-type Carbapenamases

The first report of KPC-1 enzymes emerged from North Carolina, USA in 1996 from a *K. pneumoniae* isolate (Yigit et al. 2001). These enzymes were carried on plasmids and were always associated with *K. pneumoniae*. However, by 2005, the spread of *bla*$_{KPC}$ extended throughout North America and started to appear in the other regions of the world (Bratu et al. 2005). Additionally, KPC did not remain limited to *K. pneumoniae* but also disseminated into other Enterobacteriaceae such as *Citrobacter freundii, E. coli, Enterobacter aerogenes, Enterobacter cloacae, Enterobacter gergoviae, Klebsiella oxytoca, Proteus mirabilis, Salmonella enterica*, *Serratia marcescens* and non-Enterobacteriaceae such as *P. aeruginosa, Pseudomonas putida* and Acinetobacter spp. (Arnold et al. 2011). However, *K. pneumoniae* and *E. coli* remain the most commonly isolated *bla*$_{KPC}$ carriers. Currently, there are 24 variants of KPC enzymes which differ by a few amino acids in the sequences (*bla*$_{KPC-1}$ and *bla*$_{KPC-2}$ are identical) (www.lahey.org). The antibiotic hydrolysis profiles of different variants are similar and are generally characterized based on their ability to hydrolyse penicillin, cephalosporin, aztreonam, carbapenem and inhibition by clavulanic acid (Pitout et al. 2015). The structure of KPC enzymes also shows similarities to SHV-1 and very subtle changes in the active site are responsible for generating carbapenemase activity (Nguyen et al. 2016). Although, *bla*$_{KPC}$ variants are not very strong contenders of carbapenem hydrolysis, they are still considered a major health risk because they can often go undetected by routine screening, have a potential to spread and might also co-exist with other resistance mechanisms. Furthermore, they are often associated with nosocomial spread and outbreaks (Lee and Burgess 2012; Lee et al. 2016).

1.3.3.1. Epidemiology and Genetics

Among all the variants of KPC, *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ are the most prevalent types. The first 10 years of the 21st century saw a rapid increase in KPC enzymes, at least in America. According to a report by CDC, the prevalence of KPC associated nosocomial infection in a New York, USA based medical centre increased from 9% in 2002 to 18% in 2004 and 38% in 2008 (Bratu et al. 2005; Kitchel et al. 2009). They have spread across multiple geographical locations; with endemicity in USA, Columbia, Brazil, Greece, Argentina, Italy and Chia (Figure 8) (Albiger et al. 2015). More recently, the highest prevalence rate is recorded in Greece where, according to the Greek surveillance system, the occurrence of KPC associated bacteraemia is at 40% on a national level. However, certain cities may represent a lower or higher prevalence rate depending on the population (Munoz-Price et al. 2013). For example, higher frequencies of up to 65% were recorded in the two larger teaching hospitals of Athens and Patras, Greece where the predominant strain was identified as ST258 (Giakkoupi et al. 2011; Albiger et al. 2015). Indeed, ST258 is associated with the successful spread of KPC enzymes and is usually the most dominant strain type frequently isolated in outbreaks (Pitout et al. 2015). A study report published in 2009 by CDC analysed the molecular epidemiology of KPC-producing *K. pneumoniae* isolates that were received in a period of 12 years (1996-2008) from 18 states of America, Israel and India. Their results suggest 70% of the KPC *K. pneumoniae* belonged to ST258 lineage and were carried on a variety of plasmids which were transferrable to *E. coli* (Kitchel et al. 2009; Livermore 2012). Furthermore, the gene is usually carried on a Tn*4401*, which has been shown to be capable of transposition at higher frequencies (Cuzon et al. 2011). The KPC gene is sandwiched between the two insertion sequences, IS*Kpn6* and IS*Kpn7*, which are

flanked by target site duplications on both sides (Diene and Rolain 2014). This genetic scaffold structure of bla_{KPC} is generally conserved and considered to have played an important role in its dissemination (Naas et al. 2008; Nordmann 2014). Moreover, the bla_{KPC} plasmids can be easily transferred to other Gram-negative organisms and may carry genes responsible for providing resistance to other non-β-lactam antibiotics such as quinolone (*QnrA* and *QnrB*) and aminoglycoside (*rmtB*) and, therefore, challenging antibiotic therapy (Lee and Burgess 2012; Zhou et al. 2015).

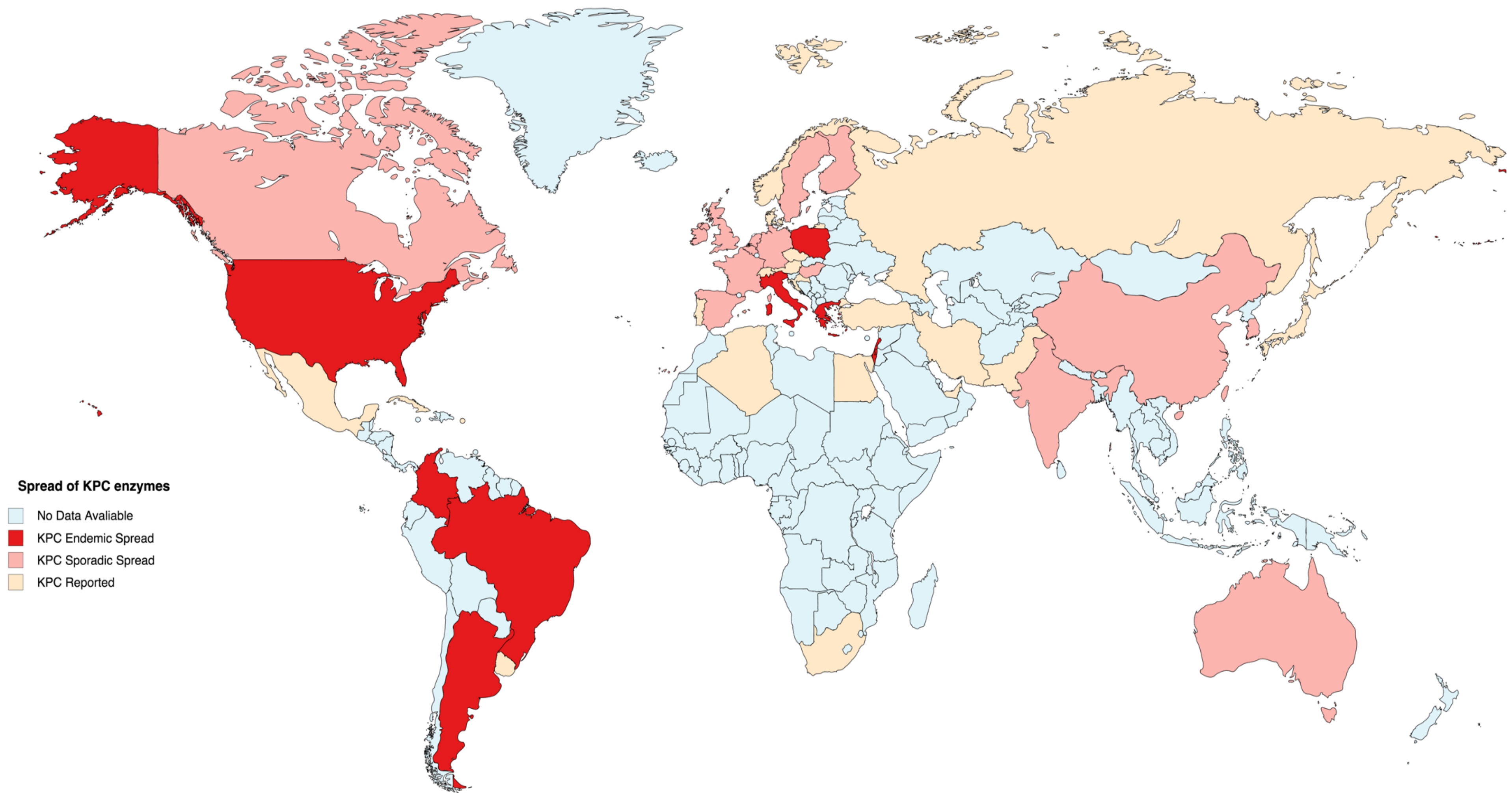


Figure 8. Distribution of KPC carbapenemases around the world. KPC-producing *K. pneumoniae* are endemic in USA, Colombia, Brazil, Argentina, Italy, Greece, Poland and Israel. Sporadic spread is observed in China, Taiwan, Canada, Spain, France, Belgium and Netherlands. All other regions, highlighted in yellow, have reported individual cases of KPC. Map created with Mapchart.net and the data derived from (Lee et al. 2016).

1.3.3.2. Clinical Significance

bla$_{KPC}$ is associated with a high mortality rate and presents a very serious health problem which is on the rise globally (Albiger et al. 2015; Lee et al. 2016). KPC enzymes are difficult to detect due to the heterogeneous antibiotic hydrolysis profile either caused by KPC or the presence of other resistance mechanisms or genes. Additionally, the routine automatic detection systems are reported to be problematic which further complicates the issue. Any misinterpretation or delay in the therapy can contribute to increased chances of treatment failure (Lee et al. 2016). A surveillance study in 2005, analysing outbreaks in two hospitals in Brooklyn, USA, observed a mortality rate of 47% with *bla*$_{KPC}$ associated bacteraemia (Bratu et al. 2005). Further studies have shown similar mortality rates such as 41.6 % in Italy, 65% in Greece, 42.9% in Brazil and 50% in Israel (Borer et al. 2009; Tumbarello et al. 2012; Fraenkel-Wandel et al. 2016; Rossi Gonçalves et al. 2016). Although it is evident that certain factors contribute to the increased mortality rate such as being immuno-compromised or having underlying medical conditions, long medical stay, transplantation, mechanical ventilation and previous antibiotic treatment (Lee and Burgess 2012). However, prompt actions to properly diagnose and treat *bla*$_{KPC}$ associated infections are essential to reduce mortality and morbidity (Munoz-Price et al. 2013). A comparison of different cases in the literature by Lee and Burges showed that combination therapy may be the treatment of choice for *bla*$_{KPC}$ associated infections since increased treatment failures were observed with monotherapy of carbapenem (60%) and polymyxin (73%) and comparatively good results (25% treatment failure) were achieved with a combination therapy regime with carbapenem (carbapenem with polymyxin/aminoglycosides/+β-lactam inhibitor)

or polymyxin (polymyxin with carbapenem/aminoglycosides or combination tigecycline and colistin (Lee and Burgess 2012).

1.3.4. General Characteristics and Properties of NDM-type Carbapenamases

NDM-1 enzyme was described for the first time in 2009 from a Swedish patient of Indian origin who was hospitalized in Sweden returning from New Delhi, India. (Yong et al. 2009). Shortly after, it was detected from different sites in India, Pakistan, Bangladesh and 17 hospitals in the UK and the region was termed as an epicentre for *bla*$_{\text{NDM-1}}$ (Al. 2010; Timothy R. Walsh et al. 2011), although there has been some controversy over the origin of the gene, as Balkan states also reported cases of *bla*$_{NDM-1}$ at the same time (Livermore et al. 2011). Initially identified in *K. pneumoniae*, *bla*$_{\text{NDM}}$ has spread to many other bacterial species. However, *K. pneumoniae*, *E. coli* and *Acinetobacter spp.* remain the most frequently isolated organisms (Livermore et al. 2011; Dortet et al. 2014). Different studies have also identified *bla*$_{\text{NDM}}$ in Acinetobacter spp. from clinical samples isolated from India collected in 2005 suggesting Acinetobacter as the origin of the gene. The enzymes do not share high similarity with other carbapenemases genes and only show ~32% amino acid similarity with VIM-1 and VIM-2 enzymes (Yong et al. 2009). So far, 16 variants of NDM gene have been identified, with only a few amino acid differences and similar antibiotic hydrolysis profiles (www.lahey.org) (Diene and Rolain 2014; Zmarlicka et al. 2015). NDM enzymes are active against a broad spectrum of substrates and resistant to all β-lactam antibiotics except monobactams. In addition, like other carbapenemases, *bla*$_{\text{NDM}}$-positive bacteria almost always carry *bla*$_{\text{CTX-M-15}}$ and are commonly associated with other β-lactam and non-β-lactam antibiotic

resistance mechanisms and close association with 16s methylase genes giving pan aminoglycoside resistance and hence complicating detection and therapy regime (Zmarlicka et al. 2015).

1.3.4.1. Epidemiology and Genetics

The spread of *bla*$_{NDM}$ can be regarded the most rapid ever seen for any carbapenemases. India, Pakistan and Bangladesh remain the hotspot regions and the increased distribution and spread in those regions is attributed to poor sanitation and waste treatment, in addition to increased carbapenem use (Dortet et al. 2014; Voulgari et al. 2014; Zmarlicka et al. 2015). Similarly, European countries with strong ties to the south Asian countries are also presented with higher incidences of *bla*$_{NDM}$. For example, Just a year after their first description, *bla*$_{NDM}$ was the predominant carbapenemase-producing Enterobacteriaceae accounting for 44 % of the carbapenemase producers (Figure 9) (Kumarasamy et al. 2010). A global systemic case review identified the majority (63%) of reports with *bla*$_{NDM-1}$ had exposure or links with south Asia (Lee et al. 2016). In China, *bla*$_{NDM}$ was only limited to *Acinetobacter spp.* until 2013 at a very low incidence rate of less than 1.5% (Chen et al. 2011; Yang et al. 2012). However, in 2013 the first *E. coli* and *K. pneumoniae* containing *bla*$_{NDM}$ was isolated and since then the prevalence seems to have escalated. A more recent study, in 2014 from China, reported the carriage of *bla*$_{NDM}$ in 14.8% of clinical faecal samples (Hu et al. 2013; Liu et al. 2013; Wang et al. 2014). In Hennai province, China, *bla*$_{NDM}$ accounted for 33.3% of all the carbapenem resistance observed (Qin et al. 2014). Moreover, *bla*$_{NDM}$ has increasingly been isolated from the environment and animal sources (Wang et al. 2017). Unlike *bla*$_{KPC}$, the spread of *bla*$_{NDM}$ cannot be attributed to a specific species, rather its ability to

colonize a wide variety of host organisms including many different environmental bacteria which may have no clinical relevance. It is generally perceived that the gene originated from an environmental bacterium, presumably *Acinetobacter baumanii,* by a fusion event between a progenitor MBL gene and aminoglycoside resistance gene *aphA6.* An alignment of bla_{NDM-1} and *aphA6* genes shows that IS*Aba125* is inserted at a point upstream of *aphA6* and exhibits complete identity to the upstream region of bla_{NDM-1} continuing to the first 19 bp within the bla_{NDM-1} gene.

Some STs are more commonly associated with bla_{NDM} than others, for example *E. coli* ST101 and *K. pneumoniae* ST11 or ST14 and, to a lesser extent, ST149, ST231 and ST147 (Giske et al. 2012; Toleman et al. 2015). Nonetheless, in all species and STs the immediate genetic background of bla_{NDM} is generally highly conserved with IS*Aba125* at the 5′-end providing promoter for bla_{NDM} and a gene encoding resistance to the anticancer drug bleomycin (ble_{MBL}) at the 3′-end (Diene and Rolain 2014; Dortet et al. 2014). In *A. baumannii*, bla_{NDM} is placed in the middle of the IS*Aba125* and ble_{MBL} composing a Tn*125* composite transposon. In Enterobacteriaceae and *P. aeruginosa*, two different versions of a truncated form of this composite transposon also exist where either IS*Aba125* or ble_{MBL} is presented as full or truncated gene (Dortet et al. 2014). However, in all known variants of bla_{NDM}, IS*Aba125* provides a strong promotor which is highly conserved. Furthermore, bla_{NDM} has been linked with multiple plasmids of broad-host-range such as IncA/C, IncFII, IncN, IncH, and IncL/M types but IncA/C remains the most common type (Giske et al. 2012; Pitout et al. 2015). IncA/C has shown to carry other genes conferring resistance to multiple non-β-lactam antibiotics such as aminoglycodes (RmtA and RmtC) and quinolones (QnrA) and β-lactam antibiotic cephalosporin (CMY-type β-lactamases) (Pitout et al. 2015).

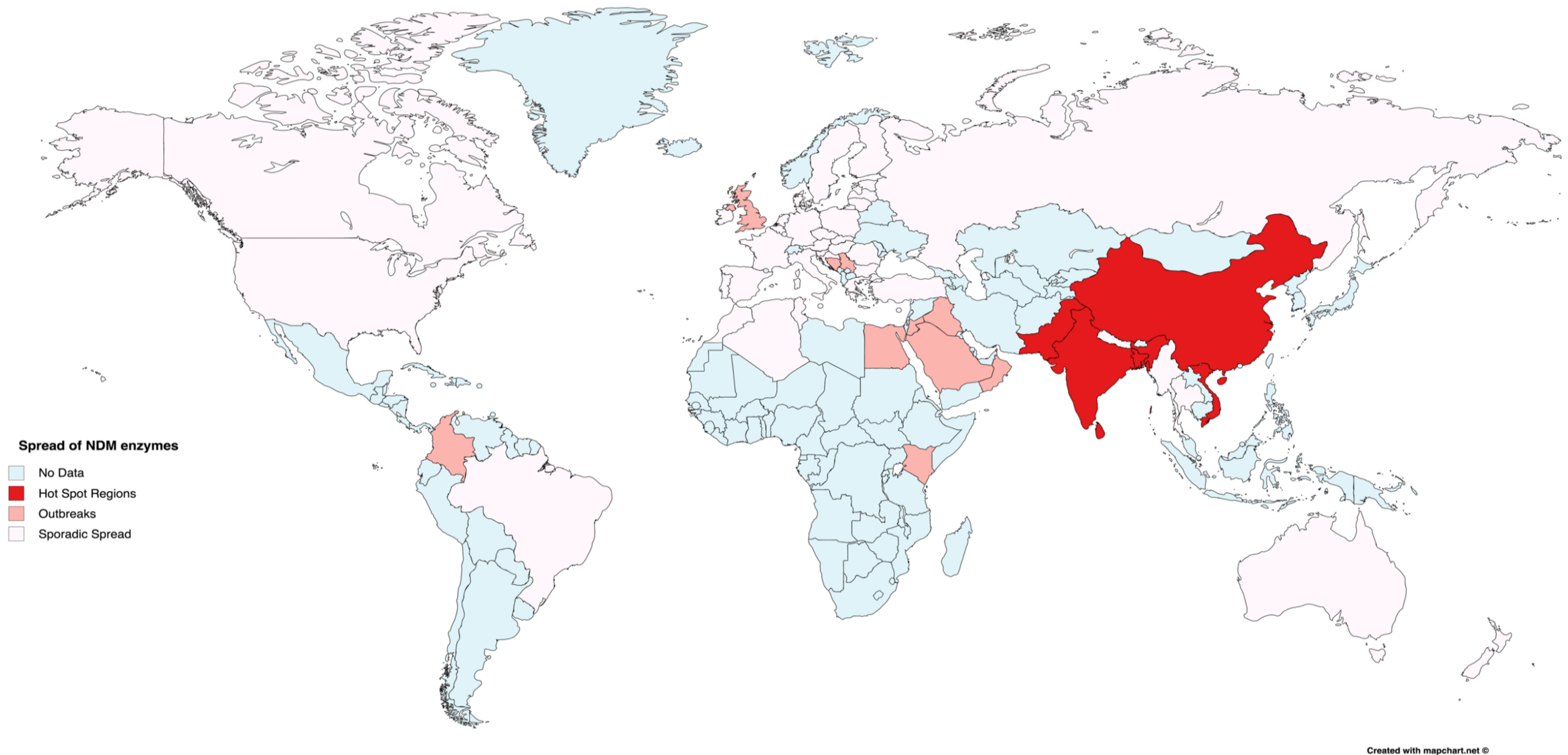


Figure 9. Worldwide distribution of NDM enzymes. The Map shows that the NDM gene is highly prevalent in India, Pakistan, Bangladesh, Sri Lanka, Vietnam and China. It has also been associated with outbreaks in the UK, the Balkan region, Saudi Arabia, Kenya, Columbia, Egypt, Oman and Jordan. Sporadic spread has been reported elsewhere (highlighted in pink). Map created with Mapchart.net and the data derived from (Zmarlicka et al. 2015).

1.3.4.2. Clinical significance

As explained earlier, *bla*$_{NDM}$ is commonly associated with other resistance genes conferring resistance to β-lactam and non-β-lactam antibiotics. Among them include; β-lactamases genes such as AmpC cephalosporinases, ESBLs especially CTX-M-15 and other carbapenemases such as OXA-48, whereas resistance against non-β-lactam antibiotics includes aminoglycosides, quinolones and macrolides. Therefore, the treatment options with *bla*$_{NDM}$ associated infections becomes very limited. Colistin, fosfomycin and tigecycline are used as a treatment of last resort (Nordmann and Poirel 2013; Lee et al. 2016). Generally, the strain types, for example ST101, usually regarded as typical for *bla*$_{NDM}$ do not shown association with significant virulence factors (Peirano et al. 2011; Fuursted et al. 2012; Peirano et al. 2013; Esterly et al. 2014). However, it should be noted that reports of virulent STs such as *E. coli* ST131 from clinical cases are emerging (Peirano et al. 2013).

1.3.5. General Characteristics and Properties of VIM Type Carbapenamases

Another type of MBL, VIM, has spread worldwide. So far, 46 variants of this enzyme have been identified with 0.4 to 27.1% amino acid difference (Zhao and Hu 2011; Dortet et al. 2014). The general fold structure of VIM enzyme variants are very similar. However, subtle changes in the active site architecture between them can initiate changes in their binding ability and, as a result, produce alternative carbapenem hydrolysis profiles (Meini et al. 2014). VIM-1 enzymes have been identified in *P. aeruginosa* and species of Enterobacteriaceae, especially *K. pneumoniae*, whereas VIM-2 is almost exclusively associated with *P. aeruginosa*.

Furthermore, *bla*$_{VIM-1}$ and particularly *bla*$_{VIM-2}$ are the most prevalent among all VIM gene variants and are commonly isolated from outbreaks and nosocomial infections (Sánchez-Romero et al. 2012; Gaibani et al. 2013; Jeannot et al. 2013; Douka et al. 2015).

1.3.5.1. Epidemiology and Genetics

Probably the most interesting feature of *bla*$_{VIM}$ is the association with class 1 integron coded on a transposon, especially Tn*402*, making them proficient expressers and distributors of the resistance genes (Tato et al. 2010). Furthermore, multiple gene cassettes are usually co-expressed on a single integron and, until 2011, at least 110 different structures of class1 integrons harbouring *bla*$_{VIM,}$ in association with other antibiotic resistance genes, had been identified (Zhao and Hu 2011). *bla*$_{VIM-2}$ is also found to be carried on the chromosome rather than a plasmid but still mobilized either by *ISCR* elements or inserted as a ene cassette into class 1 integrons (Tato et al. 2010; Zhao and Hu 2011; Meini et al. 2014). Perhaps, due to this feature of *bla*$_{VIM}$, they have been reported all over the world, although certain geographical locations have higher prevalence than others (Nordmann and Poirel 2013; Lai et al. 2014; Spyropoulou et al. 2016). There are no specific STs related to the spread of VIM enzymes but in *P. aeruginosa* they have shown a strong association with serotype O11 and O12 (Woodford et al. 2011). Furthermore, the origin of VIM is also debatable. However, it is almost certain that the initial enzyme appeared in *P. aeruginosa* and likely to have spread from one region to another through population dynamics (Nordmann 2014).

1.3.5.2. Clinical Significance

Like other MBLs, co-resistance of *bla*$_{\text{VIM}}$ with other resistance genes presents a problematic scenario for clinicians. In addition to other resistance mechanisms, *bla*$_{\text{VIM}}$ containing integrons are frequently isolated from cassette arrays in combination with other antibiotic resistance determinants such as aminoglycoside modifying enzymes (*aacA4, aadA1*, and/or *aadB*), other carbapenamases especially *bla*$_{\text{OXA-48}}$ and the chloramphenicol resistance, *catB* (Meini et al. 2014; Garneau-Tsodikova and Labby 2015); thus making the therapy regime complicated and introducing a high chance of treatment failure. The strain specific virulence characteristics may also be important factors in determining the choice of treatment and consequently influence the outcomes. A study observed the impact of such variations on the treatment outcomes when the mortality rate increased from 13.3% to 53.8% with an increase in strain specific MICs of bloodstream *bla*$_{VIM-1}$ harbouring *K. pneumoniae* isolates (Daikos et al. 2007). Generally, combination therapies of carbapenem and colistin or tigecycline or an active aminoglycoside have shown good results. However, tigecycline is not active against *P. aeruginosa per se* and, hence, colistin is often regarded as the ultimate treatment despite its unfavourable pharmacokinetics and toxic effects (Pitout et al. 2015).

1.4. Overcoming AMR

1.4.1. Drivers of AMR

To combat the threat of the emerging antibiotic resistance, it is essential to understand the factors and features of bacteria and their association with society and the environment that are considered the driving forces for the observed AMR. Although AMR is a natural phenomenon and presents as a biological defence mechanism in some bacteria, it can be propagated by the multiple driving forces related to human activities. The complex interaction of bacteria and their surroundings, and stimulation of different multifaceted mechanisms under different conditions, means that these driving forces could be different depending on the bacterial species and the class of drugs involved (Turnidge and Christiansen 2005). Nevertheless, it can be argued that the extensive use of antibiotics in livestock, agriculture, aquaculture and human and veterinary medicine has given bacteria the opportunity to evolve and survive by becoming resistant. The onward transmission and spread is attributed to several socio-demographic and population dynamic factors such as travel, sanitation, quality of food and living standards, inadequate infection control and surveillance and, more importantly, misdiagnosis and unnecessary antibiotic use (Turnidge and Christiansen 2005; Holmes et al. 2016c).

Antibiotic consumption has increased over the years (Figure 10). Although some sectors are using more than others, all have a collective role in driving AMR (Laxminarayan et al. 2013; Boeckel et al. 2014). There have been increased efforts in high income countries to reduce the consumption rate and tight regulations are imposed for prescription of antibiotics. However, the system is not flawless and there

are still reports of unnecessary use. The situation is much worse in developing countries where there are efforts to implement proper guidelines and non-prescribed over-the-counter sale is commonplace. Even if prescription drugs are needed, the lack of onward testing to ensure the appropriate therapy is often neglected (Laxminarayan et al. 2013; Laxminarayan and Chaudhury 2016). However, currently the food production industry accounts for the highest antibiotic consumption and pollutes the environment with antibiotics if waste products are not treated accordingly, which is the true for most developing countries. LMICs lack proper sewage, sanitation and waste treatment facilities (Andremont and Walsh 2015). In addition, there is a general increase in population densities and the sanitation and waste disposal systems have not been developed sufficiently to accommodate for the increasing waste (Quintela-Baluja et al. 2015). More than 36% of the world's population lack adequate sanitation and 1.1 billion are practising open defecation (Morgan et al. 2012). Generally, wastewater and animal and human waste is recognized as major sources of AMR where genes are excreted in the environment. Due to lack of proper disposal and sewage systems, resistance genes may find their way to spread to distinct environments and become a part of a continuous cycle, with the transfer of genes between bacteria, humans, animals and the environment (Andremont and Walsh 2015).

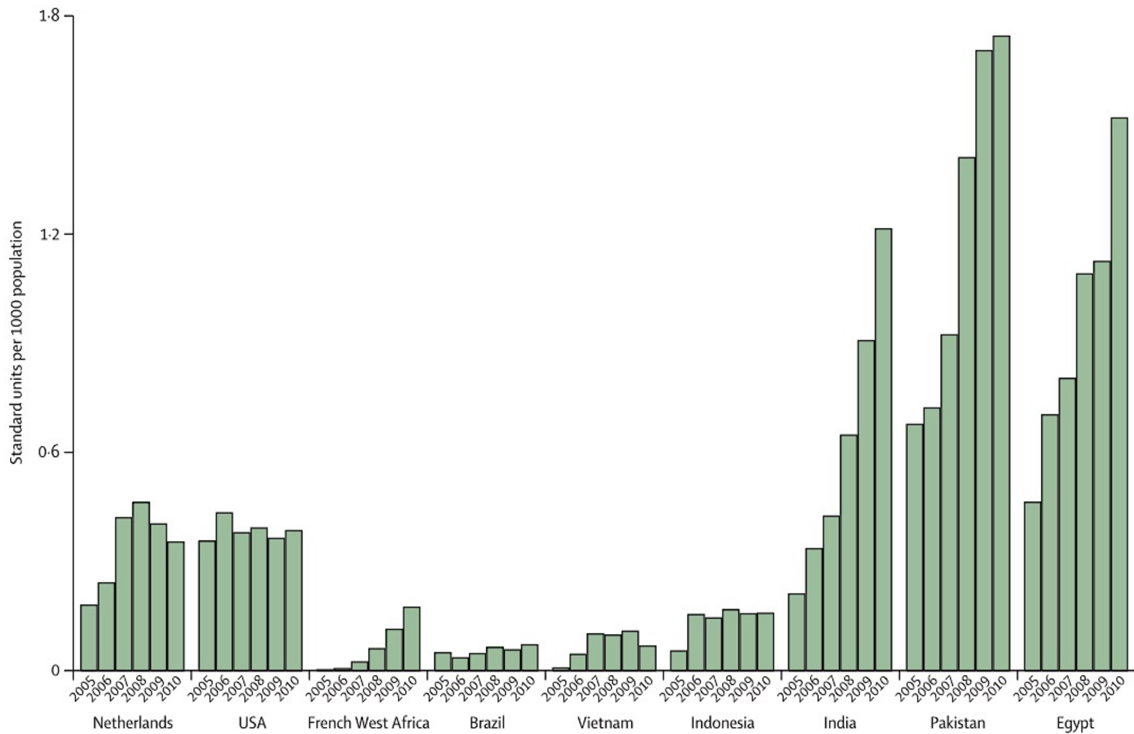


Figure 10. The increasing retail sales of carbapenems. The figure shows that an increasing sale trend is observed in developing countries of Pakistan, India and Egypt. Reproduced with permission from (Laxminarayan et al. 2013)**.**

1.4.2. Global Efforts Against AMR

Efforts are being made to reduce MDR rate and humanitarian organisations are encouraging governments to prepare policies concerning prevention and spread of MDR. Increased funding has been allocated by organisations such as the Bill and Melinda Gates foundation and improved surveillance by organizations such as centres for disease control and prevention (CDC) provide more information about the current status of AMR (Marston et al. 2016). Initiatives by the UK government, such as the Newton and Fleming funds, are providing a platform for other countries to join forces against AMR and support community engagement and control implementation

policies. AMR has been discussed on high profile forums and some of them include the G7 summit in 2015, and G20 in 2017, where AMR was one of the top agendas for G20 health ministers, the United Nations agenda and political declaration on AMR in 2016 and the WHO Global Action Plan on AMR in 2015 (WHO 2015a; Marston et al. 2016). In all those gatherings, AMR has been recognised as a major public health concern with detrimental consequences on the global health and economy. Tackling AMR is declared as an urgent aim to avoid a post-antibiotic era (O 'Neill 2015; WHO 2015a; Marston et al. 2016; European Comission 2017). To achieve this goal, collaborations of multiple governing bodies, research centres, policy makers and, to a much greater extent, public coordination are also acknowledged. In addition, a more global approach is necessary to effectively monitor and initiate appropriate responses to the emerging unprecedented threat of AMR. The global action plan on antimicrobial resistance was also supported by the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE). An increased collaboration between animal and human medicine is suggested to provide the essential information required to monitor the AMR situation in all sectors (WHO 2013a; WHO 2015a). The major goal of the global action plan is ensuring the accessibility of required medication to where it's needed most whilst still maintaining the efficiency by preventing the spread of infectious diseases. The plan also focuses on initiating a coordination on the emergence and spread of AMR between humans and animals and increasing surveillance on a global scale. The five strategic objectives were;

a) To increase awareness through education and training
b) Increase AMR surveillance globally
c) Optimise the use of antibiotics in humans and animals

d) Reduce the incidence of infection by introducing effective hygiene and implementing sanitation
e) Increase investment in new medicines, diagnostic and other intervention

There are challenges to following the strategies presented by global action plans especially in LMIC. The main problem lies in the education, or behaviour change, which would be required to achieve any successful results. In addition, the state of the health system presents challenges in improvising effective treatment due to lack of diagnosis, funding staff and resources (O'Neill 2016a). Furthermore, the hospitals usually lack basic hygiene and there is limited knowledge of infection control procedures among healthcare workers (Timilshina et al. 2011b). Effective infection control measures can help control the spread of MDRB between patients, healthcare workers and visitors and the community, whereas interventions like antibiotic stewardship can help to deliver the necessary antibiotic therapy to where it's needed most and limit the excessive use of antibiotics. Merging these strategies can also reduce the increasing selective pressure associated with the emergence of new resistance mechanisms (Carling and Polk 2011).

Currently, a major information lapse in AMR is the surveillance of MDRB in the environment. A comprehensive national surveillance and essential laboratory capacity to detect, analyse and track resistant microorganisms in clinical and environmental settings is an essential component of the "One Health" approach. Effective surveillance systems can identify the trends and patterns of AMR and outbreaks and facilitate the development of future strategies. Establishing an effective national surveillance system would also help to identify the scope of the problem (WHO 2015a), especially when there is no other existing or new treatment

option in the near future. However, there are increasing investments in the new or alternative therapies against AMR but, for now, surveillance and prevention is the best strategy.

1.4.3. Alternative or Pipeline Antimicrobials Therapeutics

Currently, antibiotic resistance is on the ris and no new class of antibiotic has been brought to the market for over 30 years (WHO 2013a). Big pharmaceutical companies have lost interest in investing in AMR drug development research. The loss of interest is somewhat attributed to the continued failure in new antibiotic discovery in addition to the extensive use or rather abuse of antibiotics resulting in generating resistance rapidly giving companies a short window for profit (Boeckel et al. 2014). In retrospect, as antibiotic resistance emerged, different analogues of the existing antibiotics were introduced and, for several years, infectious diseases were thought to have been conquered. However, genetic plasticity has equipped bacteria with enhanced adaptive systems which are perfected by the application of constant selective pressure through human antibiotic use and the continuum of which results in generating pathogens with the ability to evade all existing antibiotics. To stay ahead of this arms race against antibiotic resistance, new or alternative therapeutics are absolute essential.

At the end of 20th century, the genomic revolution unravelled a magnitude of information about bacteria. The traditional drug discovery strategies of screening natural compounds have been improved with the help of genomics, proteomics and high throughput screening. Genomics and proteomics have provided valuable insight in to the pathogenesis, virulence and regulatory mechanisms and metabolic pathways and potential new targets have been identified (Clatworthy et al. 2007;

Coates and Hu 2007; Tillotson and Theriault 2013; Allen et al. 2014). High throughput screening, combined with natural and synthetic libraries of compounds, is used to evaluated the novel compounds for antimicrobial properties (Tillotson and Theriault 2013). More specific targeted inhibition of virulence or pathogenesis, targeting pathogenic bacteria by bacteriophages, inhibiting bacterial growth by surplus nanoparticles and natural or synthetic antimicrobial peptides (AMPs) are some antimicrobial strategies currently being investigated (Clatworthy et al. 2007; Coates and Hu 2007; Azam et al. 2012; Tillotson and Theriault 2013; Allen et al. 2014). Metal oxide nanoparticles, specifically ZnO, have shown bactericidal activity against *E. coli* and *S. aureus*. The activity of nanoparticles greatly depends on the size and stability of the compound. The current advancements in nano-biotechnology has equipped the scientist with manipulation technologies to generate an ideal nano-particle with antimicrobial properties (Azam et al. 2012). A smaller size nano-particle is preferred due to its increased antibacterial properties and ease of entry across the cell membrane; however, it also presents a stability problem and generating a nanoparticle to be stable enough and restrict bacterial growth significantly is challenging (Azam et al. 2012; Tillotson and Theriault 2013).

A different approach is to target the virulence factors or the pathogenesis pathway such as quorum sensing in *Pseudomonas* and *FimH* in *E. coli*. Uropathogenic *E. coli* uses *FimH* to adhere to the mannose receptors in the epithelial cells of the urinary tract system and cause chronic urinary tract infection (UTI). Extracellular *FimH* inhibitor derived from mannosides were used to inhibit bacterial binding to the epithelial cells and reduce the virulence (Han et al. 2012). Quorum sensing in *Pseudomonas spp.* is a population density-dependent stimulatory response for regulating pathogenesis pathways through multiple virulence factor regulator (MvfR).

Inhibiting quorum sensing by inhibition of intracellular MvfR signals can break the pathogenesis cycle and limit the colonization and hence the associated infection (Lesic et al. 2007; Tillotson and Theriault 2013).

1.4.3.1. Bacteriophage Therapy

Bacteriophages are viruses of bacteria able to infect and induce cell death as it replicates in the bacterial host cells. In the early 1900s, phage therapy received much attention and some early trials generated impressive results; however, the following studies were controversial and the unreliable outcomes resulted in creating an uncertainty for the therapeutic potential of bacteriophages (Lin et al. 2017). Furthermore, the discovery of antibiotics such as penicillin shifted the attention from phage therapy until the discovery of AMR and the need for alternative treatment methods to antibiotics. However, the Soviet Union, Eastern Europe, and France continued to invest in phage therapies and still use lytic phages to target pathogens and are being used in poultry, cattle, agriculture, aquaculture and sewage in the United States (Międzybrodzki et al. 2012; Tillotson and Theriault 2013; Allen et al. 2014). In some Eastern European countries, they have also been used to treat chronic infection in humans but haven’t been approved for clinical use elsewhere. There have been several, small-scale, clinical trials; however, no rigorous studies have been performed (Andremont and Walsh 2015). However, in recent years new research is increasing the background information available for bacteriophages and their therapeutic potential is increasingly being investigated (Doss et al. 2017).

Phage therapy offers a few advantages and disadvantage over traditional antibiotic therapy when used to treat infections. They are increasingly abundant and present almost everywhere where bacteria are normally found to maintain the natural

balance of bacterial population (Loc-Carrillo and Abedon 2011). Their ability to eliminate MDR bacteria is particularly interesting due to increasing number of treatment failures through traditional antibiotics. Most phages exhibit a high specificity to their host and hence can only be effective against a certain species of bacteria, limiting their spectrum of activity, but could be useful in the elimination of a targeted species and save on the unnecessary disruption to normal microbiota. Nevertheless, an immune response could be initiated if the human defence system recognizes phages as foreign antigens and respond by producing phage-neutralizing antibodies whereas toxic effects could also be generated by the release of cellular components or endotoxins during phage mediated bacterial cell lysis. However, a lysin-deficient phage, or lysin on its own, is under investigated for its antibacterial properties to circumvent the immunogenic response (Andremont and Walsh 2015). Phage protein may also be preferred due to avoidance of other drawbacks associated with the phage's life cycle (Międzybrodzki et al. 2012; Doss et al. 2017; Lin et al. 2017), for example, horizontal gene transfer and environmental containment issues. Genetic material gained through horizontal gene transfer may increase the bacterial host's virulence especially when an antibiotic-resistance gene is involved. Similarly, environmental contamination of phages may occur since it reproduces constantly as long as the host is present. Hence, the introduction of a phage protein could be beneficial in conserving the natural ecosystems (Doss et al. 2017).

Another factor which has limited the use of phage therapy is the development of resistance (Międzybrodzki et al. 2012; Allen et al. 2014). Bacteria have developed multiple mechanisms of defence against bacteriophage infection by either intercepting phage life cycle or through cell death as a scarifier to limit further

infections (Seed 2015). However, phages have evolved to overcome these resistance mechanisms and, therefore, new phages can be re-isolated from the environment. Additionally, a cocktail of phages could be used to reduce the development of resistance with ease and also to target a broad host range (Janis Doss, 2017).

1.4.3.2. Antimicrobial Peptides and Antisense Oligonucleotides (AOs)

AMPs are found naturally in all organisms with a role in the innate immunity. They are generally positively charged and interact with the lipopolysaccharide layer of microbial cell membranes. AMPs are extensively evaluated for their role as a potential antimicrobial agent and have shown a broad spectrum of activities against bacteria, fungi, viruses and parasites (Bahar and Ren 2013; Mohamed et al. 2016). Example of AMPs include bacteriocins, such as nisin, which is produced by lactic acid bacteria and been approved for commercial use in food preservation. Similarly, Polymyxin is an old class of cyclic AMPs that were discovered in 1947. However, the clinical use was limited due to the associated nephrotoxicity and neurotoxicity (Gupta et al. 2014). Though the issues with safety still remain, the two major cationic polypeptides, namely Colistin and polymyxin B, have been used now as last resort antibiotics due to the wide spread of carbapenem resistance. They are bacteriocidal against all Gram-negative bacteria with the exception of a few intrinsically resistant species of *Burkholderia, Proteus, Providencia, Morganella morganii*, *Serratia spp,*, *Brucella spp.*, *Neisseria ssp.*, and *Chromobacterium spp.*. (Falagas and Kasiakou 2006; Garg et al. 2017). Bacterial growth is inhibited by interacting with the anionic lipopolysaccharide in the cell membrane leading to displacement of calcium (Ca^{2+})

and magnesium (Mg^{2+}) ions causing increased cell permeability followed by leakage of cell contents and, subsequently, cell death (Falagas and Kasiakou 2006; Gupta et al. 2014). Recently, resistance to colistin has also been emerging and plasmid mediated *mcr* gene variants (*mcr-1.1 to mcr-1.7, mcr-2 and mcr-3*) have been identified from more than 18 countries over the world in a short period of two years (Mammina et al. 2012; Gao et al. 2016; Liu et al. 2016; Yin et al. 2017). The spread of *mcr* is pushing the need for research to identify other AMPs' antimicrobial properties like polymixin. However, naturally-derived AMPs are usually present similar issues of toxicity in addition to other drawbacks such as degradation by proteases, sensitivity to salt and pH and high cost of production. Therefore, synthetic derivatives are currently being investigated as possible alternatives to natural AMPs (Mohamed et al. 2016).

Like AMPs, antisense RNAs are also naturally produced by bacteria, but regulate the gene expression of the producer rather than inhibiting the competitors. Based on the concept of antisense RNA, synthetic antisense oligonucleotides (AO), that mimic the structure of DNA or RNA, are constructed and used to regulate gene expression (Madani et al. 2011). AOs can potentially be used as antimicrobial agents by inhibiting gene expression through binding with complementary mRNA (Woodford et al. 2009). In nature, antisense RNA exists both in eukaryotes and prokaryotes to regulate or silence gene expression; for example, RNA interference (RNAi) in eukaryotes is the process of inhibiting gene expression by neutralizing targeted mRNA. A similar task is carried out by small, non-coding RNA's (sRNA) of 100-500bp in bacteria. sRNA can be trans-encoded or sis-encoded based on their genomic location. Trans-encoded sRNAs are located at a distant from the mRNAs they regulate and are not a complete match to the target sequences, whereas cis-

encoded sRNAs are located on the opposite DNA strand of another gene and have perfect complementarity with the target mRNA sequences (Good and Stach 2011; Lundin et al. 2015). Another bacterial RNA based gene-editing mechanism is clustered regularly inter-spaced short palindromic repeats (CRISPR) which is naturally used as a defence system against bacteriophages (Good and Stach 2011). All these systems have been evolved and used as gene editing tools in both eukaryotes and prokaryotes.

With the development in genomics, multiple AO targets can be identified according to the disease or pathogen to generate a more specific response, although, initially, the technology suffered a drawback due to issues with the target affinity, stability and the delivery of compounds in the cells. However, due to advancements in synthetic chemistry and genomics, different structures with multiple alterations were introduced to eliminate these problems (Lundin et al. 2015). It is generally accepted that, based on the genomic sizes of the species, the optimum length of antisense sequence to initiate an inhibitory effect is 11bp for bacteria and more than 15 for mammalian cells (Good et al., 2001). Naturally occurring antisense RNAs also share 12–30bp similarity with the target mRNA but can also be longer; however, the presence of mismatches and secondary structures within the genome limit their binding capacity. Synthetic AOs have the benefit of being more stable with desired sequences to provide exact matches and initiate the maximum response within target mRNA. Certain AOs, such as locked nucleic acids (LNA), phosphorodiamidate morpholino-oligomers (PMO) and peptide nucleic acids (PNA), have gained greater attention due to improvements in stability and target hybridization affinities (Sully and Geller 2016).

1.4.4. Antisense PNA as an Alternative Therapy?

Peptide nucleic acid (PNA) was developed by Nielsen et al (1991) as a ligand for DNA (Nielsen et al. 1991). The structure of PNA is unique, consisting of oligonucleotide bases and a neutral pseudo-peptide N-(2-aminoethyl)-glycine backbone; hence a hybrid between DNA and protein (Figure 11) (Sugiyama and Kittaka 2012). PNA molecules mimic DNA and are capable of binding to the complementary sequence of DNA or RNA, through Watson Crick base pairing, to inhibit gene synthesis at transcription or translational level (Geary et al. 2015). PNA shows increased thermal stability, higher affinity and specificity due to their neutral backbone. These properties of PNA, together with resistance to degradation by protease and nucleases, makes PNA ideal for antisense activity (Sugiyama and Kittaka 2012). The only known limitation is entry into the cell. PNA, being a large hydrophobic molecule, cannot enter the cell on its own and is dependent on a delivery system for its transport across the membrane. Recently though, this issue has been resolved by conjugating PNA with a cationic peptide (Good et al. 2001; Lehto et al. 2016).

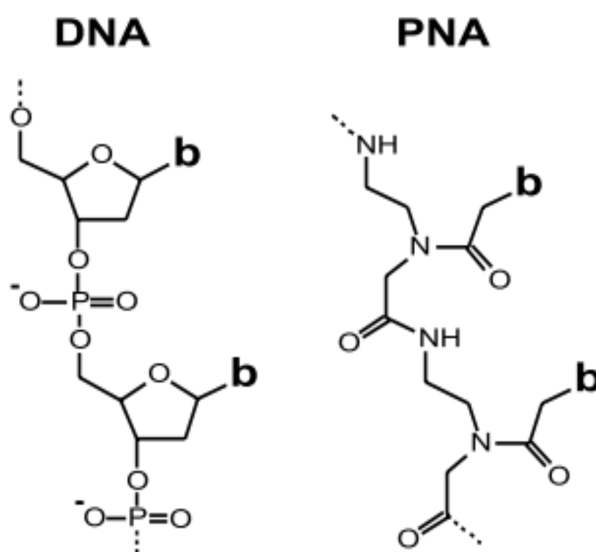


Figure 11. Comparison between the chemical structure of PNA and DNA. The above diagram represents the structure of PNA in comparison to the structure of DNA. Image copied with permission from (Good et al., 2001).

1.4.4.1. Conjugated Peptide Assisted PNA Cell Entry

Synthetic CPPs were originally derived from naturally occurring AMPs and were investigated for their antimicrobial potential in several studies. Initially, cationic lysine Lysine (K) and hydrophobic Phenylalanine (F) based synthetic peptide motif, KFFKFFKFFK, was used in synergy with other antibiotics to decrease the MIC (Vaara and Porro 1996; Good et al. 2001). Good et al. (2001) attached the same peptide motif to 9-12-mer PNAs targeting the essential acyl carrier protein (*acpP*), which is involved in fatty acid biosynthesis, to facilitate the transport across the cell membrane. The antisense peptide PNA conjugates (AP- PNA-C) resulted in improved antimicrobial potency in *E. coli* when compared to the naked PNA and no

apparent toxicity to the human cells was observed (Good et al. 2001; Hansen et al. 2016).

Shortly after, the role of arginine in certain naturally occurring AMPs led to the development of arginine rich CPPs that were not only effective in Enterobacteriaceae but also *Pseudomonas* (Strøm et al. 2002). It was also observed that changes in the chain length and the sequence motifs can alter the properties of the CPPs and hence opening up new potentials for a better design (Liu et al. 2007). It was also observed that the addition of 6-aminohexanoic acid (X) and β-alanine (B) residues increase the potency and the serum-binding profile of the attached antisense peptides (Wu et al. 2007). Furthermore, the incorporation of repeating cationic and non-polar residues in arginine based CPP motif, (RXR)4-XB was found to be a more effective strategy that could cargo PNAs across the cell membrane (Mellbye et al. 2009). However, the mechanism of entry is still controversial and several theories are proposed such as transport via endosomal compartmentalization or direct cell membrane penetration. A concentration dependent mechanism is also suggested where low concentration of CPP enters the cell through endocytosis and, upon increasing the concentration (more than 20 μMa), a rapid cytoplasmic entry is achieved (Brock 2014). Nevertheless, further research in this field would assist in optimizing CPPs for their potential use as ideal transporters for antisense peptides therapies.

1.4.4.2. Antisense Activity of PNA Conjugates

Several studies have shown the bactericidal activities of AP- PNA-C by targeting the mRNA of essential bacterial genes. So far, the antisense activities of AP-PNA-C

have been observed in multiple Gram-negative and Gram-positive bacteria when used at micro-molar quantities (Table 1). However, only limited studies have reported in vivo analysis and mostly limited to using either lysine and phenylalanine or arginine based CPPs. Very recently, Hansen et al. (2016) reported successful PNA transmembrane entry and potent antibacterial activity with anti-*acpP* PNA conjugated to AMPs (Hansen et al. 2016).

Although the exact mechanism of AP-PNA-C is not known, it is believed to bind to the complementary mRNA sequence blocking ribosome assembly on the target site. Short AP-PNA-C of 9-12 units complementary to the 5' terminus UTR region of mRNA has shown potent antisense activities compared to AP-PNA-C targeted towards the coding sites within mRNA (Dryselius et al. 2003; Ghosal and Nielsen 2012; Mohamed et al. 2016). Since PNA is synthetic, it’s unlikely that a resistance enzyme may already exist within the environment. Resistance could arise by bacteria producing enzymes to modify or degrade AP-PNA-C or possibly by mutation of the mRNA sequence. Up till now, no target mutation or modification resistance has been identified against antisense PNA and the possibility of efflux pumps is also reported to be minimal (Nikravesh et al. 2007; Ghosal et al. 2013). Furthermore, good activity against MDRB is also observed. A study evaluating the antibacterial effects of PNA on bacteria possessing ESBLs and MDR showed minimum inhibitory concentrations (MIC) of more than 512 µg/ml to β-lactam antibiotics and anti-*acpP* PNA of 5 µMol and 25 µMol to ESBLs and MDR, respectively (Bai et al. 2012). Furthermore, no resistance was developed after 5 serial passages for PNA, whereas all β-lactam antibiotics (ampicillin, ceftazidime, cefoperazone) developed resistance. Synergy of antibacterial PNA with other antibiotics is also reported. In 2007, Xue-Wen et al. observed synergetic bacterial inhibitory effects of PNA targeting 23S

rRNA domain II and tetracycline, although the activity of PNA was comparable to tetracycline but initiated a slightly slower inhibitory response than tetracycline (Xue-Wen et al. 2007).

Table 1. Studies reporting the application of AP-PNA-C as a potential antibacterial agent.

Target	CPP	Organism	Study type	Reference
23S rRNA domain ii	$(KFF)_3$	*E. coli DH5α*	vitro	(Xue-Wen et al. 2007)
rpoD	$(RXR)_4XB$	*E. coli MG1655, 25922,* MDR *Salmonella enterica, Shigella flexneri,* ESBL and MDR *E. coli*	vitro/vivo	(Bai et al. 2012)
Ftsz, acpP	$(RXR)_4XB$	ESBL *K. pneumoniae, E. coli* and *P. aeruginosa*	vitro	(Ghosal and Nielsen 2012)
rpoA, rpoD	$(KFF)_3$	Intracellular *Listeria monocytogenes*	vitro/vivo	(Alajlouni and Seleem 2013)
acpP, Ftsz	$(KFF)_3$	*E. coli MG1655*	vitro	(Ghosal et al. 2013)
gyrA	$(KFF)_3$	*Streptococcus pyogenes*	vitro	(Patenge et al. 2013)
polA, asd, dnaG	$(KFF)_3$	*Brucella suis*	vitro	(Rajasekaran et al. 2013)
MecA, Ftsz	$(KFF)_3$	*Methicillin resistant staphylococcus aureus (MRSA), Methicillin resistant staphylococcus pseudintermedius (MRSP)*	vitro	(Goh et al. 2015)
ftsz	$(RXR)_4XB$	*MRSA*	vitro	(Liang et al. 2015)
rpoA	$(KFF)_3$, $(RXR)_4XB$	Intracellular *L. monocytogenes*	vitro/vivo	(Abushahba et al. 2016)
acpP	AMPs	*E. coli MG1655*	vitro	(Hansen et al. 2016)
$bla_{\text{CTX-M-15}}$	$(KFF)_3$	*E. coli AS19*(cell wall permeable mutant) $bla_{CTX\text{-}M}$ *clone*	vitro	(Readman et al. 2016)

The case studies were selected based on search results of Scopus limited by keywords; Peptide nucleic acid, antisense and antibacterial for year 2007-2017.

1.5. Concluding Remarks

It is established that the increased consumption of un-prescribed antibiotics, lack of effective sanitation and sewage systems, environmental contamination and substandard infection control strategies have detrimental effects on the spread and dissemination of antibiotic resistance, and predominantly β-lactamase resistance, has spread worldwide threatening the potency of carbapenems when there is no substitute drug available. Therefore, to avoid slipping into the post-antibiotic *era*, new or alternative therapies against MDR are desperately needed. Concurrently, epidemiological studies to identify the extent of the problem, both in clinical and non-clinical settings, are crucial to acknowledge the extent of the problem. Hence, this study is designed to focus on the two main goals of tracking and treating the resistance genes carried by MDRB both in patients and their associated environment.

Pakistan has been chosen as a sample collection region as AMR has increased in developing countries and, additionally, no extensive studies have been performed to evaluate the effects it may have on the health and the environment. Initially, the prevalence of MDRB in clinical samples will be determined. The contamination of environmental samples will be evaluated by analysing different sectors of the environment for the presence of ESBLs and carbapenemases. It will be followed by a more in-depth analysis of antibiotic resistance in clinical (patients) and non-clinical (environmental) settings and the effects of region-specific basic infection control strategies and seasonal variations on the prevalence of MDRB. Lastly, PNA is examined as an alternative therapy to sensitize resistance strains to carbapenems. Collectively, the work of this thesis will provide a more focused overview on the environmental burden of MDR in developing countries and will highlight the

importance of infection control strategies in controlling their spread and dissemination. Furthermore, it is also hoped that my project will help provide an alternative approach towards the treatment of AMR and highlight the potential of PNA therapies in the conservation of carbapenem antibiotics.

2. Materials and Methods

2.1. Collection of samples

All samples evaluated in this thesis were collected from Pakistan. In the first results chapter 3, the samples were collected from Karachi whereas the samples in the second and third result chapters (chapter 4 and 5) were collected from Peshawar. Aimes charcoal swabs (Liofilchem®) were used for all samples except insects which were transported in sealed sterile Eppendorfs (Sigma-Aldrich, St. Louis, USA). Samples were sent to Cardiff University (CU), UK, in UN3373 containers, in accordance with the guidelines and regulations for packaging and shipment of biological and infectious substances (FedEx 2010).

2.1.1. Karachi Samples

2.1.1.1. Clinical Samples

The prospective cohort clinical study was conducted between February and October 2012. Clinical (rectal and site of infection) samples were obtained from patients visiting Civil Hospital, Karachi (CHK) which is the largest public tertiary-care hospital of Pakistan with 1900 beds and represents a diverse socio-economic background. Patients admitted to the hospital for two or more days at any of the medical, surgical, gynaecologic/obstetric, paediatric, psychiatric and allied units of CHK giving written consent were enrolled. Exclusion criteria included all forms of gastroenteritis and patients with active rectal bleeding and anal fissures. Ethical approval was granted by the Institutional Review Board of the Dow University of Health Sciences, Karachi, Pakistan (IRB-318/DUHS-12) and by the CHK ethics committee.

All patients were explained the purpose of the study at the time of admission and, upon their consent and fulfilment of inclusion criteria, a rectal swab (termed FSA) was obtained within 24 hours. In case of minors, consent was taken from a parent or guardian and, for critically ill patients, consent was taken from next-of-kin. The majority of the patients were illiterate and, thus, the consent form was verbalised in Urdu. Information on patients including socio-demographic data, hospital related information, living and sanitary conditions, and patient history were recorded onto a questionnaire. Patients were followed up to discharge where a second rectal swab was taken (termed FDA). If, during the hospital stay, the patient developed an infection, samples of the site of infection (SoI) were also taken. Bacterial isolates from SoI were identified at CHK by in-house methods.

2.1.1.2. Non-Clinical or Environmental Samples

A total of 729 samples were collected randomly over the course of the patient enrolment and included 193 insects, 92 birds' faeces, 97 samples of drinking water and 347 hospital surface swabs in the major wards e.g. ICU, paediatric, burns etc. All insects and surface and equipment swabs were collected from the CHK, whereas the birds and animal's faeces and drinking water samples were collected from the areas close to the hospital. Most of the faeces samples were derived from caged animals.

Insects samples included:

130-House Flies: *Musca domestica*:

20-Cockroaches: *Blattella asahinai*

20-Ants: *Lasius niger*

1-Honey bee: *Apis cerana*

12-Moths- Identified as *Aglossa aglossalis*

10-Mosquitoes- species of Culex

Birds or other small animals' faeces included;

4-Canary	10-Fowl	5-Peacock
5-Crow	3-Hawk	4-Pigeon
4-Deer	3-Koel	4-Rabbit
3-Dove	3-Ostrich	4-Rat Hamster
3-Duck	4-Owl	5-Sparrow
5-Eagle	20-Parrot	3-Turkey

All locations of surface samples and drinking water swabs are provided in the appendix (Table 27 and 28).

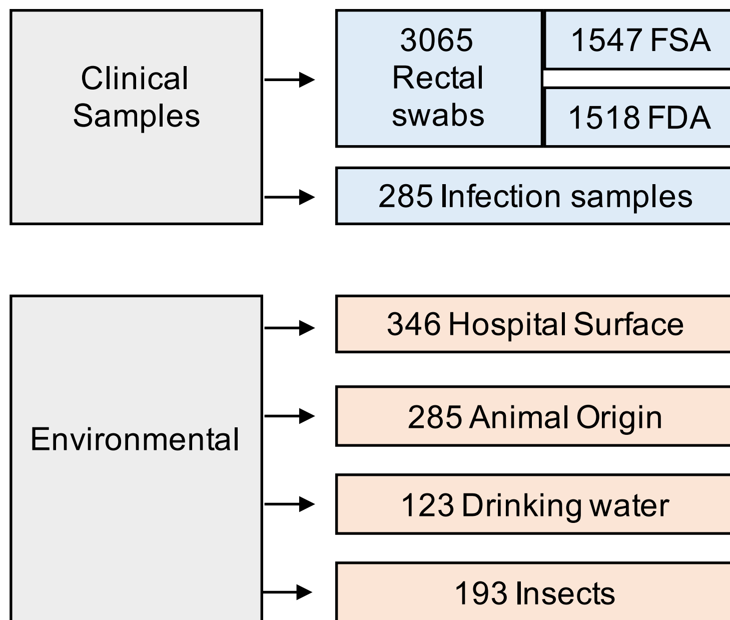


Figure 12. Karachi study design. The diagram shows the number of, and origin of, samples analysed in the Karachi study. FSA (rectal swabs at admission), FDA (rectal swabs at discharge).

2.1.2. Peshawar Samples

The Peshawar samples included surgical patients' wounds, hospital surfaces and insects, collected between January 2015 and August 2016. Two post-surgical wards were selected for the sample collection including all insects. Each ward had two separate areas and, for the purpose of this study, one of those areas was cleaned with cleaning agent containing white Phenyl (Finis S.C Jhonsons, Karachi, Pakistan) whereas the other area was left un-touched. The floors were mopped with the cleaning agent and the touch surfaces were wiped clean with damp clothes using the same cleaning agent. The Peshawar samples (surgical patients' wound swabs, touch surface and insects) were collected from these wards and the study was followed for a duration of three weeks in the Winter month of January 2015. The

same procedure was repeated in Summer and the samples were collected in July-August 2016. Ethical approval was obtained from the ethical committee of the Khyber Teaching Hospital, Peshawar.

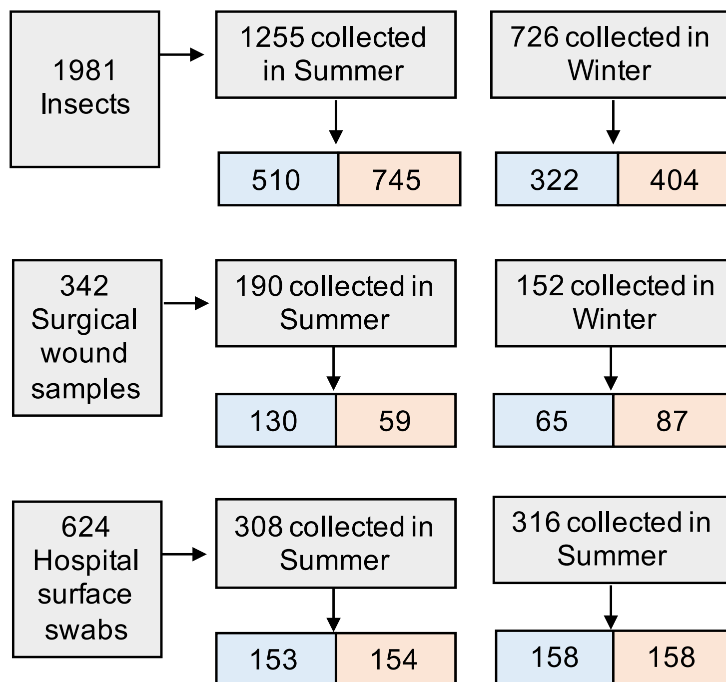


Figure 13. Peshawer study design. The diagram shows the number of and origin of samples analysed in the Peshawer study. The blue box shows the number of samples collected from cleaned wards and samples collected from un-cleaned wards are shown by orange coloured boxes.

2.1.3. Patient's Wound Swabs

The wound swabs were collected from patients from the selected wards with consent often verbalised in Urdu. Patients admitted to the hospital for surgery were asked to complete a questionnaire containing elementary patient information such as name,

age, sex, place of residence, sample site, patient history and surgery type. Subsequent information such as length of hospital stay and prescribed antibiotic therapy was also collected. An example questionnaire is supplied in appendix (Figure 60).

2.1.4. Insects

For the Winter collection of insects, most the flies were picked dead from the floor whereas the other insects, mostly cockroaches, were caught alive. However, due to the increased abundance of flies during the summer, most of them were caught alive by trapping on sticky paper.

Insects samples included;

1076-House Flies: *Musca domestica*

533-Cockroaches: *Blattella asahinai*

110-Ants: *Lasius niger*

2-Honey bee: *Apis cerana*

194-Moths- identified as *Aglossa aglossalis*

66-Spiders- Possibly *Stegodyphus pacificus*

2.1.5. Touch Surface

The locations of where the surface swabs were taken were kept constant throughout the study period i.e. the same area was swabbed during the course of the study. The table below list all swabbed sites and the wards (Table 2).

Table 2. The locations of the collected swabs from cleaned and un-cleaned wards.

Ward	Un-cleaned Surface Sites
M1-1	Exit door
	Entry door
	Entry wash room
	Wash basin sink knob
M2-1	Right sink knob
	Exit door knob
	Dustbin base
	Wash room button right
F1-1	Door exit
	Bed20 cupboard handle
	Dustbin/pillar base
F2-1	Bed13 drip hanger
	Bed14 cupboard handle
	Washroom handle
	pillar base bed 16*

Ward	Cleaned wards
M1-2	Entry Door knob
	Entry Door Washroom
	Window Surface
	Wash basin sink knob
M2-2	Right sink knob
	Exit door knob
	Red Dustbin
	Right wash button
F1-2	Right windows surface
	Door exit part
	Bed 2 cupboard handle
F2-2	Bed EX-7 drip hanger
	Bed 11 cupboard handle
	Surface under dustbin

Two sites from the list, highlighted with (*), were not selected for sampling in Summer and are excluded from result analysis. All other sampling sites were kept constant.

2.2. Other Bacterial Strains, Plasmids and Cloning Vectors

Below is the list of commercially available laboratory strains and antibiotic resistant strains used in this study (Table 3). The details of plasmid vectors are supplied in the methods where applicable.

Table 3. List of all bacterial strains used in this study.

Isolate	Origin	Specie	Resistance	Year
MG1655	Lab Strain	*E. coli*	NA	NA
ATCC 25922	Lab Strain	*E. coli*	NA	NA
DH5ALPHA	Lab Strain	*E. coli*	NA	NA
J53	Lab Strain	*E. coli*	Sodium Azide	NA
ATCC 13883	Lab Strain	*K. pneumoniae*	NA	NA
CH3490(NMI 5184/09)	EN-Secondary Panel	*E. coli*	NA	NA
CH3491(NMI 1844/06)	EN-Secondary Panel	*E. coli*	NA	NA
CH3493(NMI 7268/10)	EN-Secondary Panel	*E. coli*	NA	NA
CH3496(RYC 13053433)	EN-Secondary Panel	*K. pneumoniae*	NA	NA
NMI 1831/06	EN-Secondary Panel	*K. pneumoniae*	NA	NA
09C44	Spain	*K. pneumoniae*	KPC-3	2009
09D16	Spain	*K. pneumoniae*	KPC-3	2009
10E29	Spain	*E. coli*	KPC-3	2010
86198	Pakistan	*P. aeruginosa*	NDM	2014
86217	Pakistan	*K. pneumoniae*	NDM	2014
83092	Pakistan	*E. coli*	NDM	2014
86190	Pakistan	*P. aeruginosa*	NDM	2014
83100	Pakistan	*E. coli*	NDM	2014
KP506	Sweden	*E. coli*	NDM	2009
76207	Pakistan	*A.* baumannii	NDM	2014
12F14	Spain	*K. pneumoniae*	OXA-48	2012
12F48	Spain	*K. pneumoniae*	OXA-48	2012
12F65	Spain	*E. coli*	OXA-48/VIM-1	2012
08Y70	Spain	*K. pneumoniae*	INT-VIM-1	2008
09A69	Spain	*K. pneumoniae*	INT-VIM-1	2009
09Y79	Spain	*E. coli*	INT-VIM-1	2008
1-47	Spain	*E. coli*	INT-VIM-1	2010
RES-2074		*P. aeruginosa*	INT-VIM	
TC-NDM	Pakistan	*E. coli J53*	NDM	2014
TC-KPC	Spain	*E. coli J53*	KPC	2014
TC-OXA-48	Spain	*E. coli J53*	OXA-48	2014
ACP-P CLONE	Lab Strain	*E. coli DH5alpha*	*ACP-P*	2014

NA (Not Applicable) **.**

2.3. Peptide Nucleic Acids

All AP-PNA-C used in this study was manufactured by Copenhagen University. *acpP* PNA were designed by Copenhagen University whereas all other anti-resistance PNA sequences were selected using Geneious® (version 8.1.8). The variants of target enzymes sequences available in the NCBI database were aligned by the pairwise multiple alignment feature. Conserved ATG start site sequences were selected as targets and complementary PNAs of 9-12 PNA residues were constructed. For entry into the cytoplasm, the PNAs were attached to the two most commonly used, Lysine and Phenylalanine or Arginine based, CPPs. Below are the lists of all PNAs used in this study (Table 4 and 5).

Table 4. List of *acp-P* PNAs used in this study.

PNA	Experiment
EBL 183	Passage assay
EBL 264	Passage assay
EBL 110	FoR assay
EBL 111	FoR assay
EBL 232	FoR assay
EBL 237	FoR assay
EBL 366	Target validation
EBL 392	Target validation
EBL 506	Target validation
EBL 183	Target validation
EBL 264	Target validation

Table 5. List of all anti-resistant PNAs used in this study.

Label	Target	PNA
4703	INT Antisense	H-KFF KFF KFF K-eg1-CAT GAA AAC CGC-NH2
4704	VIM Antisense	H-KFF KFF KFF K-eg1-CAT CAA AAC TCC-NH2
4706	INT Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-CAT GAA AAC CGC-NH2
4707	VIM Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-CAT CAA AAC TCC-NH2
4708	VIM-2 Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-CAT CAA GAC TCC-NH2
4709	INT Antisense	H-KFF KFF KFF K-eg1-AGC CAT GAA AAC-NH2
4710	VIM Antisense	H-KFF KFF KFF K-eg1-GAA CAT CAA AAC-NH2
4712	INT Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-AGC CAT GAA AAC-NH2
4713	VIM Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-GAA CAT CAA AAC-NH2
4714	VIM-2 Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-TAA CAT CAA GAC-NH2
4640	KPC Mismatch	H-(R-Ahx-R)4-Ahx-(β-Ala)-TGA AAT CAC CGA-NH2
4641	OXA Mismatch	H-(R-Ahx-R)4-Ahx-(β-Ala)-ACG GAT AAC CTC-NH2
4642	INT Mismatch	H-(R-Ahx-R)4-Ahx-(β-Ala)-GCG GAT ACC CTA-NH2
4644	KPC Antisense	H-KFF KFF KFF K-eg1-TGA CAT CAA CGA-NH2
4645	OXA Antisense	H-KFF KFF KFF K-eg1-ACG CAT AAC GCT-NH2
4646	INT Antisense	H-KFF KFF KFF K-eg1-GCG CAT ACG CTA-NH2
4648	KPC Mismatch	H-KFF KFF KFF K-eg1- TGA AAT CAC CGA-NH2
4649	OXA Mismatch	H-KFF KFF KFF K-eg1- ACG GAT AAC CTC-NH2
4650	INT Mismatch	H-KFF KFF KFF K-eg1- GCG GAT ACC CTA-NH2
4476	NDM Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-TTCCATCAAGTT-NH2
4476	NDM Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-TTCCATCAAGTT-NH2
4476	NDM Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-TTCCATCAAGTT-NH2
4476	NDM Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-TTCCATCAAGTT-NH2
4477	KPC Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-TGA CAT CAA CGA-NH2
4478	OXA Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-ACG CAT AAC GTC-NH2
4479	INT Antisense	H-(R-Ahx-R)4-Ahx-(β-Ala)-GCG CAT ACG CTA-NH2
4639	NDM Mismatch	H-(R-Ahx-R)4-Ahx-(β-Ala)-TTC AAT CAC GTT-NH2
4643	NDM Antisense	H-KFF KFF KFF K-eg1-TTC CAT CAA GTT-NH2
4647	NDM Mismatch	H-KFF KFF KFF K-eg1- TTC AAT CAC GTT-NH2

2.4. General Antibiotics, Chemicals Reagents and Growth Medium

Throughout the study, custom ready-made chromogenic plates with antibiotics were purchased from Liofilchem®, Roseto degli Abruzzi, Italy. The details of which are listed below:

Chromogenic with 10mg/L Vancomycin (V plates)
Chromogenic with 10mg/L Vancomycin and 1mg/L Cefotaxime (VC plates)
Chromogenic with 10mg/L Vancomycin and 2mg/L Ertapenem (VE plates)

Cefotaxime and ertapenem were used at 2mg/L for the Karachi study whereas the concentration was reduced to 1mg/L for Peshawar samples (to capture all carbapenemases including the weakly expressed bla_{OXA181} and bla_{OXA323}). All other ready-made reagents, supplied directly from the manufacturers, are given in the main text of this chapter where applicable. Recipes for common reagents made up locally and a full list of media used is included in Appendix (Section 7.2).

2.5. Culturing clinical and non-clinical samples

The clinical (patient samples) and non-clinical (environmental samples) such as hospital surface, birds' droppings, sewage and drinking water samples were collected on charcoal swabs. The swabs were dipped in sterile water before sampling and were transported at room temperature where, on arrival, they were stored at 4°C. The swabs were directly plated on V, VC and VE selective plates and incubated overnight at 37°C. Incubation time was extended by another day where no growth was observed.

2.5.1. Culturing Insects Samples

All insects' samples in this study were collected from Karachi civil hospital or Khyber teaching hospital, Peshawar. Transportation was arranged in sterile eppendorfs without medium and the samples from both sites were processed as described below:

From Karachi, the insects were macerated with a loop and incubated in 2ml of LB at 37°C for 18hrs. The overnight broth culture was plated on V, VC and VE plates. Any growth on the plates were screened by PCR for the presence of bla_{NDM} and $bla_{CTX\text{-}M\text{-}15}$ and, in case of no growth, the incubation time was extended by another day. The original broth was also subject to PCR.

The Peshawar insects' samples were macerated in 1ml of water and screened for the presence of bla_{NDM}, bla_{OXA48} like, bla_{KPC} and $bla_{CTX\text{-}M\text{-}15}$ by PCR. 500µl of water was used to inoculate 2ml of MHB and 500µl was saved in -80°C for future genome sequence analysis. After overnight incubation at 37°C, a 10µl loop-full culture was plated on V, VC and VE plates and was grown at 37°C for 18hrs. Growth on selective agar media was processed by PCR for bla_{NDM}, bla_{OXA48} like, bla_{KPC} and $bla_{CTX\text{-}M\text{-}15}$. The incubation period was extended for another day if no growth was observed after 18hrs.

2.6. Species Identification

Isolated bacterial strains were identified by matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF, Bruker, Billerica, USA) in

the Specialist Antimicrobial Chemotherapy Unit, University Hospital Wales, Cardiff, UK.

Single isolated colonies from overnight VE or VC plates were directly applied to a Bruker MSP 96 well steel plate. One microliter of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker) was added to each sample and allowed to air-dry. The plates were then loaded onto the MALDI biotyper. Reliability scores of ≥2.0 for clinical bacteria and ≥ 1.70 for bacteria from insect samples were required for species level identification. For those samples which yielded lower reliability scores, the MALDI-TOF was repeated. If no possible identification was recorded after two MALDI-TOF repeats, 16s ribosomal RNA sequencing was undertaken.

2.7. Antimicrobial Susceptibility Testing

Antibiotic resistance profiles were established for NDM-positive isolates from Karachi hospital surface and drinking water samples using antibiotic discs and E-Test strips. Bacterial cultures were prepared to 0.5% McFarland standard in 0.85 % saline and spread on MH plates with a sterile cotton swab. Antibiotic discs (Oxoid Limited) or E-test strips (Liofilchem®, Roseto degli Abruzzi, Italy) were placed on the plate and results were recorded after incubation at 37°C for 18hrs.The results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (version 5.0) or CLSI (Clinical and Laboratory Standards Institute 2015). Where no breakpoint guidelines for specific antibiotics/bacterial species were found (for both EUCAST and CLSI) then EUCAST guidelines for Enterobacteriaceae were used.

2.8. Micro-broth Minimum Inhibitory Concentration

All isolates used in the PNA study were assessed for antibiotic MIC levels using micro-broth dilution method in 96-well plates. For optimum PNA activity, defined plates (260895, Fisher Scientific - Arendalsvägen Göteborg, Sweden) were used to reduce PNAs attachment to the 96-well plate. MICs were determined using EUCAST guidelines. Stock antibiotic solution was prepared at two times the required concentration whereas PNA stock solution was prepared at 20 times the required concentration. The inoculum was prepared to McFarland standard 0.5 (Approximately 1.0-2.0 $x10^8$ cells/ml) in MHB and diluted to approximately $1x10^6$ cells/ml.

2.8.1. MIC Meropenem

To prepare meropenem MIC plates, 100 µl of MHB was pipetted in all columns except the first, followed by pipetting 100 µl of the prepared antibiotic solution in the first two columns and serially diluting across the rest of the columns until the desired antibiotic concentration had been reached. 100 µl of prepared bacterial culture were added to each well giving a final inoculum density of $1\text{-}5x10^5$ cells per well. The plate was incubated at 37°C for 18hrs, shaking at 150rpm.

2.8.2. MIC PNA

To prepare a MIC plate with PNA, 90µl of MHB was mixed with 100µl of bacterial suspension and 10µl of PNA. The plate was incubated at 37°C for 18hrs, shaking at 150rpm.

2.8.3. PNA and Meropenem MICs

To prepare PNA and meropenem MIC plates, 100µl of MHB was pipetted in all columns except the first, followed by pipetting 100µl of the prepared antibiotic solution in the first two columns and serially diluting across the rest of the columns until the desired antibiotic concentration had been reached.
Each well was inoculated with 90µl of the corresponding bacterial culture and 10µl of the prepared PNA suspension to make a final volume of 200µl per well; approximately $5x10^5$ cells per well. The plate was incubated at 37°C for 18hrs, shaking at 150rpm, and results were recorded by visual observation of growth. Optical density at 595 was also measured for some experiments.

2.8.4. MIC of the cleaning agent

MIC to the cleaning agent was performed using the same method in section 2.8.1 except different concentration of cleaning solution was added instead of antibiotics. Selected isolates of *E. coli*, *E. cloacae* and *K. pneumoniae* from cleaned wards were choosen for the experiment. To obtain a contaminant-free cleaning agent, the solution was filtered through 0.45µm syringe membrane filter (Sigma-Aldrich, St. Louis, USA) and MICs repeated. The growth was measured by measurimg the optical density at 595 using EZ microplate reader 400 (Biochrom Ltd, Cambridge, UK).

2.9. Polymerase Chain Reaction

In this study, PCR experiments were used to detect the presence of MDR genes. All Karachi samples were assessed by PCR to detect bla_{NDM} and $bla_{\text{CTX-M-15}}$, whereas the Peshawar samples were assessed by a multiplex PCR method to detect bla_{NDM}, bla_{KPC} and bla_{OXA48} like genes; additionally, $bla_{\text{CTX-M-15}}$ was detection by standard PCR. Standard primers were used for the Karachi study; however, primers for the multiplex were designed by myself using PrimerPlex software (Premier Biosoft California, USA). Furthermore, PCRs were used for other applications such as cloning, target validation, sequence typing, confirmation of resistance genes before experiments and 16s gene amplification for identification. Optimising conditions for all new primers were determined by gradient PCR to refine annealing and extension temperature which varied accordingly with the primers and the amplicon length. PCRs were run on a G-Storm thermal cycler (G-STORM, Somerset, UK). The list of primers, their target and conditions are supplied in appendix (Table 28).

2.9.1. Classic PCR

Standard PCRs were performed using Extensor Hi-Fidelity PCR Master Mix (AB-0794/B, Thermo Fisher Scientific, Waltham, USA) or puReTaq Ready-To-Go PCR Beads (Illustra 27-9557-02, GE Healthcare Life Sciences, Buckinghamshire, UK). Template DNA was prepared by suspending a 10µl loop of bacteria (grown overnight at 37ºC) in 200 µl of water. For PCR beads, the loading dye was prepared by mixing 1000µl of 20mg/ml of Orange G (Sigma-Aldrich, St. Louis, USA) with 200µl of 0.5% xylene cyanol (Sigma-Aldrich, St. Louis, USA) prepared in 40% sucrose solution (Sigma-Aldrich, St. Louis, USA).

Each reaction mix contained 12.5µl ready master-mix, 0.5µl of each primer, 10.5µl of molecular grade water and 1µl of template when using Extensor Hi-Fidelity PCR Master Mix or 18µl molecular grade water, 0.5µl of each primer, 1µl of loading dye and 1µl of template when using puReTaq Ready-To-Go PCR Beads.

The general PCR conditions used are listed below:

Initial Denaturation	95°C	5 Minutes	
Denaturation	95°C	1 Minute	30-40 Cycles
Annealing	50-60°C	1 Minutes	
Extension	72°C	1-2 Minutes	
Final extension	72°C	5 Minutes	
Store 4°C			

2.9.2. Multiplex PCR reaction for *bla*$_{OXA-48}$-like, *bla*$_{NDM}$ and *bla*$_{KPC}$

A multiplex PCR method was used to detect the presence of *bla*$_{NDM}$, *bla*$_{KPC}$ and *bla*$_{OXA48}$ like in clinical and non-clinical samples.

Overnight grown bacterial colonies were suspended in 200ul of water and 1µl was added to the 18µl of puReTaq Ready-To-Go PCR Beads (Illustra 27-9557-02) mixed with 1µl of dye. The PCR conditions used are listed below:

Initial Denaturation	95°C	5 Minutes	
Denaturation	95°C	30 Seconds	30 Cycles
Annealing	61°C	30 Seconds	
Extension	72°C	1 Minutes	

Final extension 72°C 5 Minutes

Store 4°C

2.10. Gel electrophoresis

The PCR amplicons were run on an agarose gel (1.5% DNA, 0.8% RNA) to separate the amplified bands. Two-grams of HiRes standard agarose (AGTC Bio products Ltd., Hull, England) was added to 200ml of 1% TBE (recipe in Appendix Section 7.2) and boiled in the microwave for 2-3 minutes at 900 volts until completely dissolved. Gel was casted in the mould after addition of 30µl of 100% ethidium bromide (Thermo Fisher Scientific, Waltham, USA). The samples were injected in the wells of the agarose gel and separated using 260 Volts for 30 minutes.

RNA samples were also analysed on agarose gels to check integrity and purity. The gels and buffers were prepared by the same method explained above but DEPC-treated water was used; instead of autoclaved sterile water as used for DNA gels. Pure RNA samples were heated to 65°C for 5 minutes before being loaded onto the gels. RNA bands were separated at between 50 and 90v for 50 minutes. Both DNA and RNA gels were visualized under UV using a G-BOX (Syngene, Cambridge, UK).

2.11. TOPO cloning and transformation

bla_{NDM}, bla_{KPC}, bla_{OXA-48} and *acpP* constructs were prepared using topo TOPO™ TA Cloning™ Kit (Thermo Fisher Scientific, Waltham, USA). For *acpP*, the full gene was synthesized with DNA Strings™ from Invitrogen, Thermofisher (Thermo Fisher

Scientific, Waltham, USA). Initially, PCR was performed using Mastermix and the instruction described in Section 2.9.1. A total volume of 6µl of cloning reaction mix was prepared by mixing 2µl of the PCR product with 1 µl of salt containing 1.2 M NaCl and 0.06 M $MgCl_2$, 1µl of TOPO vector (pCR™2.1-TOPO®) and 2µl of water. After incubating at room temperature for 5 minutes the salt was filtered by placing the cloning mixture on 0.2µM Millipore filter discs (Merck, Nottingham, UK) placed on a petri dish filled with water for 10 minutes. Concurrently, competent cells were prepared by spinning down 2 ml of overnight grown DH5α in MHB at 13,000 rpm for 1 minute. The cells were washed by removing the supernatant and suspending the pellet in 1ml of ice-cold MG water. The process was repeated twice and the final pellet re-suspend in 80µl of water and immediately added to the filtered TOPO cloning mixture. The cells were transferred to a gene pulsar cuvette and exposed to pulse under bacteria Ec3 setting (3.00 KV for 5.90 miliseconds) using a MicroPulser electroporator (Bio-Rad, California , USA). Pre-warmed 400 ml LB medium was immediately added to the cuvette and the cells were allowed to recover for 60 minutes at 37°C. Various volumes (50µl, 100µl and 200µl) were plated on LB Agar plates containing 65µg/ml ampicillin, 100mM IPTG and 40mg/ml Xgal (Sigma-Aldrich, St. Louis, USA). Approximatly 10 single colonies per reaction were propagated by re-plating and the cloning frequency confirmed by PCR and sequencing.

2.12. Qiagen DNA extraction and sequencing

The PCR amplified DNA was extracted using Qiagen DNA extraction kits (Qiagen, Limburg, Netherlands). The desired band was cut from the agarose gel and placed in

a bijou and immersed in QG buffer of an equivalent volume to approximately 3x the volume of the agarose gel containing the amplicon. The agarose and buffer were incubated for 30 minutes in QG buffer and 700µl was transferred to QIAquick columns placed in 2ml collection tubes. After centrifugation for 1 minute at 13,000rpm, the flow-through was discarded and the process repeated by adding the remaining QG buffer. The columns were washed by adding 750µl of Buffer PE, with resting for 2 minutes at room temperature and centrifuging for 1 minute at 13,000 rpm. The columns were washed/centrifuged again and the cartridge transferred to a sterile 1.5 ml Eppendorf. The DNA was eluted from the cartridge by applying 50µl of molecular grade water (pH 7.0) to the column and centrifuging for 2 minutes at 13,000rpm. The concentration of purified DNA was determined using a spectrophotometer (Jenway, Staffordshire, UK) and sent to Eurofins MWG operon (Eurofins, Ebersberg, Germany) following submission guidelines. The DNA was diluted to concentrations of 2ng/µl for amplicons of length 150-300bp, 5ng/µl for amplicons 300-1000bp and 10ng/µl for amplicons of over 1000 bp). 15µl of the purified DNA was mixed with 2µl of the primer at concentration (10µM) and sent to Eurofins for sequencing. The quality of the sequences was analysed by the supplied Chromatograms and further analysed using NCBI and Geneious (version 8.0).

2.13. RNA isolation

For *acpP* expression analysis, an overnight culture was grown in MHB, diluted 10-fold, and treated with a sub-inhibitory concentration of PNA. The cells were harvested after an additional growth period of 4 hours at 37°C, shaking 200 rpm and the RNA was extracted using the RNeasy Mini Kit (Qiagen, Limburg, Netherlands).

Two ml of cell culture was vortexed briefly and centrifuged for 5 minutes at 13,000rpm. The supernatant was discarded and the pellet was re-suspended in 600 μl of buffer RLT with 1% (V/V) of β-mercaptoethanol. After adding 600μl of 70% ethanol, the suspension was mixed by gently pipetting up and down and up to 700 μl was transferred to an RNeasy spin column which was placed in 2 ml collection tube. The columns were centrifuged for 15 seconds at 8000g and the flow-through was discarded. The process was repeated by adding the remaining suspension to the columns, centrifuging and discarding the supernatant. Another round of centrifugation for 15 seconds at 8000g was followed by the addition of 350μl buffer RW1.DNase incubation mix, which was prepared by mixing 10μl of DNase stock solution with 70μl buffer RDD, was added to the columns. After incubation at ambient temperature for 30 minutes, 350μl of buffer RW1 was added and centrifuged for 15 seconds at 8000g. Three rounds of centrifugations were performed with 500μl RPE to remove the residual RPE. The RNA was eluted by adding 50μl MG water (pH 7.0) and centrifuging the spin columns for 2 minutes at 8000g. The purified RNA concentration was determined using a spectrophotometer (Jenway, Staffordshire, UK) and the integrity was assessed by running the RNA on an agarose gel. The samples were kept at -80.

2.14. Reverse Transcription and cDNA synthesis

The purified RNA was reverse transcribed to cDNA using the High Capacity RNA to cDNA kit (Applied Biosystems, California, United States). The concentration of all samples was adjusted prior to the reverse transcription reaction. For each sample, a 20μl reverse transcription reaction mix was prepared in 200μl PCR tubes by adding

10μl of 2 x RT buffer, 1μl of 20 x enzyme mix, up to 2μg of RNA sample and adjusting the remaining volume with water. A negative control (-RT) reaction was also prepared by the addition of enzyme mix. All samples were incubated at 37°C for 60 minutes and the reaction was stopped by heating to 95°C for 5 minutes. The resultant cDNA was saved at -20°C for long term storage.

2.15. Quantitative Real-Time PCR

Real-Time Quantitative Reverse Transcription PCR (RT-qRT-PCR) was used to evaluate the differential expression levels between the clone and wild-type *acpP* strain.The 16s RNA housekeeping gene was used as an internal control in all experiments. Primers, probes and assays were designed by primer express software (full list provided in Appendices). "Dual-labelled" probes with quencher and fluorescence dye FAM-MGB probes (Applied Biosystems, Carlsbad, CA) were used in all experiments. The optimum concentration of cDNA was determined by amplifying different dilutions and 2-10μg was found to be the ideal concentration. with the supplied condition of Taqman fast advanced mastermix. The reaction mix was prepared by adding 10μl of 20 x Taqman fast advanced mastermix (Applied Biosystems, Carlsbad, USA) to one-microliter of 20x Custom TaqMan® Gene Expression Assay and adjusting the remaining 9 ul with 2-10μg of DNA and DNase/RNase free water. The experiments were performed in ViiA™ 7 (Applied Biosystems, Carlsbad, CA) by initially holding at 50°C for 2 minutes and then 95°C for 20 seconds, followed by 40 cycles at 95°C (denaturing) for 1 second and 60 °C annealing and extension for 20 seconds. Each reading was measured in triplicates and the average cycle threshold (Ct) values were calculated for each gene

expression system and normalised by subtracting the Ct values of 16s RNA. The relative mRNA difference between treated and untreated or cloned and-wild type was calculated by 2-Δct method using Microsoft Excel 2015-16 (Microsoft, Redmond, USA).

2.16. Conjugation experiments

Conjugation experiments were performed using *E. coli* 83092 (bla_{NDM}) *E. coli* 12F65 (bla_{OXA}) and *E. coli* 10E29 (bla_{KPC}) as donors and *E. coli J53* (azide^{R}) as a recipient. Prior to the mating experiments, the presence of resistance genes was confirmed by PCR analysis. Carbapenem resistant samples were grown on chromogenic media plates with 0.5µg/ml meropenem (AstraZeneca, London, UK) and *E. coli J53* on chromogenic media with 100µg/ml sodium azide. Pure cultures were propagated by picking a single colony and inoculating in 10ml of LB broth for incubation at 37°C for 18hrs, with shaking at 200rpm. Mating was undertaken by mixing 1.5 ml of overnight culture of the carbapenem resistant strain with 1ml of *E. coli J53* bacterial culture and 2ml of LB broth. After incubation for 18hrs at 37°C, 10µl was used to inoculate plates containing 100µg/ml sodium azide and 0.5µg/ml meropenem to select for transconjugates. Single colonies were subcultured and analysed for the presence of resistance genes by PCR.

2.17. Passage experiments

Bacterial cultures were processed through a series of serial passages to assess for the development of resistance by exposing to increasing concentration of PNAs.

Initially, micro-broth MIC assays with MH broth were performed. Subsequently, the last well with growth and the first well with no growth were mixed and incubated for 3 hours at 37°C which was then used to inoculate the next micro-titre plate. The culture was used to inoculate chromogenic agar after every micro-broth MIC experiment to check for purity. The process was repeated for 7 days or until the MIC reached 128μMol. The resultant resistant strains and the original starting culture were sent to Uppsala University, Sweden for sequencing and were analysed by Douglas Hughes for identification of acquired mutations and further analysed by myself using Geneious R8 version 8.1 (Biomatters Ltd, Auckland, New Zealand) as explained in section 2.21.

2.18. FoR assay

Frequency of resistance (FOR) rate was determined in micro-well plates as an alternative approach to "Luria–Delbrück" experiment (the fluctuation test) due to PNA's binding nature. Initially, 20 independent bacterial cell cultures of *E. coli ATCC 25922* and *K. pneumoniae ATCC13883* were grown overnight without selection in 10ml MHB. The cultures were used to inoculate a micro-well plate with approximately 10^7 cfu/well and PNAs (EBL110, EBL111, EBL232 and EBL237) were added at X4-8 higher concentrations than the original MICs. The results were recorded over a 2-day period of incubation at 37°C where an OD of >0.4 was considered as growth. Serial dilutions of each culture were inoculated onto drug-free MH media to count the number of colonies in each well and ciprofloxacin FoR assays were performed in parallel as a control. The mutation rate was calculated using equation below:

mutation rate = - (1/N) x Natural log of P0

mutation rate is per cell per replication cycle (generation)

N is the number of viable cells

P0 is the proportion of cultures giving rise to no mutants of the type being scored (i.e. number of wells where no significant net growth occurs).

2.19. REP PCR typing

Selected similar isolates, recovered from Karachi and Peshawar samples, were analysed by REP-PCR for phylogenetic relationships. Overnight grown cultures were acquired by heating at 95°C for 10 minutes and centrifuging for 5 minutes at 13,000 rpm and the supernatant used as a DNA template. The PCR reaction mix was prepared in ready-to go beads by adding 1μl of DNA suspension to 16μl molecular grade water, 1.5μl Primer 2L and primer 1R (each at 20nmol concentration) and 1μl loading dye. The conditions used were as described in Versalovic et al. 1991 with the following modifications:

Initial Denaturation	95°C	5 minutes	
Denaturation	95°C	30 seconds	35 Cycles
Annealing	40°C	1 minutes	
Extension	65°C	8 minutes	
Final extension	65°C	16 minutes	
Store 4°C			

Electrophoresis gel was prepared using 2% agarose gel at 240 V and run for 140 min in 1xTBE buffer containing 30µL of ethidium bromide. Gels were visualised and photographs recorded under UV light.

For analysis, gel images were uploaded to GelCompar II version 6.6.11 (Applied Maths NV [available from http://www.applied-maths.com/bionumerics]). All amplicons were normalised using the SmartLadder MW 1700-02 molecular weight marker (Eurogentec, UK). The variations in band intensity were not considered to be significant and DNA fragments detected by the software were carefully verified by visual examination. Levels of similarity between the banding patterns were calculated by the Dice coefficient. Dendrograms for each species were produced by the unweighted pair group method with arithmetic averages (UPGMA).

2.20 Sequence Typing

FumC-FimH (CH) typing was used for sequence type assignments of *E. coli* as previously described (Weissman et al. 2012). Full MLST of seven house-keeping genes including *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) *and recA* (ATP/GTP binding motif) was performed whenever CH typing did not resolve the ST assignment (Wirth et al. 2006). PCR analysis was performed using Extensor Hi-Fidelity PCR Master Mix (Thermofisher AB-0794/B) and further processed by following the

protocol described in section 2.8 and 2.9 of this chapter. Purified DNA concentration was measured using a NanoPhotometer (IMPLEN, Germany) and products with concentrations above 5 ng/µL were sent to Eurofins MWG Operon (Eurofins, Germany) for sequencing. Geneious was used to assign a sequence number by comparing with *FumC* and *FimH* database which was acquired from webpage http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/ and Veronika Tchesnokova (Evgeni Sokurenko lab; veronika@uw.edu). Veronika also assigned the sequence types based on CH typing.

For *K. pneumoniae*, whole genomic DNA was sequenced following the protocol in section 1.19 and sequence typing was assigned using scheme defined by (Diancourt et al. 2005; Brisse et al. 2009). The protocol used seven housekeeping genes: *rpoB* (beta-subunit of RNA polymerase), *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *infB* (translation initiation factor 2) and *tonB* (periplasmic energy transducer). Geneious software version 8.1 (Biomatters Ltd, Auckland, New Zealand) was used to assign the ST by blasting against the *K. pneumoniae* database which was acquired from web page http://bigsdb.pasteur.fr. The new ST types for both *K. pneumoniae* and *E. coli* were added to the database following instructions from the curators.

2.20. Whole genome sequencing and analysis

Whole genomic sequencing was carried out by in-house sequencing facility (MiSeq). Total genomic DNA (gDNA) was extracted from an overnight culture (2ml) using commercially available column-based Qiagen DNA extraction kits. Following

extraction gDNA was eluted in molecular-grade water and quantified by fluorometric methods using a Qubit (ThermoFisher Scientific). Quality ratios of gDNA (A260/280 and 260/230) were determined using the Nanodrop (ThermoFisher Scientific). gDNA libraries were prepared for whole genome sequencing using the NexteraXT kit (Illumina). Briefly, input gDNA was normalised (to ensure all bacterial DNA samples were the same concentration) and the DNA tagmented. Following tagmentation of the DNA, the samples were prepared for PCR amplification, whereby each bacterial DNA sample was allocated individual tags for downstream multiplexing. Using AMPure beads to allow size selection, the PCR clean-up step effectively removes unwanted fragments of DNA. After the remaining DNA libraries were normalised each individually tagged sample was pooled and a known quantity of sequencing control (PhiX) added. Paired end sequencing was performed using the Illumina MiSeq platform (MiSeq Reagent V3 Kit; 2x 300 cycles). For each *E. coli* isolate, at least 80x coverage was generated. Raw sequence reads were trimmed using Trim Galore and the genomes were *de novo* assembled into contigs using SPAdes (3.9.0) with pre-defined kmers set. Further analysis was performed using Geneious R8 software version 8.1 (Biomatters Ltd, Auckland, New Zealand).

2.21. Sequence alignment and comparison

All the bioinformatic functions required for this study were performed by Geneious software version 8.1 (Biomatters Ltd, Auckland, New Zealand). The contigs were assembled by the de-novo assembly tool using the default settings defined in Geneious. For PNA mutation analysis, the mutated strains were sequenced by

Uppsala University, Sweden, and analysed by mapping the sequences to the reference genes.

The target sequence of PNA was selected by multiple alignment of all the gene variants and selecting a common complementary ATP start region as target for PNA synthesis. Though the sequences were provided by myself, the specific 12bp PNA sequence was selected by our collaborators in Copenhagen (Prof. Peter Nielson). For NDM and KPC PNA's, all the available gene variants on Lahey web-page (https://www.lahey.org/studies/other.asp#table1) were aligned using the multiple alignment function available in Geneious R8 with default settings. For class 1 integrons expressing VIM genes, PNA's were synthesised by aligning VIM-1 and VIM-2 genes and the promotor and start site of integrases. Due to the abundance of OXA48-like genes, only a set of representative genes were selected and aligned to extract complementary regions for PNA synthesis.

2.22. Statistical Analysis

The statistical analysis for Peshawar study was performed using IBM SPSS statistics (Version. 23). Quantitative variables such as age, duration of hospital stay, length of stay in hospital were grouped based on the values obtained for each variable in the questionnaires. Analysis was performed on the detection of bla_{NDM}, $bla_{CTX-M-15}$ and bla_{OXA-48} like gebes. bla_{KPC} was exclude for all analysis as no positive samples were detected. Crosstabulations with Pearson chi-square and Fisher's exact test (when

appropriate) were used to perform univariate analysis on the distribution of the resistance genes and their association with different variables. Variables with statistically significant associations to the resistance genes were selected for multivariate analysis by binary logistic regression. Variables such as residence and type of surgery were excluded from statistical analysis since those couldn’t be grouped in sub-categories. P- values of <0.05 were considered as significant.

3. Environmental Contamination of MDR (bla_{NDM} and bla_{CTX-M}) in Karachi, Pakistan

3.1. Introduction

Earlier studies on β-lactam resistance focused on the presence of MDRB in healthcare settings and it was generally believed that patients are the main carrier and distributors of AMR genes (Carmeli et al. 2010). The increasing incidence of MDR found in normal faecal flora is concerning as MDR can widely spread in the normal microbiota and has disseminated in large populations and act as reservoirs (Woerther et al. 2013). In addition, the increased contamination of antibiotics in the environment has led to AMR genes spreading in non-clinical settings with direct and indirect effects on the overall emergence and distribution of AMR. For example, besides humans, β-lactams are widely consumed in veterinary medicine and the resistance arising in livestock has great potential to spread to insect pests and other wild animals (Woodford et al. 2014). Indeed, several studies have found that wild-birds, animals and insects carry antibiotic resistance genes and could transfer it to distinct environments. For example, a study on wild birds in the Netherlands found that 65 birds (15.7%) from 21 different species contained ESBL or AmpC-type genes (Veldman et al. 2013). In Bangladesh, a higher resistance rate of approximately 30% ESBLs was reported in wild and domestic birds (Hasan et al. 2012).

Environmental antibiotic contamination is a global issue; however, in LMICs it is more concerning due to the general increase in antibiotic consumption and poor health infrastructure, unhygienic living conditions and lack of waste management which could leads to unprecedented MDR spread (Raka 2010; WHO 2014b). Therefore, an environmental prevalence study combined with clinical data on the status of MDR in LMICs is important. Furthermore, it is also not known to what extend all these region-specific features influence the MDR rate and in-depth

analyse of these factors could be essential in determining the major risk factors associated with the carriage of antibiotic resistance. A few small studies have reported the characterisation of AMR bacteria from LMIC non-clinical and hospital environmental samples, but an extensive study exploring AMR in an holistic and systematic manner has not been reported (Rahuma et al. 2005; Macovei and Zurek 2006).

In this chapter, samples are collected from Karachi which is one of the largest cities in Pakistan with a population of over 21 million. Besides overcrowding, Karachi faces additional problems with waste management, sanitation and poor infection control. Clinical samples were collected from patients visiting the Civil Hospital Karachi whereas non-clinical samples were collected from insects, hospital surfaces, drinking water supplies and birds or small animal droppings. CTX-M-15 genes were used as marker for ESBLs prevalence whereas NDM genes were used as a marker for the resistance rate to carbapenems. The results will provide an overview of MDR contamination in different environments of a representative city in LMIC. Furthermore, the clinical data will provide vital information about the prevalence rate of MDR in hospital settings and the effects of environmental contamination on the carriage of MDR in patients.

The clinical samples of this chapter were processed by Ammara Mushtaq and Maria Carvalho and colleagues whereas the environmental samples were processed by myself.

3.2. Results

3.2.1. Molecular detection of *bla*$_{NDM}$ and *bla*$_{CTX-M}$ in clinical samples from Civil Hospital Karachi, Pakistan

Overall, 1893 patients were enrolled in this study from which 1691 patient's rectal swabs and 285 site of infection samples from 191 patients were processed and analysed for the presence of *bla*$_{NDM}$. Site of infection samples (SOI) were also analysed for the presence of *bla*$_{CTX-M-15}$; however, as the prevalence of *bla*$_{CTX-M-15}$ among rectal samples in Karachi was unexpectantly high so we focused on *bla*$_{NDM-1}$ only for further analysis and a sub-cohort of 764 patient's rectal swabs randomly selected from the total study population of 1046 swabs with 630 samples at admission (FSA) and 416 samples at discharge (FDA) were analysed for the presence of *bla*$_{CTX-M-15}$. The results showed that 24% (n=400) FSA , 28% (n=481) FSD and 10% (n=31) SOI samples were positive for *bla*$_{NDM}$ whereas 69% (n=527) FSA , 44% (n=338) FSD and 38% (n=72) SoI samples were positive for *bla*$_{CTX-M-15}$ (Figure 14).

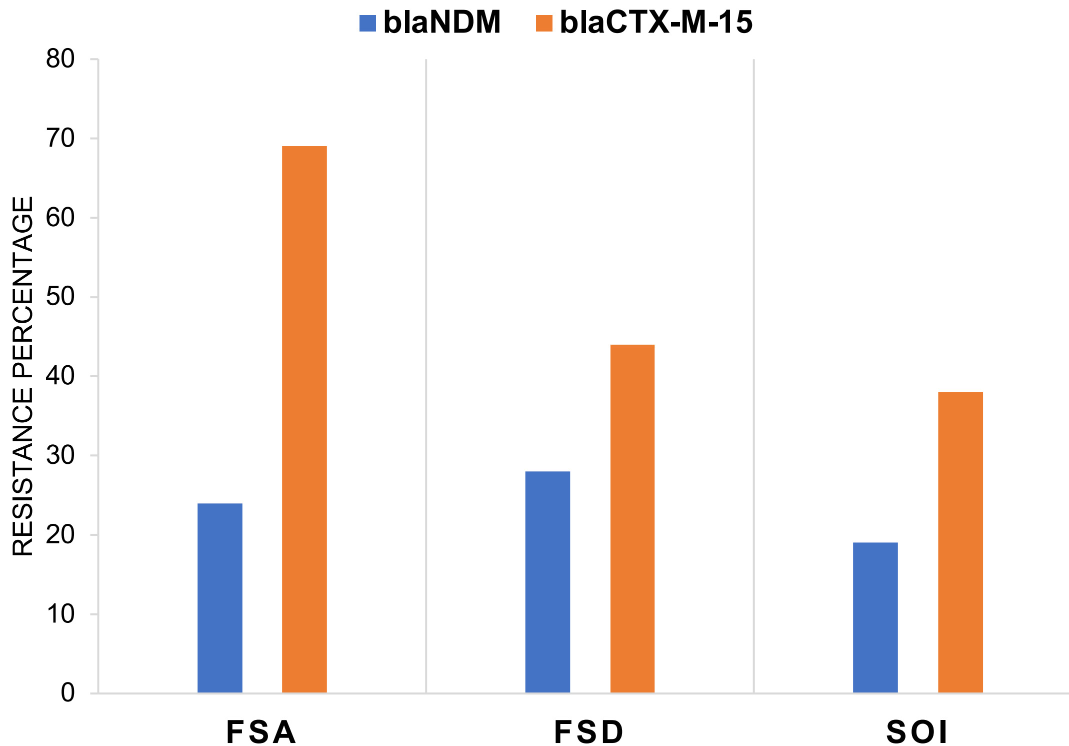


Figure 14. Occurrence of bla_{NDM} and $bla_{CTX-M-15}$ in patient's samples from Karachi, Pakistan. The graph shows percentage of PCR positive samples. Rectal swabs at admission are denoted as "Faecal Samples at Admission" (FSA), at discharge as "Faecal Samples at Discharge" (FSD) and infection samples as "Site of Infection" (SOI).

3.2.2. Molecular detection of bla_{NDM} and bla_{CTX-M} in non-clinical samples from Karachi, Pakistan

A total of 729 environmental samples were obtained from Karachi, Pakistan in 2013. Among the 347 hospital surface swabs, 22.76 % (n=79) were positive for bla_{NDM} and 11.81 % (n=41) for $bla_{CTX-M-15}$ whereas 6.34 % (n=22) samples were positive for both bla_{NDM} and $bla_{CTX-M-15}$ (Figure 15). The positive samples showed an association with a variety of places and certain wards, for example Surgery and Orthopaedics, had

the highest number of AMR bacteria but, no specific links between wards was identified (Table 6 and 7).

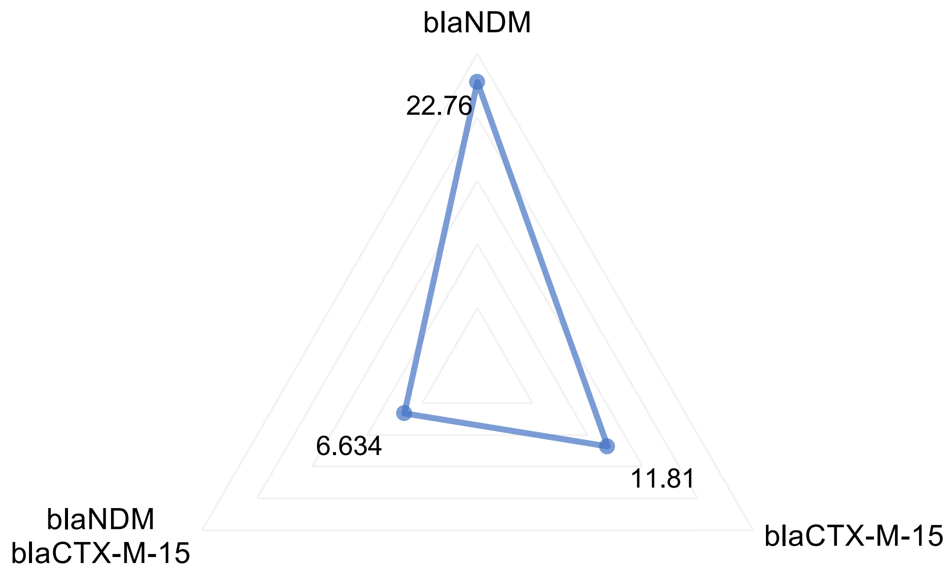


Figure 15. Occurrence of *bla*$_{NDM}$ and *bla*$_{CTX\text{-}M\text{-}15}$ in hospital surface samples from Karachi, Pakistan. The radar graph shows percentage of *bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{NDM}$ PCR-positive samples.

Table 6. Occurrence of *bla*$_{NDM}$ in surface samples from Civil Hospital, Karachi.

Ward	*bla*$_{NDM}$ PCR-positives	Sampling Site
Burns	3	Bedside table, fire extinguisher, patient file
Emergency	1	Floor
ENT	1	NG tube
ICU	1	Floor
Medicine	5	Bed linen, nursing counter, oxygen mask
MICU	3	Bed linen, Stairs, stretcher, lunch trolley
O&G	11	Dust bin, curtain
Orthopaedics	3	Bed metal, food trolley, window
Paediatrics	2	Bed linen
Surgery	11	Floor, table, medicine counter, dust bin, bedside table, envelope, CT scan, floor, bed linen, stretcher

ENT: Ears, Nose & Throat; ICU: Intensive Care Unit; MICU, Medical ICU; O&G: Obstetrics & Gynaecology.

Table 7. Occurrence of *bla*$_{CTX-M-15}$ in surface samples from Civil Hospital, Karachi.

Ward	*bla*$_{CTX-M-15}$ PCR-positive	Sampling Site
Burns	3	Bed linen, bedside table, patient file
Emergency	2	Table
ENT	1	Pillow case
ICU	4	Floor, nursing counter, weighing machine
Medicine	16	Bed linen, nursing counter, bed adjustment handle, drawer, medicine trolley, nursing counter, oxygen mask, pillow case, light during procedures, lunch trolley, storage shelf, stair railing, stretcher, switch board
MICU	1	Stairs
O&G	8	Corridor, dust bin, curtain, dust bin, stairs railing, visitor's chair, floor, nursing counter
Orthopaedics	18	Bandage on patient's leg, bed adjustment handle, blood bag, curtain, door handle, drawer, envelope x-ray, foley's catheter, kettle, medicine trolley, o2 cylinder knob, oxygen mask, phone on nursing counter, plastic for patient's support, switch board, tape to secure cannula, tape to secure cannula, trolley
Paediatrics	7	Bed linen, door handle, drip bag, lunch trolley, pillow case, switch board
Surgery	13	Floor, lunch table, medicine counter, dust bin, lunch table, bedside table, patient file, door handle, iv line, patient file
Ultrasound	6	Chair waiting area, door handle, gel bottle, stretcher, switch board, window

ENT: Ears, Nose & Throat; ICU: Intensive Care Unit; MICU, Medical ICU; O&G: Obstetrics & Gynaecology.

Among insects (n=193), five samples (3 %) carried *bla*$_{NDM}$ and 18 (9%) were positive for *bla*$_{CTX-M-15}$. *bla*$_{NDM}$ were carried by 2 ants (*Lasius niger)*, 2 cockroaches (*Blattella asahinai*) and a fly (*Musca domestica*) whereas *bla*$_{CTX-M-15}$ were detected mostly in flies (Table 8).

Table 8. Location of *bla*$_{NDM}$ and *bla*$_{CTX-M-15}$ in Insects from Civil Hospital, Karachi.

Location of samples	*bla*$_{CTX-M-15}$ PCR-positives	*bla*$_{NDM}$ PCR-positives
Common Hospital Areas	8	4
Medicine	3	0
O&G	2	0
Paediatrics	1	1
Surgery	4	0

O&G: Obstetrics & Gynaecology.

Among the 92 small animals and bird's faeces, a Hawk's sample was found to be positive for *bla*$_{NDM}$ and 22 (20 %) other different birds' faeces carried *bla*$_{CTX-M-15}$ (Table 9).

Table 9. Occurrence of $bla_{CTX\text{-}M\text{-}15}$ in birds' faeces. All birds dropping collected from either outside the hospital or caged birds in the markets near the hospital.

Species	$bla_{CTX\text{-}M\text{-}15}$ PCR-positives
Crows	3
Eagles	2
Fowls	2
Peacocks	2
Pigeon	1

Among the total 123 water supply units tested, eight (7 %) were found PCR-positive for $bla_{CTX\text{-}M\text{-}15}$ and seven (6 %) for bla_{NDM}. Two of the drinking water sites, Essa-Nagri and Jail-road, were both positive for bla_{NDM} and $bla_{CTX\text{-}M\text{-}15}$. Most of the contaminated water supply units are within proximity (approximately 6-kilometres distance) to each other (Figure 16).

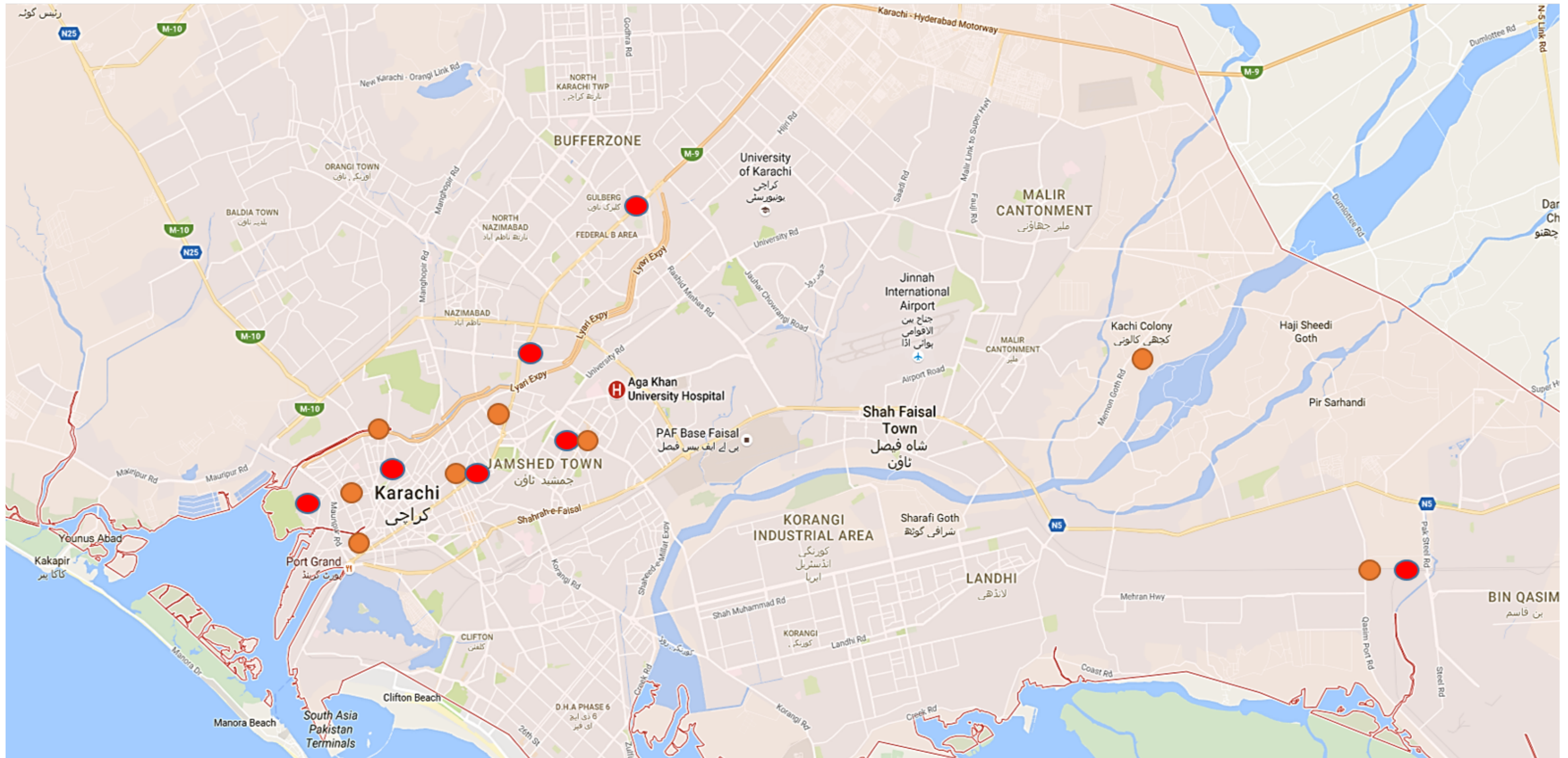


Figure 16. **Map of the areas with contaminated water supply.** The above image shows the 80-kilometer radius of Karachi city where the drinking water samples were collected. The areas highlighted in orange are the sites where *bla*$_{CTX-M-15}$ was detected and the areas in red were positive for *bla*$_{NDM}$.

3.2.3. Identification of NDM- and CTX-M15-positve bacteria

From the insects and birds, I attempted isolating bacteria carrying *bla*$_{CTX-M-15}$ and *bla*$_{NDM}$. Although there were more insects which came up positive for the carriage of NDM and CTX-M-15 genes by performing PCR on the overnight broth culture, no growth on plates were achieved and hence no MDR bacteria could be isolated. Only one *bla*$_{CTX-M-15}$ carrying sample from a fly (*Musca domestica*), caught in the gynaecology ward, was grown on selective media and subsequently isolated and subsequently shown to be *E. coli*.

A total of 13 *bla*$_{CTX-M-15}$ positive isolates were recovered from birds' faeces samples. Eight were *E. coli*, two *K. pneumoniae* and one each of *E. cloacae, Pseudomonas fulva* and *Citrobacter amalonaticus.*

From drinking water, out of the seven *bla*$_{NDM}$ positive samples, only two *K. pneumoniae* were successfully isolated. However, from the hospital surface swabs, most of the *bla*$_{NDM}$ positive isolates belonged to the species of *Acinetobacter johnsonii.* In contrast, clinical samples revealed a wide variety of species that were responsible for the carriage of resistant genes. However, *bla*$_{NDM}$ from SOI and rectal swabs were mainly *E. coli*, Enterobacter and *Klebsiella* spp. (Figure 17) whereas *bla*$_{CTX-M-15}$ from SOI was mostly recovered from *Enterobacter, Klebsiella and Citrobacter* spp. (Figure 18).

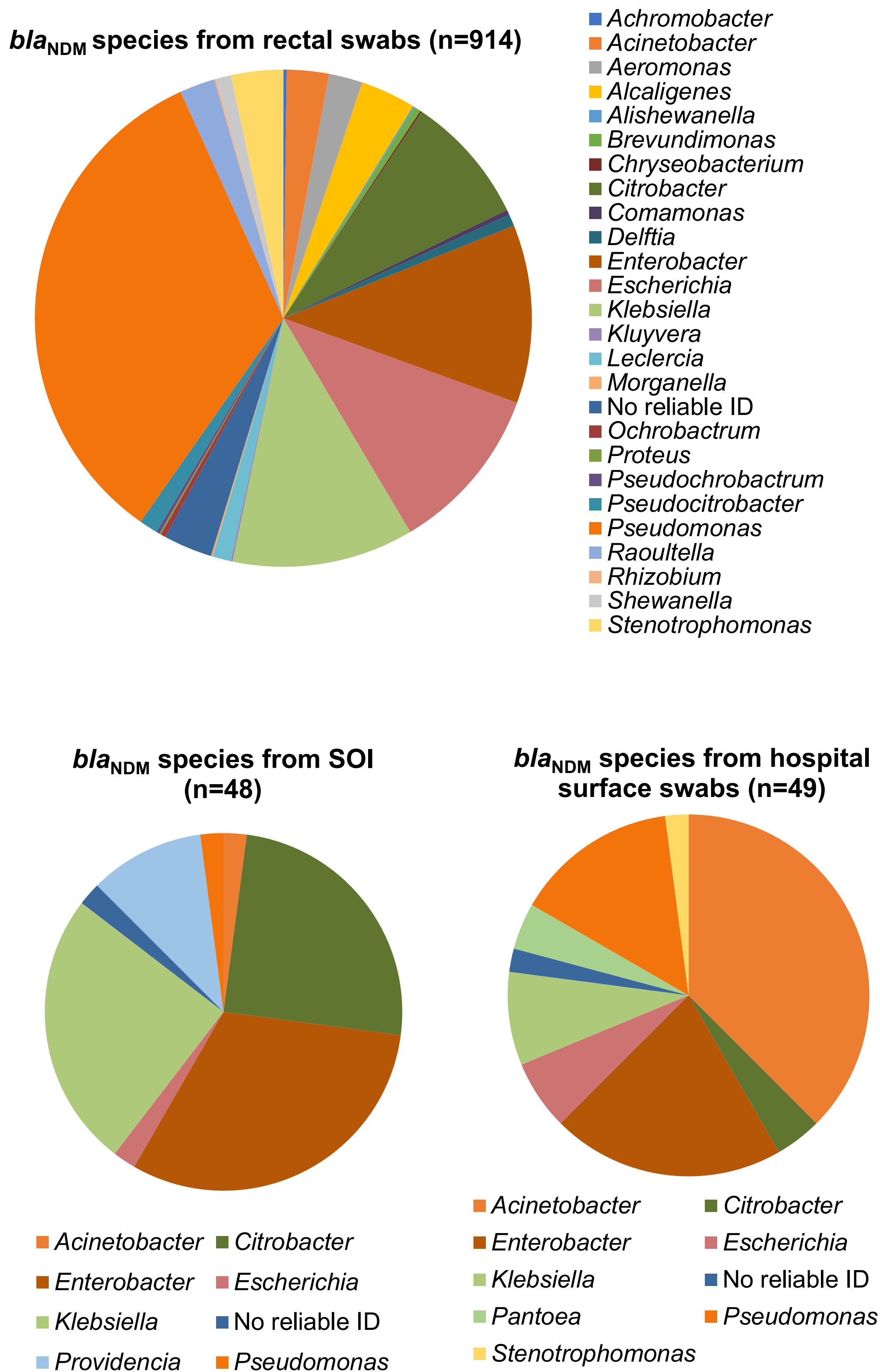
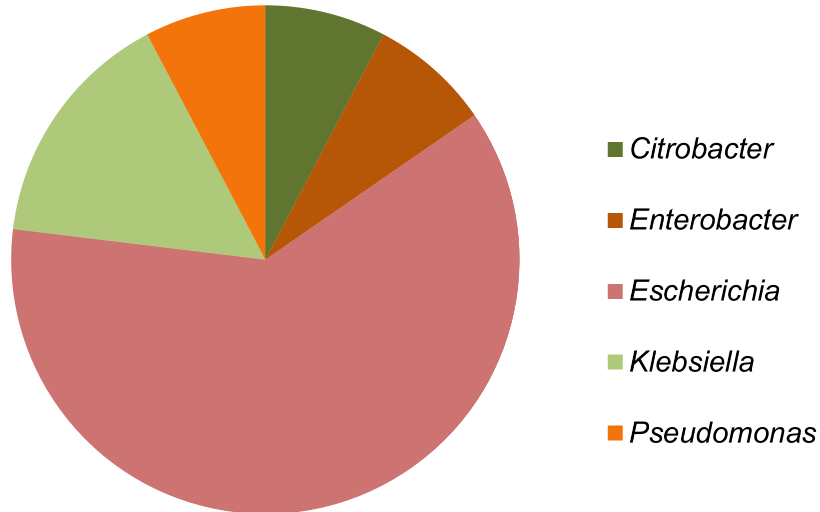


Figure 17. ***bla*$_{NDM}$ positive bacteria from Karachi clinical and non-clinical samples.** SOI (site of infection).



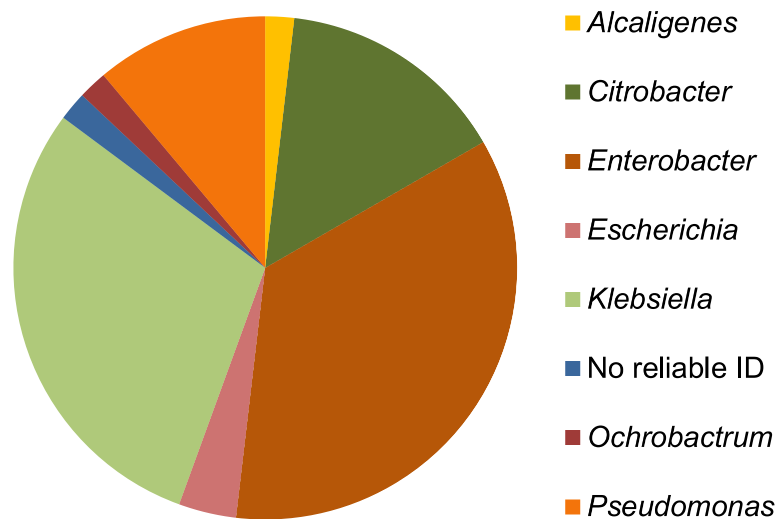
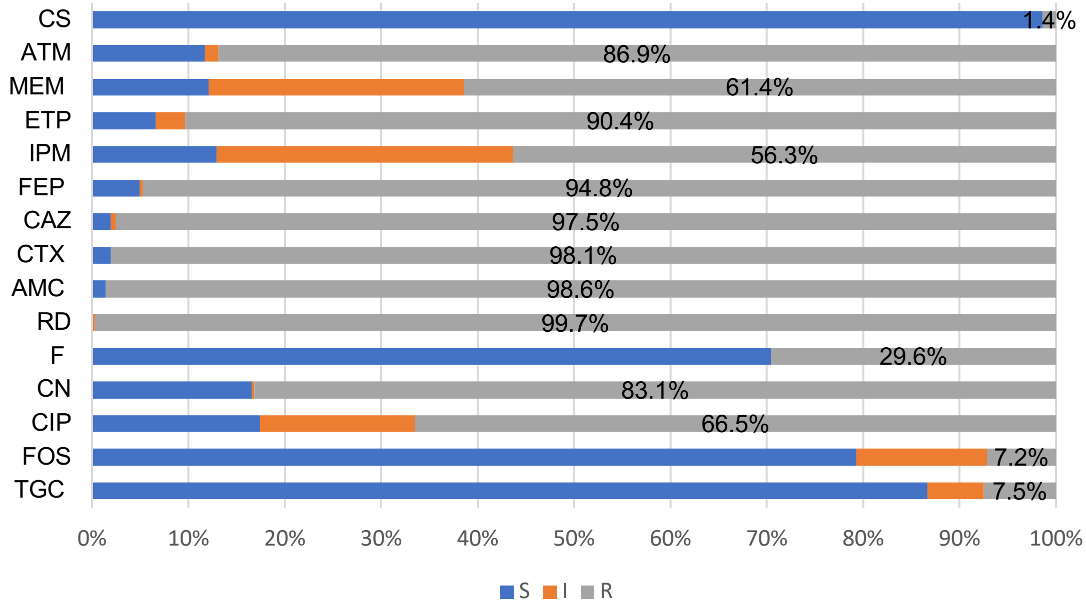


Figure 18. ***bla*$_{CTX-M-15}$ positive bacterial species from Karachi clinical and non-clinical samples.** SOI (site of infection).

3.2.4. Antimicrobial susceptibility profiles of bla_{NDM} bacteria

Antimicrobial susceptibility results for bla_{NDM} PCR-positive samples showed high-level resistance to all β-lactams. Among the carbapenems, the bacteria isolated from environmental samples were least resistant to meropenem whereas bacteria from clinical samples were most resistant to imipenem. Slightly different percentage of resistance was also observed in clinical samples to meropenem; however, bacteria from both clinical and environmental samples were found to be most resistant to ertapenem.

Isolates from all samples possessed similar resistance profiles to ciprofloxacin, rifampicin and all cephalosporins except cefepime. Bacteria isolated from clinical samples showed increased resistance cefepime, gentamicin and aztreonam compared to isolates of environmental origins. Among all antibiotics tested, bacterial isolates from both clinical and non-clinical samples were least resistant to fosfomycin, tigecycline and colistin the least resistance.



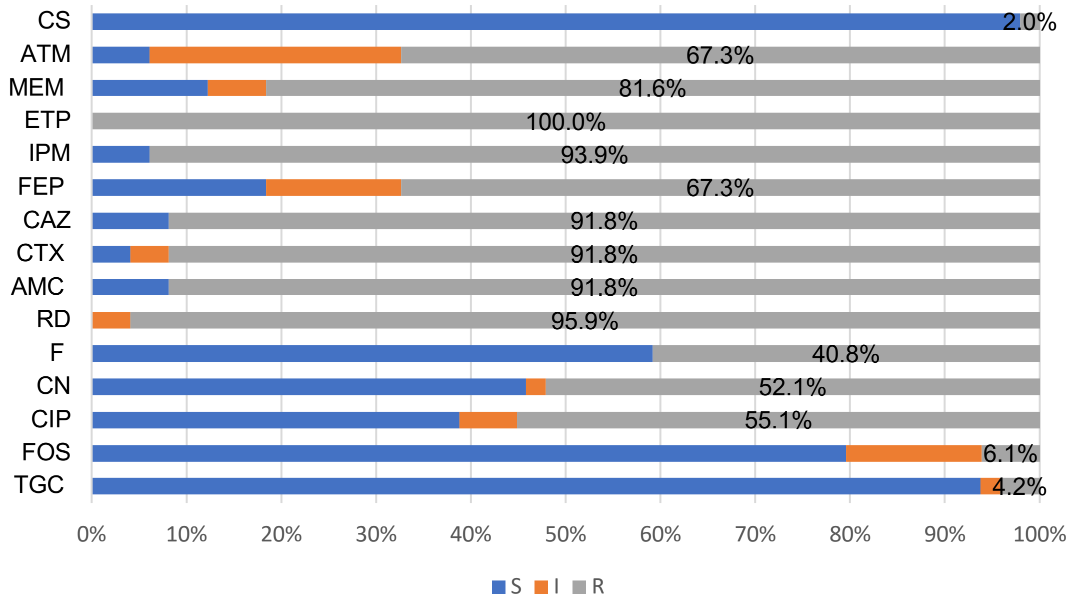
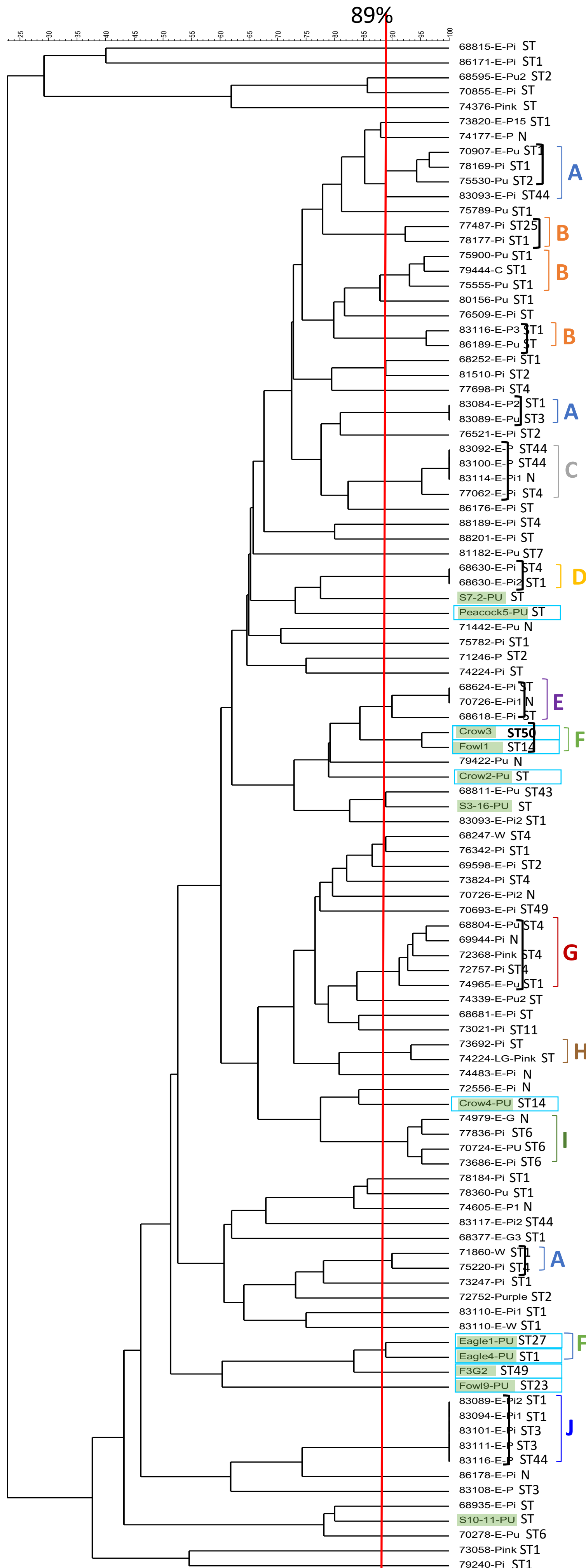


Figure 19. Antibiotic susceptibility profiles of clinical and non-clinical isolates. Percentage of resistant isolates to each antibiotic tested is labelled. TGC= tygecycline (15 µg); FOS= fosfomycin (200 µg); CIP= ciprofloxacin (5 µg); CN= gentamycin (10 µg); F= nitrofurantoin (100 µg); RD= rifampicin (5 µg); AMC= amycacin (30 µg); CTX= cefotaxime (5 µg); CAZ= ceftazidime (10 µg); FEP= cefepime (30 µg); IPM= imipenem (10 µg); MEM= meropenem (10 µg); ATM= aztreonam (30 µg); CS= colistin. (Bar colour blue=sensitive, Orange=intermediate and grey=resistant)

3.2.5. Phylogenetic analysis of environmental samples by REP-PCR typing

Similar REP-PCR profiles were shown for $bla_{CTX\text{-}M\text{-}15}$-positive *E. coli* isolates from faeces of a crow, a fowl, and two eagles. (Figure 20**)**. Two bla_{NDM}-positive *K. pneumoniae* isolates from a bed table in surgical ward-3 (S11-50) and a medical counter from a female surgical ward-1 (S7-23) also showed similar REP profiles. (Figure 21**)**. Although more than half (11 out of 17) of the Acinetobacter isolates containing bla_{NDM} had a minimum of 70% similarity, eight of the isolates were 100% identical and were mostly associated with either the orthopaedics male ward 2 or the paediatrics ward. (Figure 22**)**. The Acinetobacter isolates were cultured from bed linen (S3-18), a door handle (S3-21) in paediatric ward; a drawer (S4-13), door handle (S4-14), bed handle (S4-19), security tape of cannula (S6-2), medicine tray (S9-17) from orthopaedics male ward-2, and an ultra-sound door handle (S9-19). From the Enterobacteriacae isolates (Figure 23**)**, 3 samples from hospital surfaces had similar REP-PCR profiles with clinical samples indicating cross contamination. These isolates were cultured from stairs (SA-5), a dust bin in labour room (S11-49) and IV line in surgical ward. (S11-10).

All samples were analysed by UPGMA cluster analysis based on the Dice coefficient of REP-PCR fingerprints. Similarity is indicated as a percentage.



Escherichia coli
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Escherichia coli
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Escherichia coli
Escherichia coli

Figure 20. REP-profile of *E. coli* isolated from clinical and non-clinical samples from Karachi. The minimum similarity coefficient for two profiles being considered very similar if not the same was 89%. ST of the distinct isolates is shown

<u>Legend:</u> green squares highlight non-clinical isolates. Blue boxes indicate bla_{NDM} negative isolates of animal origin. Black brackets denote discrepancies between REP typing and sequence typing analysis. The newly identified ST5003 in an *E. coli* isolate from crow faeces is in bold.

A: Isolates with the same REP profile found in FSD from distinct patients admitted to different wards < one month.

B: Isolates with the same REP profile found in FSD from distinct patients admitted to different wards < two months.

C: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) from distinct patients admitted to the same ward at the same time and < one month; and admitted to different wards at the same time and < one month.

D: Isolates with the same REP profile found in the same sample of a patient, probably corresponding to multiple isolations of the same strain.

E: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) from distinct patients admitted to different wards < one month.

F: Isolates with the same REP profile found in bird's faeces from distinct birds.

G: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) from distinct patients admitted to the same ward and different wards < one month; and admitted to different wards < two months.

H: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) from distinct patients admitted to the same ward <1 month.

I: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) from distinct patients admitted to the same ward < two months.

J: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) from distinct patients admitted at the same time to the same ward; at the same time to different wards; to the same ward < two months.

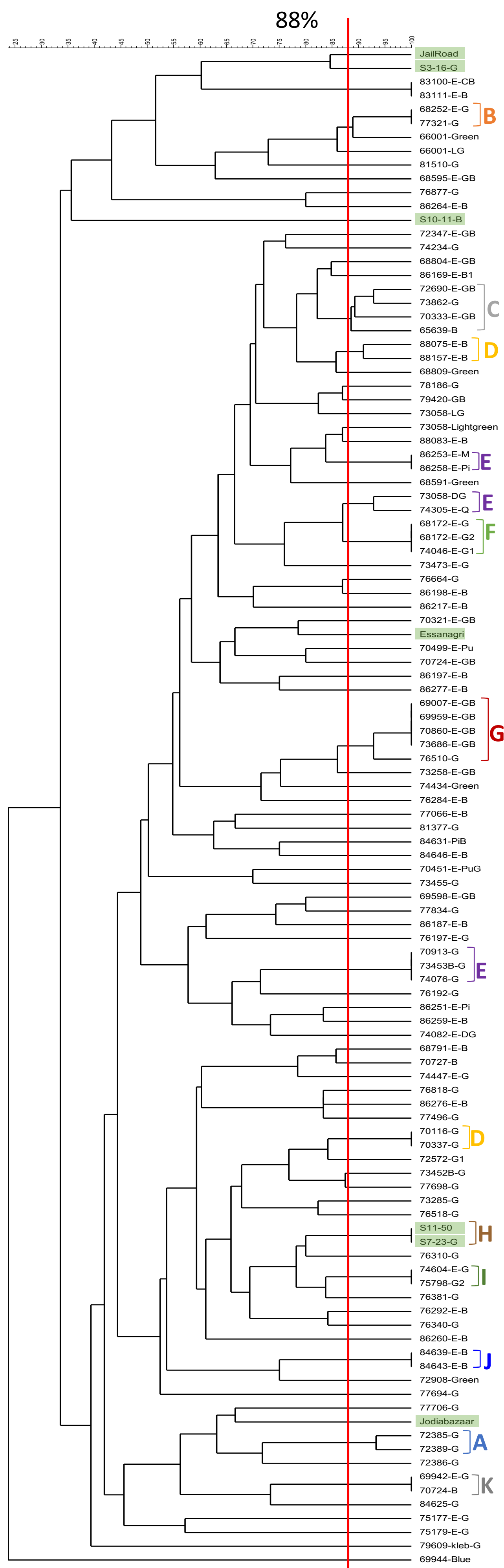

Figure 21. REP-profile of *K. pneumoniae* isolated from birds' droppings, drinking water and hospital surface samples. The minimum similarity coefficient for two profiles being considered very similar if not the same was 88%.

Legend: green squares highlight non-clinical isolates.
A: Isolates with the same REP profile found in the same type of sample (FSD or SOI) from distinct patients admitted to the same ward at the same time.
B: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) from distinct patients admitted to different wards < one/two months apart.
C: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) from distinct patients admitted to the same ward > two months; and admitted to different wards < two months apart.
D: Isolates with the same REP profile found in the same type of sample (FSA) of distinct patients admitted to different wards at the same time.
E: Isolates with the same REP profile found in the same type of sample (FSD or SOI) from distinct patients admitted to different wards < one month apart.
F: Isolates with the same REP profile found in the same sample of a patient, probably corresponding to multiple isolations of the same strain; and isolates with the same REP profile found in the same type of sample (FSA) from distinct patients admitted to different wards < two months apart.
G: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) admitted to the same or distinct wards < one month apart.
H: Isolates with the same REP profile found in distinct hospital surface from different wards.
I: Isolates with the same REP profile found in the same type of samples (SOI) from distinct patients admitted to the same ward <1 month.
J: Isolates with the same REP profile found in the same type of sample (FSD) from distinct patients admitted to different wards > two months apart.
K: Isolates with the same REP profile found in distinct types of samples (FSA and FSD) admitted to the distinct wards at the same

Klebsiella pneumoniae
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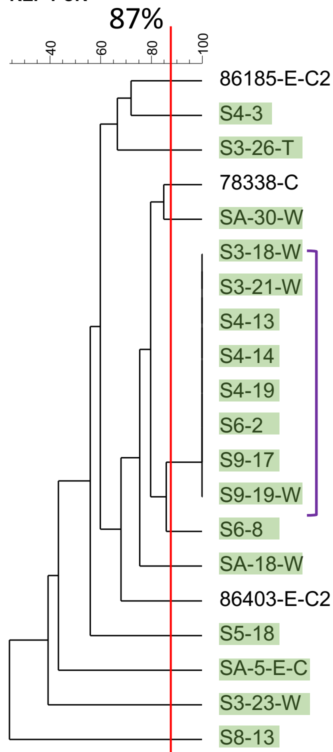


Figure 22. REP-profile of *bla*$_{NDM}$ positive *A. johnsonii* isolates. The minimum similarity coefficient for two profiles being considered very similar if not the same was 87%.

Among 20 isolates subjected to REP-PCR, 13 REP profiles were found.

Legend: green squares highlight non-clinical isolates. Purple bracket delimits isolates sharing the same REP-PCR profile that were found in related samples. These results suggest that genotypically closely related *A. johnsonii* isolates if not the same strain is present in distinct surfaces of the paediatrics, orthopaedics-2 male wards and in the ultrasound department.

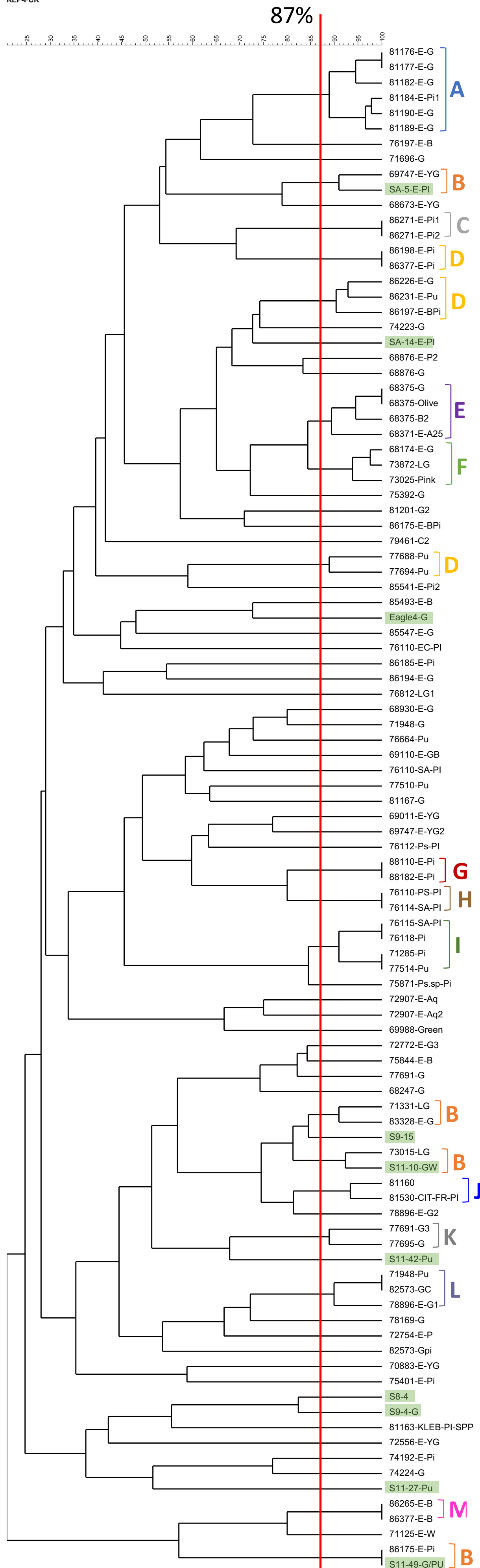


Figure 23. REP-profile of *bla*$_{NDM}$ positive *E. cloacae* isolates. The minimum similarity coefficient for two profiles being considered very similar if not the same was 87%.

Legend: green squares highlight non-clinical isolates. **A:** Isolates with the same REP profile found in different types of clinical samples (FSA and FSD) from distinct patients admitted to three different wards at the same time. **B: Isolates with the same REP profile found in different types of samples (FSA, FSD, SOI, hospital surface) in different wards and different sampling times/ < two months apart.** **C:** Isolates with the same REP profile found in the same sample of a patient, probably corresponding to multiple isolations of the same strain. **D:** Isolates with the same REP profile found in FSD from distinct patients admitted to the same ward at the same time. **E:** Isolates with the same REP profile found in the same FSA of a patient, probably corresponding to multiple isolations of the same strain and isolate from an FSA from a distinct patient admitted to a different ward at the same time. **F:** Isolates with the same REP profile found in FSA from distinct patients admitted to three different wards < two months apart. **G:** Isolates with the same REP profile found in FSA from distinct patients admitted to different wards at the same time. **H:** Isolates with the same REP profile found in SOI samples from distinct patients admitted to the same ward at the same time. **I:** Isolates with the same REP profile found in SOI samples and FSD from distinct patients; two were admitted to the same ward at the same time and the other two were admitted to different wards in the same month. **J:** Isolates with the same REP profile found in SOI samples from distinct patients admitted to different wards < one month apart. **K:** Isolates with the same REP profile found in FSD from distinct patients admitted at the same time (ward unknown). **L:** Isolates with the same REP profile found in FSD and FSA samples from distinct patients; all admitted at different wards, two at the same time and the other two months apart. **M:** Isolates with the same REP profile found in FSD from distinct patients admitted to different wards at the same time.

3.3. Discussion

South Asian countries are generally associated with increased carriage of NDM and CTX-M type genes but their spread in the environment is rarely monitored. This is the first study which is primarily associated with analysing a patient's immediate and distant environments that may contribute to their clinical impact. Different sectors in the environment may contribute differently to the dissemination of AMR but poor sanitation, waste management and lack of infection control are important factors in the increasing MDR rates that has been recently witnessed in LMICs. Based on the findings of this study, multiple pathways exist as possible transmission routes leading to the MDR bacteria transfer between patients, community and the environment. For example, hospitals lacking infection control could unwittingly augment the transfer of resistance between staff, patients and visitors. Improper waste management and poor infrastructure could lead to leakage and discharge of contaminated effluents into public drinking water supply. Animals feeding on waste or contaminated water could also retain and disperse the MDR bacteria further. All these issues are highlighted by the results of this chapter where besides patient's rectal flora and bacteria isolated from the site of infection, MDR is present in drinking water, birds, insects and the hospital environment. Remarkably, the water supply units which were contaminated with MDR bacteria (East, Korangi and central areas), were also associated with the rectal carriage of MDR bacteria (CTX-M-15 and/or NDM positives) in patients from the same area. This highlights a very serious issue of access to clean and safe water - a problem which is not uncommon for residents of highly populated cities in LMICs. Different studies have identified the presence of increased number of bacterial pathogens and virus in LMICs causing multiple diseases in public health such as cholera, diarrhoea, dysentery, hepatitis A, typhoid,

and polio (WHO 2014a; Andremont and Walsh 2015; Quintela-Baluja et al. 2015; WHO 2017). Recently, a petition was filed against the quality of water and sewage system of Karachi which was followed by a commission enquiry by the supreme court of Pakistan (Muhammad and Kalhoro 2017). The commission investigated multiple water supply units and found the majority (75 % or 251/336 samples) were unsafe for human consumption under the standards specified by WHO due to microbiological contamination or the presence of other chemicals or metals such as calcium, sodium, potassium, chloride, sulfate, fluoride, nitrate and iron (Muhammad and Kalhoro 2017). One of the main problem identified were old sewerage and drinking water supply infrastructure and the potential of mixing of sewerage and drinking water. Although the report resulted in the dismissal of the managing director for Karachi Water and Sewage Board, the city is yet to get any clean water supply and are unlikely to repair the old sewage infrastructure.

There was a considerable difference among the prevalence rate of bla_{NDM} and $bla_{CTX\text{-}M\text{-}15}$ in the samples from the hospital surface. The NDM gene showed almost double the prevalence that was observed for $bla_{CTX\text{-}M\text{-}15}$. This is not in consistent with the global trend that is generally observed with Gram-negative MDR which is almost always dominated by CTX-M type ESBLs (Kumarasamy et al., 2011b; Woerther et al. 2013). However, recently we have witnessed a considerable increase in the consumption of carbapenems, with a subsequent change in the pattern of Gram-negative resistance. According to Boeckel et al. the global consumption of carbapenems increased by 45% between 2000 and 2010 and LMICs with large populations such as India and Pakistan witnessed a 6-fold increase from 2005 to 2010 (Boeckel et al. 2014). In the same period, several species of bacteria belonging to the family of Enterobacteriaceae acquired bla_{NDM} on a broad range plasmid

(Walsh et al., 2011) which resulted in its successful dissemination worldwide (Boeckel et al. 2014). This study also identified the NDM gene in a variety of clinically relevant and environmental bacteria. Although, it can be argued that the origin of the resistant genes was the environment, their dissemination to such a variety of species is nonetheless interesting. A large proportion of *bla*$_{NDM}$ isolates (37%) recovered from surface samples belonged to the species of *Acinetobacter* and predominantly *A. johnsonii* (Figure 17). *Acinetobacter* spp. are generally found in the environment and are common commensal organisms of the microbiota. However, they can cause HAIs specially in immune compromised patients and are frequently associated with outbreaks (Manchanda et al. 2010). The increase in antibiotic resistance of *Acinetobacter species* can be linked to the increased consumption of carbapenems whereas a gradual reduction is achieved when carbapenem use is withdrawn (Ogutlu et al. 2014; Tan et al. 2015). A study by Ogutlu et al. observed a 2-fold reduction in Acinetobacter associated infections when carbapenem consumption was restricted in ICU patients for 8-months (Ogutlu et al. 2014). Regrettably, in this chapter we were unable to obtain each patient's specific therapy or generic hospital data on carbapenem consumption, and thus it is difficult to determine if the increased carbapenem usage may have caused higher incidence of *Acinetobacter* resistance. One other factor that may have effected this observation is the possibility of an outbreak - from the total 17 *bla*$_{NDM}$ *A. johnsonii* recovered, eight had identical REP-PCR profiles. Indistinguishable *A. johnsonii* isolates were identified from paediatrics, orthopaedics and surgical wards suggesting the possibility of an outbreak and highlighting the issue of inadequate infection control practices. One reason could also be the ability of *Acinetobacter spp.* to survive in dry and moist conditions for longer periods. Studies investigating the desiccation

tolerance of *Acinetobacter spp.* revealed that they can survive on dry surfaces of up to 30 days and hence, increasing the possibility of cross-contamination and spread (Jawad et al. 1996; Jawad et al. 1998). The spread of MDR strains between birds and surface was also observed; however, none of the samples from different environments had similar REP profiles suggesting an intra- rather than inter-environmental spread.

The potential of flies as a vector for MDR couldn't be fully confirmed to its full extent because of complications with the growth of bacteria as most of the bacteria from flies failed to grow on selective media despite being tested PCR-positive for the carriage of *bla*$_{NDM}$ and *bla*$_{CTXM-15}$. Similar findings were reported by Wang et al. when they screened samples from environmental and animal sources including hatcheries, commercial farms, slaughterhouse and supermarkets for *bla*$_{NDM}$ and colistin resistance gene (*mcr-1)* and direct sample testing revealed a higher number of resistance carriage compared to those seen in isolated species (Wang et al. 2017). There could be multiple reasons for this observation. Firstly, the organism carrying the gene could not have been grown because of its unknown nutrient requirement and secondly, the gene could have been unstable and the resistance might have been lost during culturing. Nevertheless, it won't be completely wrong to term them as "phantom-resistomes" (Wang et al. 2017). The role of environmental resistomes is greatly underestimated even though the origin of antimicrobial resistance genes is invariably traced back to the environment (Hawkey 1998; Jayaraman 2009). The possibility of interspecies spread of clonal MDRB indicate a possible vertical transmission route which could be expanded across different environments providing a co-ordinated system for the development and transfer of new resistance mechanisms.

4. Clinical Prevalence of MDRB:The Effects of Cleaning and Seasonal Variations on the Carriage and Transmission Rates of β-lactam Resistance among Patient's Surgical Wounds

4.1. Introduction

Infections associated with Gram-negative bacteria are considered difficult to treat due to their inherited physiological resilience and acquired MDR (Walsh and Toleman 2012; Cox and Wright 2013). They are one of the major causes of mortality and morbidity in hospitals and frequently associated with outbreaks. There is also a considerable cost involved in treating the effected patients and controlling the spread of Gram-negative MDRB associated infections in hospitals (Stone 2010; Marston et al. 2016; O'Neill 2016a). For example, the containment of vancomycin-resistant Enterococcus (VRE) outbreak in a hospital in Netherland costed more than €2,000,000 (AMRNext 2016). The report by O' Neil suggested that MDR associated infections could claim 10 million lives a year costing an estimated 100 trillion US dollars globally by 2050 (O'Neill 2016b). Therefore, infection control strategies are implemented to reduce the spread of MDRB between patients, clinicians and the community and to limit the distribution of resistant genes to other bacteria (Raka 2010; Carling and Polk 2011; O'Neill 2016a). However, infection control strategies are practically non-existent in various parts of the LMIC and as a result there is an increased burden of HAI. An estimated 7% patients admitted to hospital in high-income countries will develop a nosocomial infection which is increased to 35% in LMIC (Raka 2010; Stone 2010; O'Neill 2016a).

LMICs of South Asia such as India and Pakistan are also presented with increased prevalence rate of AMR (Hawkey 2017). Most parts of these countries usually have higher average annual temperature (30°C) which is also preferred for the growth of bacterial pathogens (Shah et al. 2013). Warm climate together with unsatisfactory infection control and waste management could potentially provide the ideal

conditions to enhance the propagation of MDRB and potentially infectious diseases. However, there are very limited studies to acknowledge the effects of various independent co-factors that may contribute to the amplification of MDR rate for example; region specific ecological, environmental, climate, humidity and sociodemographic influence such as human activities and behaviour. To investigate and evaluate the role of region specific infection control strategies in an already compromised environment, a basic cleaning regime was implemented in a set of surgical wards and compared with that of an “un-touched” ward. In addition, the effects of seasonal variations were also monitored by sampling different seasons and the results were compared to evaluate their effects on the spread and dissemination of MDRB among surgical wound patients.

4.2. Results

4.2.1. Molecular detection of *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$ like and *bla*$_{CTX-M-15}$ in surgical wound swabs

A total of 342 surgical wound samples were collected over three weeks of summer and winter. One hundred ninety samples were collected in Summer, 130 of them were taken from patients in clean wards and 59 from un-cleaned wards. In winter, 65 samples were collected from cleaned wards and 87 from un-cleaned wards, making a total of 152 samples. The initial PCR on those samples revealed that 35.1% (120/342) patient's samples were positive for the carriage of resistance either one or multiple resistance genes belonging to the family of *bla*$_{NDM}$, *bla*$_{OXA-48}$ like and *bla*$_{CTX-M-15}$. The samples collected in winter displayed an increase resistance rate of 46.0 % compared to 26.3% in summer. *bla*$_{KPC}$ was not detected in any of the samples and hence it has been excluded from the result tables and graphs.

The distribution of individual genes according to the seasons and wards revealed an increased resistance prevalence rate in winter. A general increase among un-cleaned wards for *bla*$_{CTX-M-15}$ (29.2% cleaned: 48.2% un-cleaned) and *bla*$_{NDM}$ (16.9% cleaned: 24.1% un-cleaned) was observed. In contrast, the same trend was not observed for *bla*$_{OXA-48}$ like PCR-positive samples and a very similar resistance distribution was seen in both cleaned and un-cleaned wards (20% cleaned: 19.5% uncleaned). However, all the resistant genes revealed a very similar prevalence rate for both cleaned and uncleaned wards in summer (*bla*$_{CTX-M-15}$ 21.5% cleaned:

22.03% un-cleaned, *bla*$_{NDM}$ 10.0% cleaned: 13.5% un-cleaned, *bla*$_{OXA-48}$ *like* 7.6% cleaned: 10.1% un-cleaned) (Figure 24).

Nonetheless, statistical analysis revealed that only *bla*$_{CTX-M-15}$ was significantly associated with un-cleaned wards in univariate analysis whereas no association with, *bla*$_{NDM}$ and *bla*$_{OXA-48}$like was seen (Table 10, 11 and 12). However, there was a significant association with the carriage of *bla*$_{CTX-M-15}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$-like with hospital stay of more than 7 days of stay and seasons winter (See univariate analysis table 10, 11, 12 and multivariate analysis table 13, 14, 15). Furthermore, *bla*$_{CTX-M-15}$ and *bla*$_{NDM}$ were also associated with the development of infection in patients (Table 10).

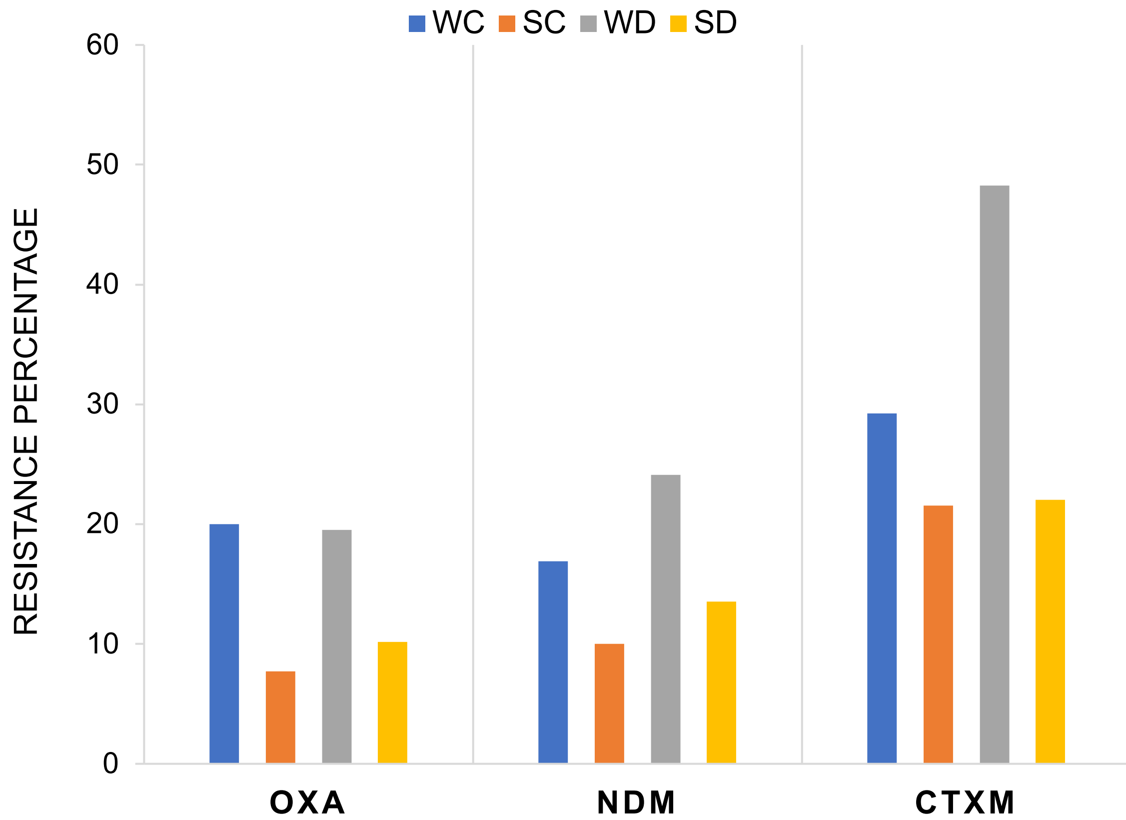


Figure 24. Occurrence Of *bla*$_{NDM}$, *bla*$_{OXA-48}$ like and *bla*$_{CTX-M-15}$ in patient's wound samples isolated during winter and summer from clean and un-clean Wards. clean wards(SC), summer un-clean wards(SD), winter clean wards(WC) and winter un-clean wards (WD).

Table 10. Univariate statistical analysis of $bla_{CTX-M-15}$ from patients' samples.

			$bla_{CTX-M-15}$			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
CD	C	N	148	47	195	0.007	0.008
		%	75.9%	24.1%	100.0%		
	D	N	91	55	146		
		%	62.3%	37.7%	100.0%		
Total		N	239	102	341		
		%	70.1%	29.9%	100.0%		
Stay	1-3	N	122	36	158	0.002	
		%	77.2%	22.8%	100.0%		
	4-7	N	86	40	126		
		%	68.3%	31.7%	100.0%		
	>7	N	20	21	41		
		%	48.8%	51.2%	100.0%		
Total		N	228	97	325		
		%	70.2%	29.8%	100.0%		
Age	0-18	N	53	24	77	0.477	
		%	68.8%	31.2%	100.0%		
	19-30	N	48	13	61		
		%	78.7%	21.3%	100.0%		
	31-45	N	53	22	75		
		%	70.7%	29.3%	100.0%		
	>46	N	71	34	105		
		%	67.6%	32.4%	100.0%		
Total		N	225	93	318		
		%	70.8%	29.2%	100.0%		
Sex	M	N	107	48	155	0.598	0.632
		%	69.0%	31.0%	100.0%		
	F	N	129	51	180		
		%	71.7%	28.3%	100.0%		
Total		N	236	99	335		
		%	70.4%	29.6%	100.0%		
Infection	N	N	149	46	195	0.002	0.002
		%	76.4%	23.6%	100.0%		
	Y	N	85	56	141		
		%	60.3%	39.7%	100.0%		
Total		N	234	102	336		
		%	69.6%	30.4%	100.0%		
Season	S	N	149	41	190	< 0.001	< 0.001
		%	78.4%	21.6%	100.0%		
	W	N	91	61	152		
		%	59.9%	40.1%	100.0%		
Total		N	240	102	342		
		%	70.2%	29.8%	100.0%		
Antibiotics	N	N	36	15	51	0.944	1.000
		%	70.6%	29.4%	100.0%		
	Y	N	204	87	291		
		%	70.1%	29.9%	100.0%		
Total		N	240	102	342		
		%	70.2%	29.8%	100.0%		

Ward shown as Clean (C), Un-cleaned (D), Stay in days, Age in years, Sex as Male (M), Female (F), Season as Summer (S), Winter (W) and development of infection and antibiotics treatment as No (N) and Yes (N). p- value of <0.05 is taken as significant and selected for multivariate analysis.

Table 11. Univariate statistical analysis of bla_{NDM} from patients' samples.

			bla_{NDM}			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
CD	C	N	171	24	195	0.057	0.070
		%	87.7%	12.3%	100.0%		
	D	N	117	29	146		
		%	80.1%	19.9%	100.0%		
Total		N	288	53	341		
		%	84.5%	15.5%	100.0%		
Stay	1-3	N	145	13	158	<0.001	
		%	91.8%	8.2%	100.0%		
	4-7	N	106	20	126		
		%	84.1%	15.9%	100.0%		
	>7	N	25	16	41		
		%	61.0%	39.0%	100.0%		
Total		N	276	49	325		
		%	84.9%	15.1%	100.0%		
Age	0-18	N	67	10	77	0.638	
		%	87.0%	13.0%	100.0%		
	19-30	N	50	11	61		
		%	82.0%	18.0%	100.0%		
	31-45	N	67	8	75		
		%	89.3%	10.7%	100.0%		
	>45	N	89	16	105		
		%	84.8%	15.2%	100.0%		
Total		N	273	45	318		
		%	85.8%	14.2%	100.0%		
Sex	M	N	128	27	155	0.134	0.160
		%	82.6%	17.4%	100.0%		
	F	N	159	21	180		
		%	88.3%	11.7%	100.0%		
Total		N	287	48	335		
		%	85.7%	14.3%	100.0%		
Infection	N	N	174	21	195	0.003	0.004
		%	89.2%	10.8%	100.0%		
	Y	N	109	32	141		
		%	77.3%	22.7%	100.0%		
Total		N	283	53	336		
		%	84.2%	15.8%	100.0%		
Season	S	N	169	21	190	0.011	0.016
		%	88.9%	11.1%	100.0%		
	W	N	120	32	152		
		%	78.9%	21.1%	100.0%		
Total		N	289	53	342		
		%	84.5%	15.5%	100.0%		
Antibiotics	N	N	42	9	51	0.646	0.675
		%	82.4%	17.6%	100.0%		
	Y	N	247	44	291		
		%	84.9%	15.1%	100.0%		
Total		N	289	53	342		
		%	84.5%	15.5%	100.0%		

Ward shown as Clean (C), Un-cleaned (D), Stay in days, Age in years, Sex as Male (M), Female (F), Season as Summer (S), Winter (W) and development of infection and antibiotics treatment as No (N) and Yes (N). p- value of <0.05 is taken as significant and selected for multivariate analysis.

Table 12. Univariate statistical analysis of bla_{OXA-48}-like from patients' samples.

			bla_{OXA-48}-like			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
CD	C	N	172	23	195	0.390	0.377
		%	88.2%	11.8%	100.0%		
	D	N	123	23	146		
		%	84.2%	15.8%	100.0%		
Total		N	295	46	341		
		%	86.5%	13.5%	100.0%		
Stay	1-3	N	148	10	158	< 0.001	
		%	93.7%	6.3%	100.0%		
	4-7	N	105	21	126		
		%	83.3%	16.7%	100.0%		
	>7	N	27	14	41		
		%	65.9%	34.1%	100.0%		
Total		N	280	45	325		
		%	86.2%	13.8%	100.0%		
Age	0-18	N	70	7	77	0.310	
		%	90.9%	9.1%	100.0%		
	19-30	N	49	12	61		
		%	80.3%	19.7%	100.0%		
	31-45	N	66	9	75		
		%	88.0%	12.0%	100.0%		
	>46	N	89	16	105		
		%	84.8%	15.2%	100.0%		
Total		N	274	44	318		
		%	86.2%	13.8%	100.0%		
Sex	M	N	130	25	155	0.237	0.267
		%	83.9%	16.1%	100.0%		
	F	N	159	21	180		
		%	88.3%	11.7%	100.0%		
Total		N	289	46	335		
		%	86.3%	13.7%	100.0%		
Infection	N	N	173	22	195	0.131	0.149
		%	88.7%	11.3%	100.0%		
	Y	N	117	24	141		
		%	83.0%	17.0%	100.0%		
Total		N	290	46	336		
		%	86.3%	13.7%	100.0%		
Season	S	N	174	16	190	0.002	0.004
		%	91.6%	8.4%	100.0%		
	W	N	122	30	152		
		%	80.3%	19.7%	100.0%		
Total		N	296	46	342		
		%	86.5%	13.5%	100.0%		
Antibiotics	N	N	47	4	51	0.203	0.267
		%	92.2%	7.8%	100.0%		
	Y	N	249	42	291		
		%	85.6%	14.4%	100.0%		
Total		N	296	46	342		
		%	86.5%	13.5%	100.0%		

Ward shown as Clean (C), Un-cleaned (D), Stay in days, Age in years, Sex as Male (M), Female (F), Season as Summer (S), Winter (W) and development of infection and antibiotics treatment as No (N) and Yes (N). p- value of <0.05 is taken as significant and selected for multivariate analysis.

Table 13. Multiivariate statistical analysis of *bla*$_{CTX-M-15}$ from patients' samples

	B	S.E.	Wald	df	Sig.	Odd ratio	95% C.I. for Odd ratio	
							Lower	Upper
Stay (4-7)	.333	.284	1.378	1	.240	1.396	.800	2.436
Stay (>7)	1.022	.389	6.914	1	.009	2.779	1.297	5.953
Infection (Y)	.779	.275	8.027	1	.005	2.180	1.272	3.739
Season (W)	.989	.278	12.665	1	.000	2.689	1.560	4.637
CD (D)	.343	.268	1.634	1	.201	1.409	.833	2.382
Constant	-2.087	.287	52.831	1	.000	.124		

bla$_{CTX-M-15}$ variable(s) entered for analysis are Ward as Clean (C), Un-cleaned (D), Stay in days, Season as Summer (S), Winter (W) and development of infection as No (N) and Yes (N). Reference set as Stay 1-3, Infection (N), Season (S) and ward (C). P- value of <0.05 is taken as significant.

Table 14. Multiivariate statistical analysis of *bla*$_{NDM}$ from patients' samples

	B	S.E.	Wald	df	Sig.	Odd ratio	95% C.I. for Odd ratio	
							Lower	Upper
Stay (4-7)	.636	.388	2.678	1	.102	1.888	.882	4.043
Stay (>7)	1.764	.449	15.462	1	.000	5.836	2.423	14.062
Infection (Y)	.907	.345	6.919	1	.009	2.478	1.260	4.871
Season (W)	.919	.346	7.069	1	.008	2.507	1.273	4.937
Constant	-3.211	.397	65.419	1	.000	.040		

bla$_{NDM}$ variable(s) entered for analysis are Stay in days, Season as Summer (S), Winter (W) and development of infection as No (N) and Yes (N). Reference set as Stay 1-3, Infection (N) and Season (S). P- value of <0.05 is taken as significant.

Table 15. Multiivariate statistical analysis of *bla*$_{OXA-48}$-like from patients' samples

	B	S.E.	Wald	df	Sig.	Odd ratio	95% C.I. for Odd ratio	
							Lower	Upper
Stay (4-7)	1.064	.409	6.779	1	.009	2.898	1.301	6.456
Stay (>7)	1.967	.471	17.450	1	.000	7.150	2.841	17.993
Season (W)	.927	.345	7.222	1	.007	2.528	1.285	4.972
Constant	-3.157	.388	66.129	1	.000	.043		

bla$_{OXA-48}$-like variable(s) entered for analysis are Stay in days, Season as Summer (S) and Winter (W). Reference set as Stay 1-3 and Season (S). P- value of <0.05 is taken as significant.

4.2.2. Investigating the efficiency of the cleaning agent used in the study

The efficacy of the cleaning was examined which resulted in producing growth in all wells including wells with no bacterial culture. However, the media controls wells were still clear. Therefore, the cleaning agent was plated onto UTI media which resulted in heavy growth and upon isolation, the contaminating bacteria was releveled to be *P. aeruginosa* by MALDI-Toff analysis. Accordingly, the solution was passed through filter membrane to get rid of the contaminating *P. aeruginos*a and the efficacy of the cleaning solution retested. All selected *E. coli* samples had growth inhibited at 50% (v/v) of the cleaning solution (0/31) and a small number at 25% (v/v) (6%: 2/31); whereas 48% (15/31) grew at 12.5% (28/31) and 90% at 6.25% of the original concentration. *E. cloacae* and *K. pneumoniae* were least effected by the cleaning agent and most *E. cloacae* (80%: 17/21) and *K. pneumoniae* (75%: 15/20) showed growth at 50% of the cleaning agent concentration (Figure 25, 26 and 27).

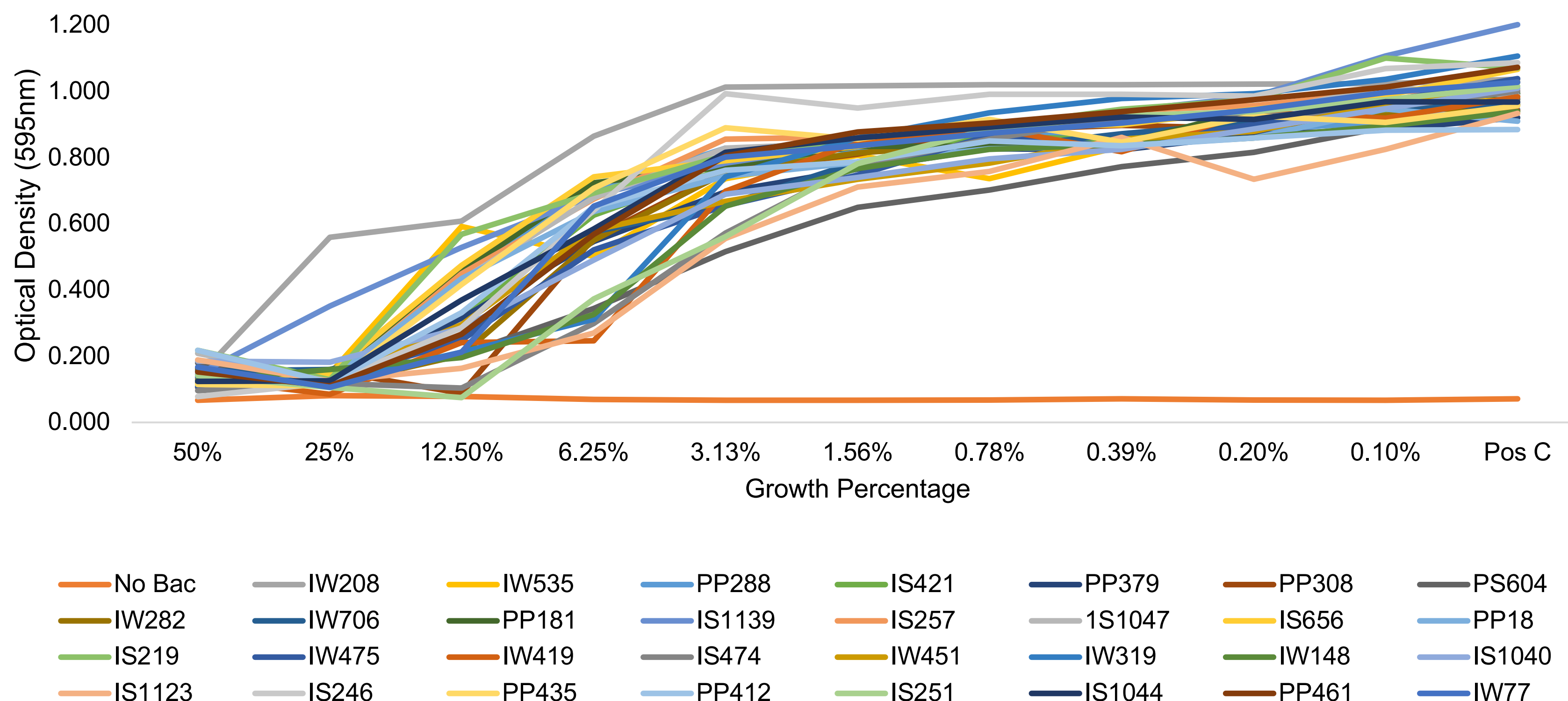


Figure 25. MIC of the cleaning agent to resistant *E. coli* (n=31) isolates. The line graph represents MICs of strains isolated from insects collected in winter (IW), insects collected in summer (IS), surface samples (PS) and patients (PP).

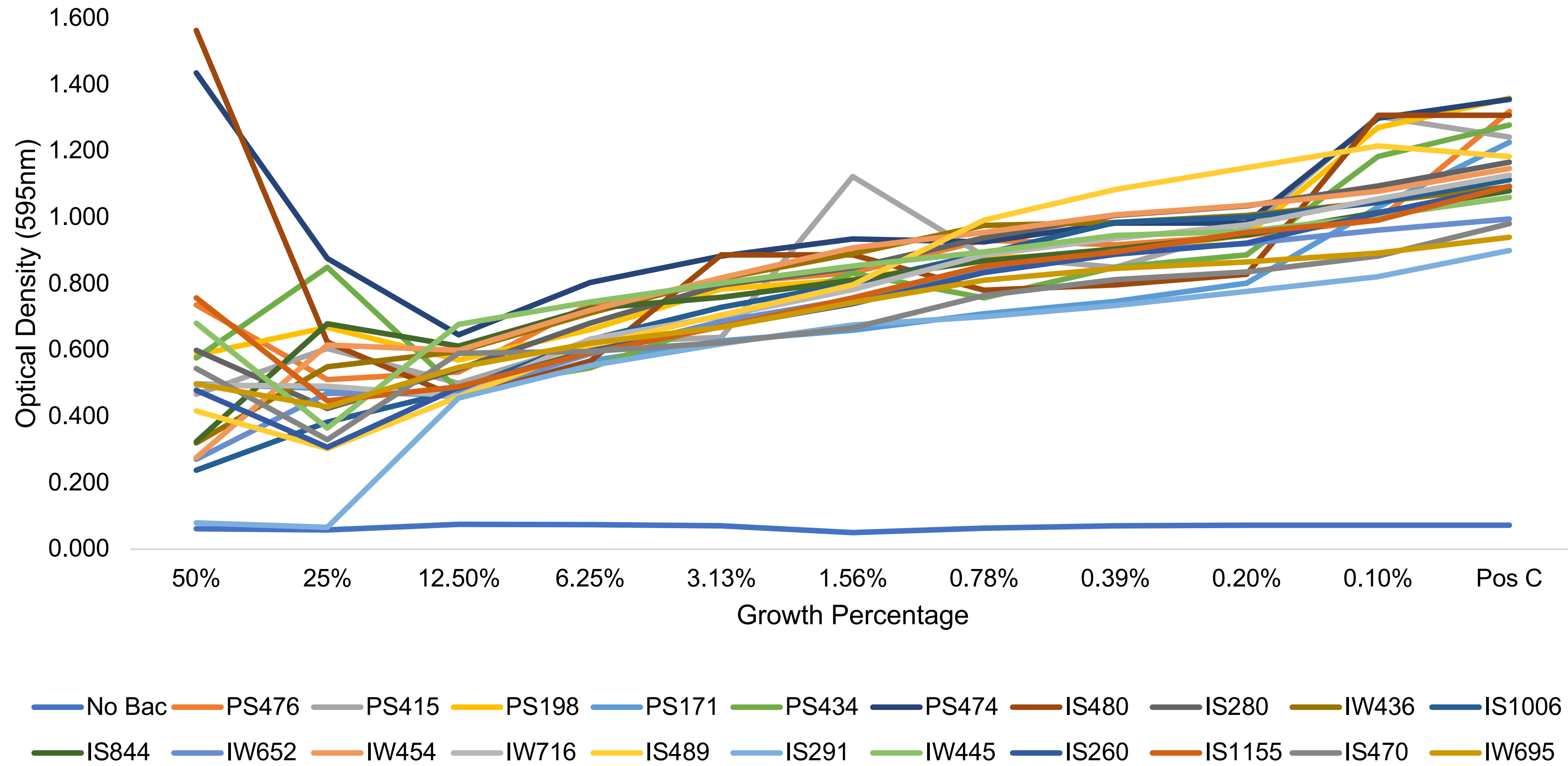


Figure 26. MIC of the cleaning agent to resistant *E. cloacae* (n=21) isolates. The line graph represents MICs of strains isolated from insects collected in winter (IW), insects collected in summer (IS), surface samples (PS) and patients (PP).

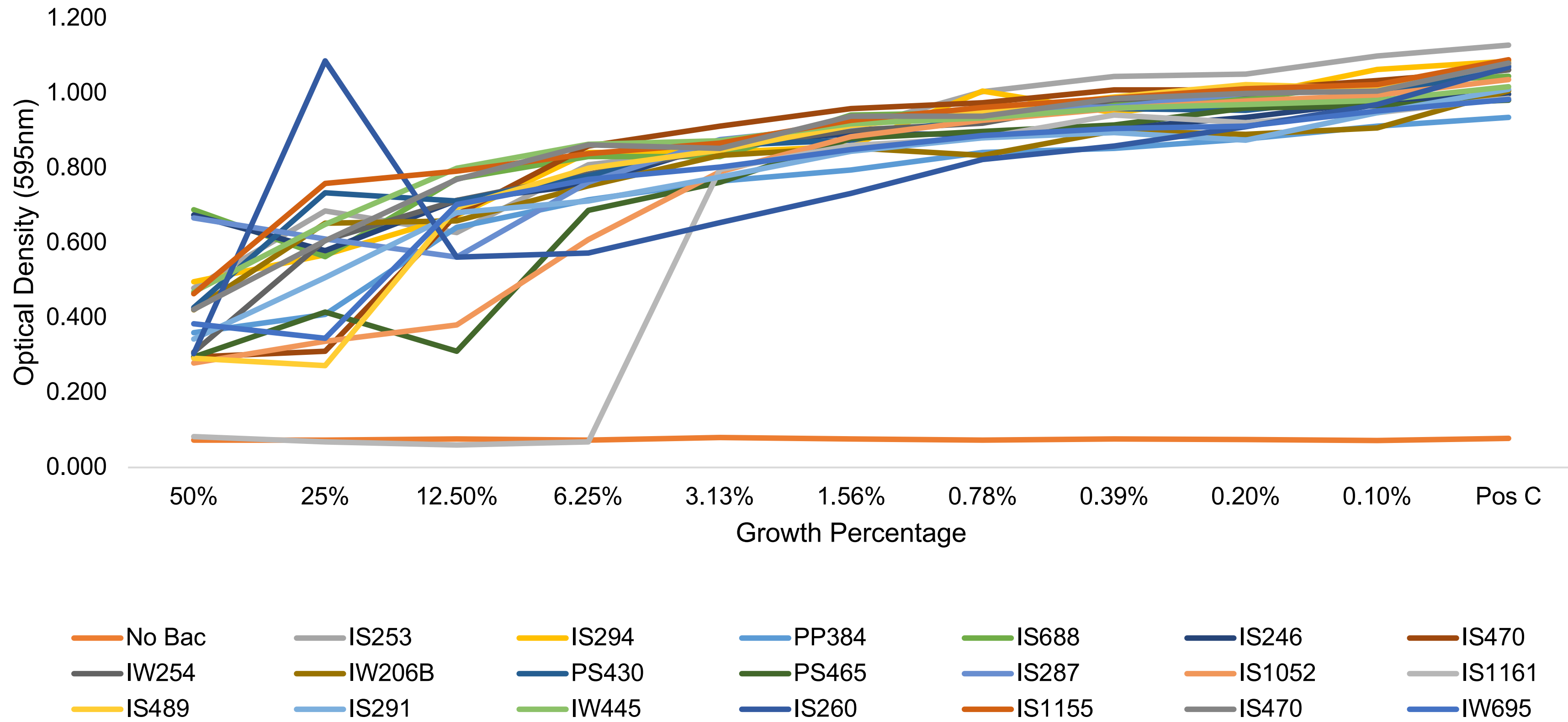


Figure 27. MIC of the cleaning agent to resistant *K. pneumoniae* (n=20) isolates. The line graph represents MICs of strains isolated from insects collected in winter (IW), insects collected in summer (IS), surface samples (PS) and patients (PP).

4.2.3. Identification of resistant gene carrying bacterial species from patients' samples

One hundred and sixty-six PCR-positive strains from patients were isolated and identified by MALDI-TOF and *16s* RNA. Only genus level identification was achieved by *16s* RNA whereas species were identified by MALDI-TOF. *E. coli* was the most prevalent (n=66), followed by *E. cloacae* (n=34) and *K. pneumoniae* (n=31). Other species included a variety of bacteria, primarily of environmental origin (Figure 28).

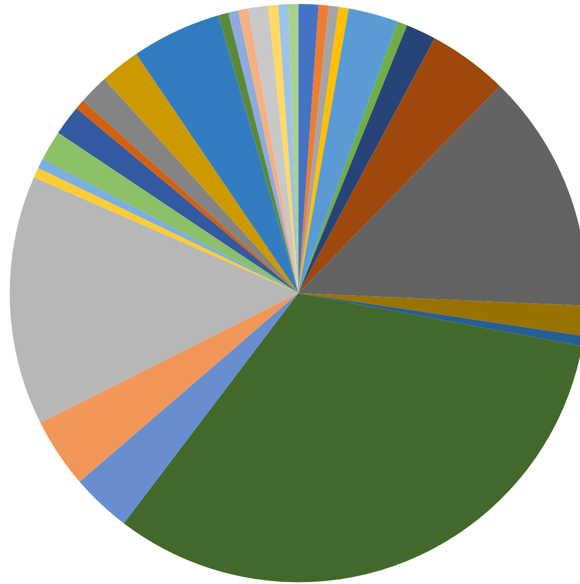


Figure 28. Bacterial species isolated from patient's wound's samples, Peshawar, Pakistan (n=179).

4.2.4. Antimicrobial susceptibility profiles of bacteria carrying *bla_{NDM}* and *bla_{OXA-48}*-like from patients' wound samples

Antimicrobial susceptibility testing of *bla*$_{NDM}$ and *bla*$_{OXA-48}$ like PCR-positive samples showed increased resistance to a variety of antibiotics tested. Bacteria isolated from all samples (n=48) were resistant to amoxicillin-clavulanic acid and more than 70% of strains were resistant to cefotaxime, erthapenem, rifampicin, aztreonam and ciprofloxacin. Samples were least resistant to Tigecycline and fosfomycin. Among colistin resistant isolates, 10 out of 20 samples belonged to inherently colistin resistant species of Proteus and Providencia. Therefore, it can be concluded that acquired resistance to colistin was 10% (n=5) which included three *K. pneumoniae*, an *E. cloacae* and a *P. aeruginosa* (Figure 29).

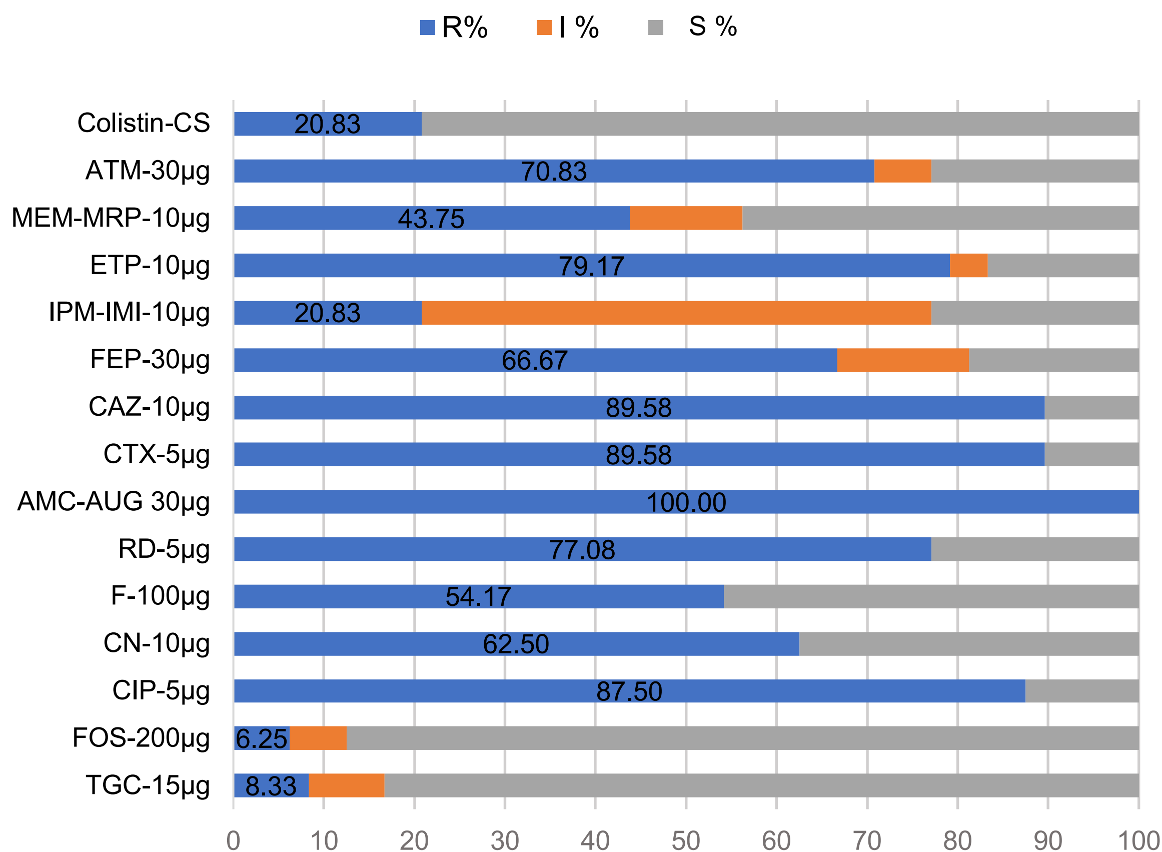


Figure 29. Antimicrobial susceptibility profile of *bla*$_{NDM}$ and *bla*$_{OXA-48}$ like PCR-positive samples. Table representing the percentage resistance to antibiotics tested. Tigecycline (TGC), Fosfomycin (FOS), Ciprofloxacin (CIP), Gentamicin (CN), Nitrofurantoin (F), Rifampicin (RD), Amoxicillin-clavulanic acid (AMC-AUG), Cefotaxime (CTX), Ceftazidime (CAZ), Cefepime (FEP), Imipenem (IPM-IMI), Erthapenem (ETP), Meropenem (MEM-MRP), Aztreonam (ATM), Colistin. (Bar colour blue=resistant, Orange=intermediate and grey=sensitive).

4.3. Discussion

In this study, resistance carriage showed a significant association with the winter season. Perhaps the reason for increased resistance in winter could be the local extreme weather conditions. For example, summers in Peshawar could get extremely hot and temperature of up to 45°C was the average day temperature recorded during the study period (Figure 30). Extreme weathers are not ideal for bacterial growth. The optimal growth temperature of *K. pneumoniae* and *E. coli* is 37°C where growth starts to reduce at 40°C and a considerable reduction is seen at 45°C (Esener et al. 1981; Nguyen 2006). Hence, this might explain the increased resistance rate that has been observed in winters compared to summers in this study.

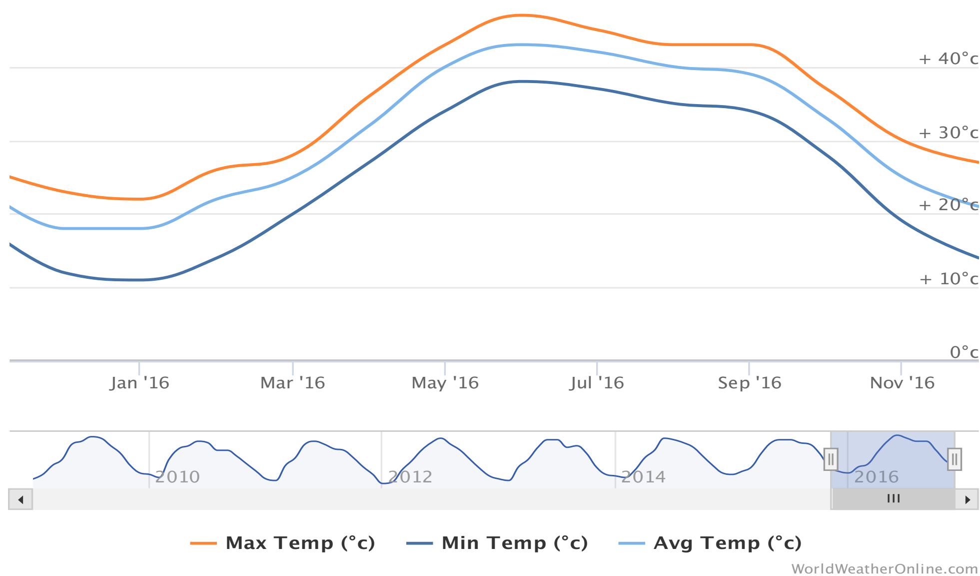


Figure 30. Weather chart of Peshawar, Pakistan in 2016. Graph showing the temperature for study period of 18th July to 6th of August in Summer: 6th January to 26th January in Winter (derived from webpage: worldweatheronline).

Furthermore, the prevalence of resistance genes obtained from cleaned and uncleaned wards was also rather unexpected. Apart from a small insignificant decrease in *bla*$_{CTX-M-15}$ and *bla*$_{NDM}$ resistance rate in winter from clean ward, there was no significant difference between the resistance rate from cleaned or un-cleaned wards. This question whether the cleaning technique was ineffective or the cleaning agent itself was unsuccessful in eliminating the surface contaminant. The cleaning agent used in this study is commercially available solution known as "FINIS" manufactured by Jhonson company Karachi, Pakistan. The active ingredient is stated as "white phenyl". These types of cleaning agents are commonly used in India and Pakistan for cleaning hospital, domestic and industrial cleaning purposes; however, there is very little information available about their antimicrobial or disinfectant efficiency (Taneja et al. 2012; Zaman and Siddiqui 2015). Two different studies compared the efficiency of disinfectants routinely used in hospitals of India and Pakistan. Their results showed that phenyl based cleaning agents were less effective then chloride, alcohol and quaternary ammonium compound based cleaning agents when tested on surfaces with similar structures to walls and floors (Taneja et al. 2012; Zaman and Siddiqui 2015). Similarly, our results have shown that the cleaning agent was ineffective and bacterial species of *E. coli, K. pneumoniae* and *E. cloacae* were shown to grow at high concentrations of 50%. Moreover, the contamination of the cleaning agent with *P. aeruginosa* is concerning since it is well adapted to survive in hospital settings and can have devastating effects for immune compromised patients if infected (Elias et al. 2010; CDC 2013). It also questions the appropriateness of the healthcare systems in LMICs.

The actual cleaning/disinfection technique is also important to consider. Byers et al. investigated the efficiency of a new cleaning technique after they found that 16% of

the sites that were initially positive for VRE remained positive after three attempted cleaning and complete disinfection was only achieved after fourth session of spray disinfection. They introduced a 'bucket method" which involved wiping all the surfaces with a cloth soaked with a solution of a quaternary ammonium compound (Byers et al. 1998; Talon 1999). Although, in this study the same bucket method was employed and the only difference was the type of cleaning agent that was used to disinfect the floors and other touch surfaces. Even so, irregularity with the cleaning procedure cannot be overruled as it is challenging to carry out such a job without disrupting the normal hospital proceedings. Furthermore, since the cleaning was only attempted once, recontamination in a busy environment is un-avoidable. Perhaps a more suitable approach would have been constant cleaning at multiple time points and using a suitable dis-infective agent with proven antimicrobial properties. Furthermore, introduction of intervention such educating the staff, patients and other health care workers about the importance of cleaning, hygiene and infection control procedures could have presented a more stable solution in the form of behaviour changes as a long term strategy (Collins 2008; Carling and Polk 2011). A similar study was designed by Hayden et al, where they investigated the effects of environmental cleaning on the carriage and transmission rate of VRE in a hospital ICU. The 9-month study period was divided in to four parts starting with a baseline period, followed by a cleaning period with education, cleaning without education and a hand hygiene intervention period. The results revealed that improved cleaning in addition to educating staff about importance of infection control contributed to a significant reduction in VRE environmental contamination and transmission rates (Hayden et al. 2006; Boyce 2007). Nevertheless, the results of this study highlight

the inefficiency of the current cleaning regimes in the hospital of Pakistan which requires urgent consideration.

5. Environmental Prevalence of MDRB: The Effects of Cleaning and Seasonal Variations on the Carriage and Transmission Rates of β-lactam Resistance in Insects and Hospital Surfaces

5.1. Introduction

As described in the introduction to chapter 3 and 4, LMICs usually lack basic cleaning and essential waste management systems. Hospital are presented with un-satisfactory sanitation and the waste is frequently discarded outside creating waste grounds which could become breeding sites for insects and animals. Hospital waste are often found to contain antibiotic residues which could give to rise to antibiotic resistance (Berglund 2015). Furthermore, this waste might already contain infectious agents or contaminants with AMR/MDR bacteria from the hospital sources (Laxminarayan et al. 2013; Munoz-Price et al. 2013; Laxminarayan and Chaudhury 2016). Insects and animals may acquire MDRB from the contaminated grounds and disseminate to other areas (Wang et al. 2017). Generally, the carriage of MDRB by insects is associated with animal husbandry and the effects on human's health or the environment are rarely considered (Zurek and Ghosh 2014). In Peshawar, Pakistan, the number of insects, especially flies (*Musca domestica*), increase considerably in summer months (personal observations). Their movement is not restricted and including the hospital. Furthermore, there are no regulation in place for pest control and no routine treatments are carried out resulting in increased numbers of insects in the hospital in close proximity to patients (discussion with collaborators in Peshawar, Pakistan).

Nevertheless, a patient's health is dependent on the immediate environment which is constantly being contaminated by insects, infected patients or healthcare workers if not disinfected routinely. Several studies have identified VRE, methicillin-resistant *S. aureus*, *Clostridium difficile*, *A. baumannii* and *P. aeruginosa* among the typical contaminants and locations such as the floor, furniture, mattress and pillows etc. are

the common sources (Talon 1999). Studies have shown that bacterial pathogens can survive for days or even weeks on these dry surfaces and may survive even longer on damp, moist and unclean surfaces (Jawad et al. 1998; Paterson and Bonomo 2005). Therefore, cleaning and disinfection of the rooms and changing of bed-sheets and pillow-cases are recommended when the infected patients leave the hospital (Talon 1999). However, these recommendations are rarely followed especially in LMICs where MDR has recently increased (Raka 2010; WHO 2015c; Laxminarayan and Chaudhury 2016). This situation is worsened by the ambient temperature which supports the growth of pest and insects (Eber et al. 2011; Richet 2012). Vector-borne diseases are common and frequent outbreaks of diseases are reported (WHO 2014a). Recently, Pakistan has seen several outbreaks of dengue and chikungunya virus and experts have no hesitation in blaming the waste management for the existing situation (Rauf et al. 2017). Water-borne diseases such as cholera, dysentery and especially typhoid are very common among the community (WHO 2014b). These infections are becoming difficult to treat due to the spread of AMR (WHO 2013a).

This study aims to provide better understanding of patients' relationship with their unique surrounding environment in a hospital settings. Furthermore, the introduction of a cleaning regime and sampling in different seasons will provide information about the effects of basic cleaning and seasonal variations on the spread and dissemination of MDRB among hospital touch surface and insects that are commonly found in the hospital.

5.2. Results

5.2.1. Molecular detection of *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$ like and *bla*$_{CTX-M-15}$ in insects samples

In this study, 1981 insects were collected in summer and winter spanning over six different species of local abundance. Those included ants (n=110), bees (n=2), cockroaches (n=533), flies (1076), moths (n=194) and spiders (n=66). Their movement was not restricted and, besides cleaning one ward in the hospital, other conditions were not changed. A total of 39.6% insects carried one or multiple types of the target resistance genes (*bla*$_{NDM}$, *bla*$_{CTX-M-15}$ and *bla*$_{OXA-48}$ like) and similar resistance rate was observed in summer (39.7%) and winter (39.4%).

The distribution of genes independently revealed that *bla*$_{NDM}$ and *bla*$_{OXA-48}$-like positive samples collected from uncleaned wards in winter were generally associated with higher resistance rate (*bla*$_{NDM}$ 13.4% cleaned wards: 27.0% un-cleaned wards, *bla*$_{OXA-48}$ like 3.4% cleaned wards: 4.5% un-cleaned wards) but very little difference was observed in the resistance rates from the summer (*bla*$_{NDM}$ 10.9%-cleaned: 13.2%-un-cleaned, *bla*$_{OXA-48}$-like 2.8%-cleaned: 1.07%-un-cleaned). However, *bla*$_{CTX-M-15}$ showed an increased resistance rate in uncleaned wards from both seasons. In Summer, 32.35% (165/510) insects from cleaned wards were *bla*$_{CTX-M-15}$ PCR-positive compared to 39.06% (291/745) from un-cleaned wards. Similarly, winter samples had a prevalence rate of 21.43% (69/322) from cleaned wards and 43.32% (175/404) from uncleaned wards (Figure 31).

Univariate statistical analysis showed that *bla*$_{CTX-M-15}$, *bla*$_{NDM}$ and *bla*$_{OXA-48}$ like were significantly associated with insects; *bla*$_{CTX-M-15}$, *bla*$_{NDM}$ with un-cleaned wards and

bla$_{NDM}$ and *bla*$_{OXA-48}$ like with winter season (Table 16, 18 and 20). However, multivariate analysis no significant association of *bla*$_{OXA-48}$ like with any of the variants and significant association of *bla*$_{NDM}$ with insects and *bla*$_{CTX-M-15}$ with insects and un-cleaned wards (Table 17, 19, 21).

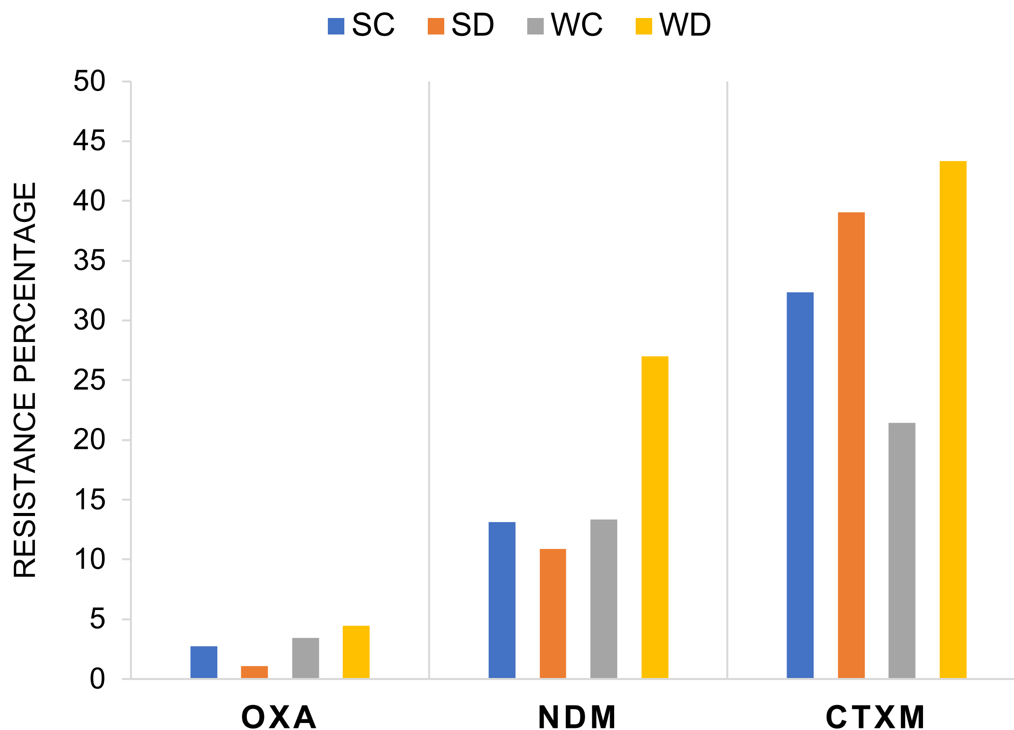


Figure 31. Occurrence of *bla*$_{NDM}$, *bla*$_{OXA-48}$ like, *bla*$_{CTX-M-15}$ in insects' samples during winter and summer from clean and un-cleaned wards. clean wards(SC), summer un-clean wards(SD), winter clean wards(WC) and winter unclean wards (WD).

Cockroaches showed the highest resistance prevalence rate at 50.84% followed by flies, spiders, mosquitos and ants at 38.2%, 42.4%, 32.5% and 10.0%, respectively. None of the bees collected were positive for the carriage of resistance genes.

Surprisingly, there was a very distinct difference in the proportion of the resistance gene carried by different species according to the season. Flies were responsible for

65.8% of all the resistance that was observed in the summer season. In contrast, cockroaches carried most of the resistance genes detected in winter (78.0%). In addition, in the summer, moths, spiders and ants possessed bacteria having a resistance rate of 12.5, 6.0% and 2.7%, respectively. Whereas in winter, moths didn't possess bacteria that carried any resistance and spiders (0.5%) and ants (0.2%) possessed bacteria that had very low resistance rate compared to summer (Figure 32).

The proportion of bla_{NDM}, bla_{OXA-48}-like and $bla_{CTX-M-15}$ were distributed among different species at a varying rate and species of insects such as cockroaches, flies, moths and spiders carried bacteria that showed a statistically significant association with bla_{NDM} and $bla_{CTX-M-15}$. (Figure 33).

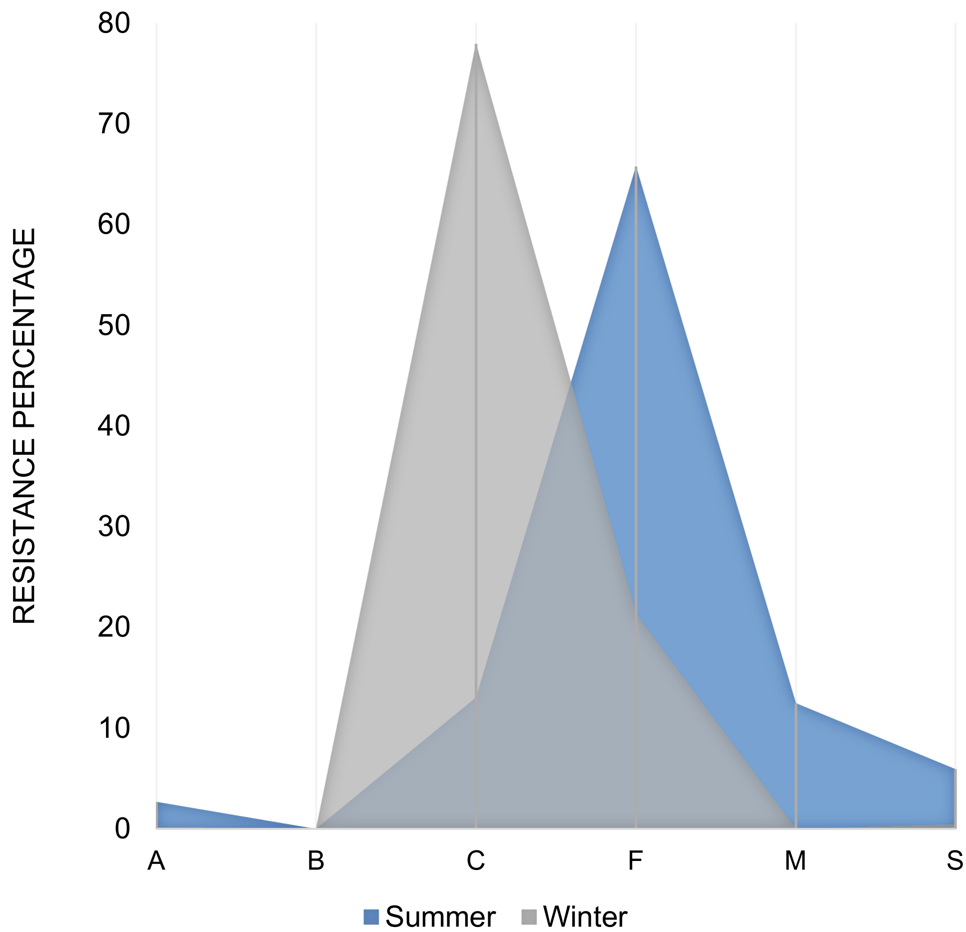
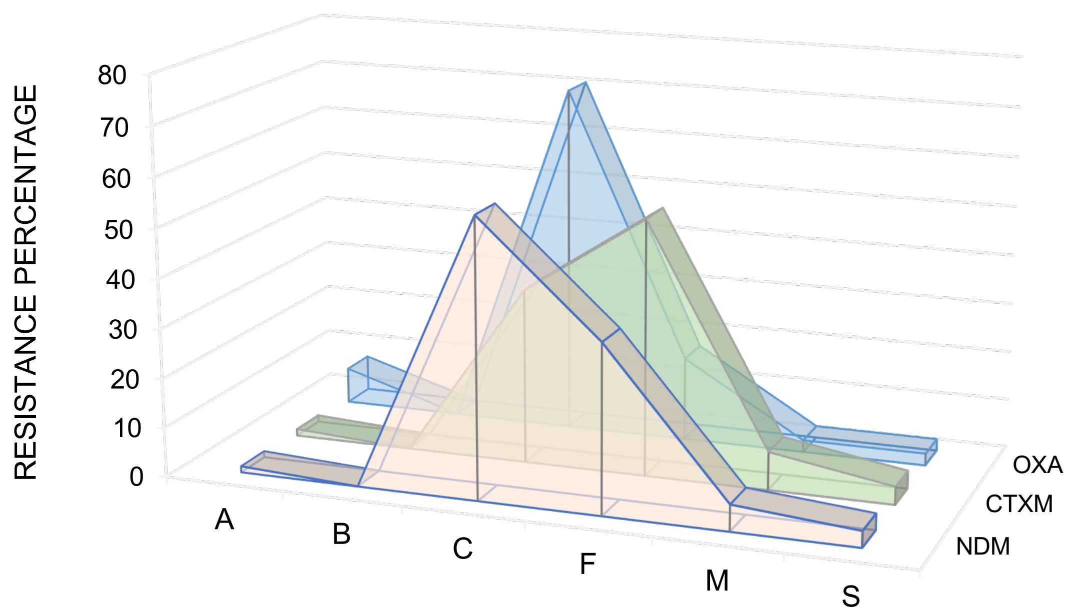


Figure 32. The prevalence of resistance genes among different species of insects. A (ants), B (bees), C (cockroaches), F (flies), M (moths), S (spiders).



	A	B	C	F	M	S
NDM	1.23	0.00	56.15	34.02	5.33	3.28
CTXM	1.36	0.00	35.45	51.82	7.73	3.64
OXA	7.32	0.00	70.73	17.07	2.44	2.44

Figure 33. The prevalence of resistance genes among different species of insects. The area graph representing the proportional percentage distribution of *bla*$_{OXA-48}$-like*, bla*$_{CTX-M-15}$ and *bla*$_{NDM}$ among different species of insects; A (ants), B (bees), C (cockroaches), F (flies), M (moths), S (spiders).

Table 16. Univariate statistical analysis of bla_{OXA-48}-like from insects samples.

			bla_{OXA-48}-like			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
Organism	Ants	N	107	3	110	< 0.001	< 0.001
		%	97.3%	2.7%	100.0%		
	Moths	N	191	3	194		
		%	98.5%	1.5%	100.0%		
	Spiders	N	65	1	66		
		%	98.5%	1.5%	100.0%		
	Cockroaches	N	502	31	533		
		%	94.2%	5.8%	100.0%		
	Flies	N	1063	13	1076		
		%	98.8%	1.2%	100.0%		
Total		N	1928	51	1979		
		%	97.4%	2.6%	100.0%		
CD	C	N	807	25	832	0.303	0.317
		%	97.0%	3.0%	100.0%		
	D	N	1123	26	1149		
		%	97.7%	2.3%	100.0%		
Total		N	1930	51	1981		
		%	97.4%	2.6%	100.0%		
Season	S	N	1233	22	1255	0.002	0.003
		%	98.2%	1.8%	100.0%		
	W	N	697	29	726		
		%	96.0%	4.0%	100.0%		
Total		N	1930	51	1981		
		%	97.4%	2.6%	100.0%		

Table shows ward as clean (C), un-cleaned (D), season as summer (S), winter (W) and organisms/insects (ants, moths, spiders, cockroaches and flies). P- value of <0.05 is taken as significant and selected for multivariate analysis.

Table 17. Multiivariate statistical analysis of bla_{OXA-48}-like from insects samples

	B	S.E.	Wald	df	Sig.	Odd Ratio	95% C.I.for Odd ratio	
							Lower	Upper
Season (W)	0.106	0.363	0.085	1	0.771	1.112	0.545	2.266
Moths	-0.573	0.826	0.482	1	0.487	0.564	0.112	2.843
Spiders	-0.606	1.166	0.27	1	0.603	0.546	0.056	5.358
Cockroaches	0.712	0.67	1.127	1	0.288	2.037	0.547	7.581
Flies	-0.854	0.654	1.703	1	0.192	0.426	0.118	1.535
Constant	-3.58	0.586	37.354	1	0	0.028		

bla_{OXA-48}-like variables entered for analysis are organisms/insects (moths, ants, spiders, cockroaches and flies) and Season as summer (S) winter (W). Reference taken as organism (ants) and season (S). P- value of <0.05 is taken as significant.

Table 18. Univariate statistical analysis of bla_{NDM} from insects samples.

			bla_{NDM}			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
Organism	Ants	N	107	3	110	< 0.001	< 0.001
		%	97.3%	2.7%	100.0%		
	Moths	N	176	18	194		
		%	90.7%	9.3%	100.0%		
	Spiders	N	56	10	66		
		%	84.8%	15.2%	100.0%		
	Cockroaches	N	389	144	533		
		%	73.0%	27.0%	100.0%		
	Flies	N	951	125	1076		
		%	88.4%	11.6%	100.0%		
Total		N	1679	300	1979		
		%	84.8%	15.2%	100.0%		
CD	C	N	722	110	832	0.042	0.042
		%	86.8%	13.2%	100.0%		
	D	N	959	190	1149		
		%	83.5%	16.5%	100.0%		
Total		N	1681	300	1981		
		%	84.9%	15.1%	100.0%		
Season	S	N	1107	148	1255	< 0.001	< 0.001
		%	88.2%	11.8%	100.0%		
	W	N	574	152	726		
		%	79.1%	20.9%	100.0%		
Total		N	1681	300	1981		
		%	84.9%	15.1%	100.0%		

Table shows ward as clean (C), un-cleaned (D), season as summer (S), winter (W) and organisms/insects (ants, moths, spiders, cockroaches and flies). P- value of <0.05 is taken as significant and selected for multivariate analysis.

Table 19. Multiivariate statistical analysis of bla_{NDM} from insects samples

	B	S.E.	Wald	df	Sig.	Odd ratio	95% C.I. for Odd ratio	
							Lower	Upper
CD (D)	.246	.135	3.347	1	.067	1.279	.983	1.665
Moths	1.387	.638	4.729	1	.030	4.001	1.147	13.961
Spiders	1.835	.679	7.303	1	.007	6.263	1.655	23.696
Cockroaches	2.517	.605	17.334	1	.000	12.392	3.789	40.526
Flies	1.535	.594	6.670	1	.010	4.640	1.448	14.871
Season (W)	.109	.157	.481	1	.488	1.115	.819	1.519
Constant	-3.748	.593	39.901	1	.000	.024		

bla_{NDM} variables entered for analysis are organisms/insects (moths, ants, spiders, cockroaches and flies), ward as Clean (C), un-cleaned (D) and Season as summer (S) winter (W). Reference taken as organism (ants), ward (C) and season (S). P- value of <0.05 is taken as significant.

Table 20. Univariate statistical analysis of $bla_{CTX-M-15}$ from insects samples.

			$bla_{CTX-M-15}$			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
Organism	Ants	N	101	9	110	< 0.001	< 0.001
		%	91.8%	8.2%	100.0%		
	Moths	N	139	55	194		
		%	71.6%	28.4%	100.0%		
	Spiders	N	42	24	66		
		%	63.6%	36.4%	100.0%		
	Cockroaches	N	293	240	533		
		%	55.0%	45.0%	100.0%		
	Flies	N	704	372	1076		
		%	65.4%	34.6%	100.0%		
Total		N	1279	700	1979		
		%	64.6%	35.4%	100.0%		
CD	C	N	598	234	832	< 0.001	< 0.001
		%	71.9%	28.1%	100.0%		
	D	N	683	466	1149		
		%	59.4%	40.6%	100.0%		
Total		N	1281	700	1981		
		%	64.7%	35.3%	100.0%		
Season	S	N	799	456	1255	0.221	0.223
		%	63.7%	36.3%	100.0%		
	w	N	482	244	726		
		%	66.4%	33.6%	100.0%		
Total		N	1281	700	1981		
		%	64.7%	35.3%	100.0%		

Table shows ward as clean (C), un-cleaned (D), season as summer (S), winter (W) and organisms/insects (ants, moths, spiders, cockroaches and flies). P- value of <0.05 is taken as significant and selected for multivariate analysis.

Table 21. Multiivariate statistical analysis of bla_{CTX-M} from insects samples

	B	S.E.	Wald	df	Sig.	Odd ratio	95% C.I.for Odd ratio	
							Lower	Upper
CD (D)	.568	.101	31.743	1	.000	1.764	1.448	2.150
Moths	1.702	.386	19.443	1	.000	5.483	2.573	11.681
Spiders	1.845	.434	18.104	1	.000	6.326	2.705	14.798
Cockroaches	2.274	.360	39.926	1	.000	9.715	4.799	19.667
Flies	1.826	.355	26.488	1	.000	6.208	3.097	12.443
Constant	-2.819	.357	62.388	1	.000	.060		

$bla_{CTX-M-15}$ variables entered for analysis are organisms/insects (moths, ants, spiders, cockroaches and flies) and ward as Clean (C), un-cleaned (D). Reference taken as organism (ants) and ward (C). P- value of <0.05 is taken as significant.

5.2.2. Molecular detection of *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{OXA-4-}$ like and *bla*$_{CTX-M-15}$ in hospital surface swabs

Six hundred and twenty-four surface samples were collected in winter and summer. PCR analysis on those samples revealed that 26.1% (n=163) were positive for the carriage of either one or multiple genes of *bla*$_{OXA-48}$like, *bla*$_{CTX-M-15}$ and *bla*$_{NDM}$. Winter was associated with slightly more resistance rate of 28.8% (91/316) compared to 23.4% (71/308) for the summer.

Analysis on the surface samples revealed no significant association of *bla*$_{NDM}$ and *bla*$_{CTX-M-15}$ with winter (Table 7). In some cases, a lower resistance rate was observed from uncleaned wards although the results were not significant. For example, *bla*$_{CTX-M-15}$ prevalence rate of 20.1% from cleaned wards and 15.6% from uncleaned in summer compared to 28.5% from cleaned wards and 22.8% from un-cleaned wards in winter.

Similarly, samples containing bacteria with *bla*$_{OXA-48-}$like genes had a prevalence rate of 4.6% from clean wards and 0.7% from unclean wards from the summer season. Whereas in winter, the cleaned wards possessed bacteria that had 5.1% of *bla*$_{OXA-48-}$like PCR-positives compared to 2.5% positives from uncleaned wards. In contrast, samples that possessed bacteria positive for *bla*$_{NDM}$ also shared similar findings to the summer season (16.9%-cleaned, 5.2%-uncleaned) but in the winter, a slightly decreased resistance rate of 14.6% in cleaned wards compared to 16.5% from uncleaned wards was observed (Figure 34).

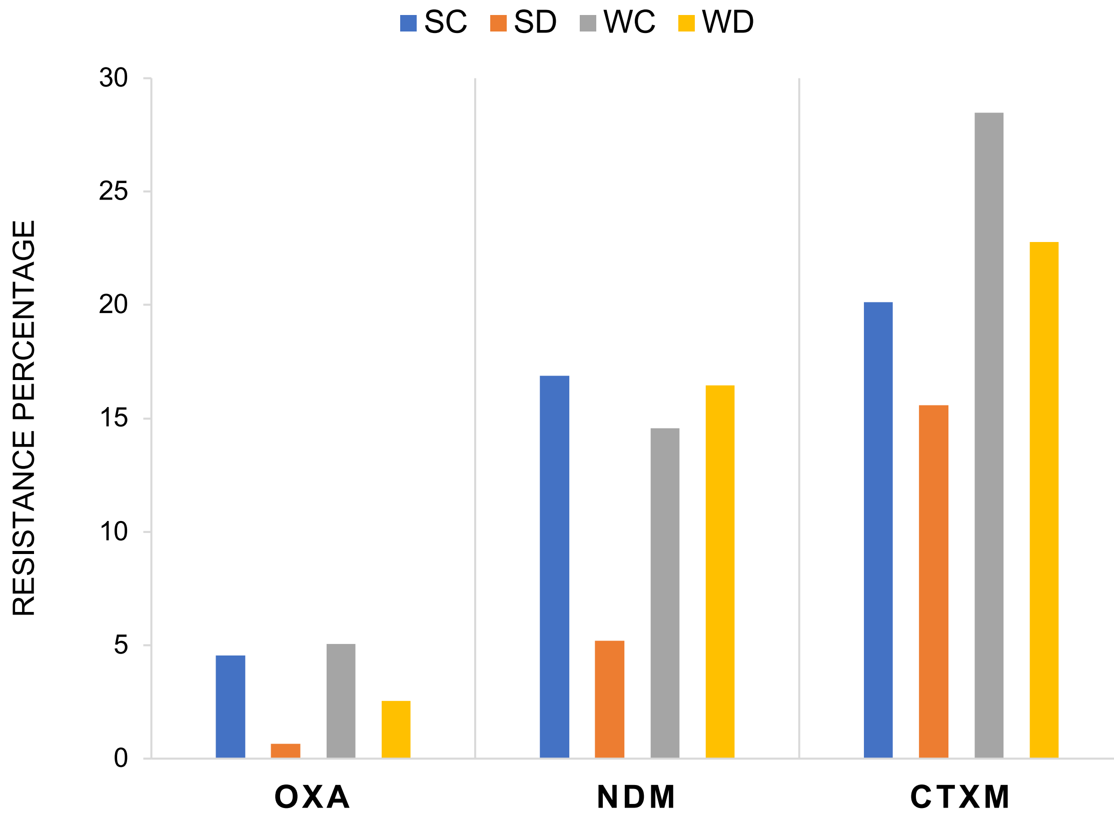


Figure 34. Occurrence of *bla*$_{NDM}$, *bla*$_{OXA-48}$-like and *bla*$_{CTX-M-15}$ among surface samples during winter and summer from clean and un-clean wards. clean wards(SC), summer un-clean wards(SD), winter clean wards(WC) and winter un-clean wards (WD).

Table 22. Univariate statistical analysis of *bla*$_{NDM}$, *bla*$_{CTX-M-15}$ and *bla*$_{OXA-48}$-like from surface samples.

			bla$_{NDM}$			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
Season	S	N	274	34	308	0.100	0.125
		%	89.0%	11.0%	100.0%		
	W	N	267	49	316		
		%	84.5%	15.5%	100.0%		
Total		N	541	83	624		
		%	86.7%	13.3%	100.0%		
CD	C	N	263	49	312	0.077	0.098
		%	84.3%	15.7%	100.0%		
	D	N	278	34	312		
		%	89.1%	10.9%	100.0%		
Total		N	541	83	624		
		%	86.7%	13.3%	100.0%		

			bla$_{CTX-M-15}$			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
Season	S	N	253	55	308	0.019	0.020
		%	82.1%	17.9%	100.0%		
	w	N	235	81	316		
		%	74.4%	25.6%	100.0%		
Total		N	488	136	624		
		%	78.2%	21.8%	100.0%		
CD	C	N	236	76	312	0.121	0.146
		%	75.6%	24.4%	100.0%		
	D	N	252	60	312		
		%	80.8%	19.2%	100.0%		
Total		N	488	136	624		
		%	78.2%	21.8%	100.0%		

			bla$_{OXA-48}$-like			Pearson Chi-Square	Fisher's Exact Test
			Neg	Pos	Total		
Season	S	N	300	8	308	0.395	0.497
		%	97.4%	2.6%	100.0%		
	W	N	304	12	316		
		%	96.2%	3.8%	100.0%		
Total		N	604	20	624		
		%	96.8%	3.2%	100.0%		
CD	C	N	297	15	312	0.023	0.038
		%	95.2%	4.8%	100.0%		
	D	N	307	5	312		
		%	98.4%	1.6%	100.0%		
Total		N	604	20	624		
		%	96.8%	3.2%	100.0%		

Table shows season as summer (S), winter (W) and ward as clean (C), un-cleaned (D). P- value of <0.05 is taken as significant.

5.2.3. Antibiotic susceptibility profiles of bacteria from *bla*$_{NDM}$ and *bla*$_{OXA-48}$ like PCR-positive samples from insects and hospital surface swabs

Antimicrobial susceptibility testing results for bacteria from *bla*$_{NDM}$ and *bla*$_{OXA-48}$ like positive samples from insects and hospital surface swabs showed resistance to a variety of antibiotics. Bacteria from all samples from insects and surface showed maximum resistance (>85 %-100 %) to rifampicin, amoxicillin-clavulanic acid, cefotaxime and ceftazidime and maximum sensitivity (>80 %-100 %) to tigecycline, fosfomycin and colistin. Among insect's, 24 samples produced bacteria that displayed resistance to colistin from which, nine samples were belonging to the species of Proteus and Providencia, known to be inherently resistant. Therefore, it can be concluded that the insects' samples possessed bacteria that had acquired colistin resistance was at 8 % (n=15) which included 10 species of *Citrobacter,* three K. pneumoniae, an E. coli and a specie of *Raoultella* (Figure 35).

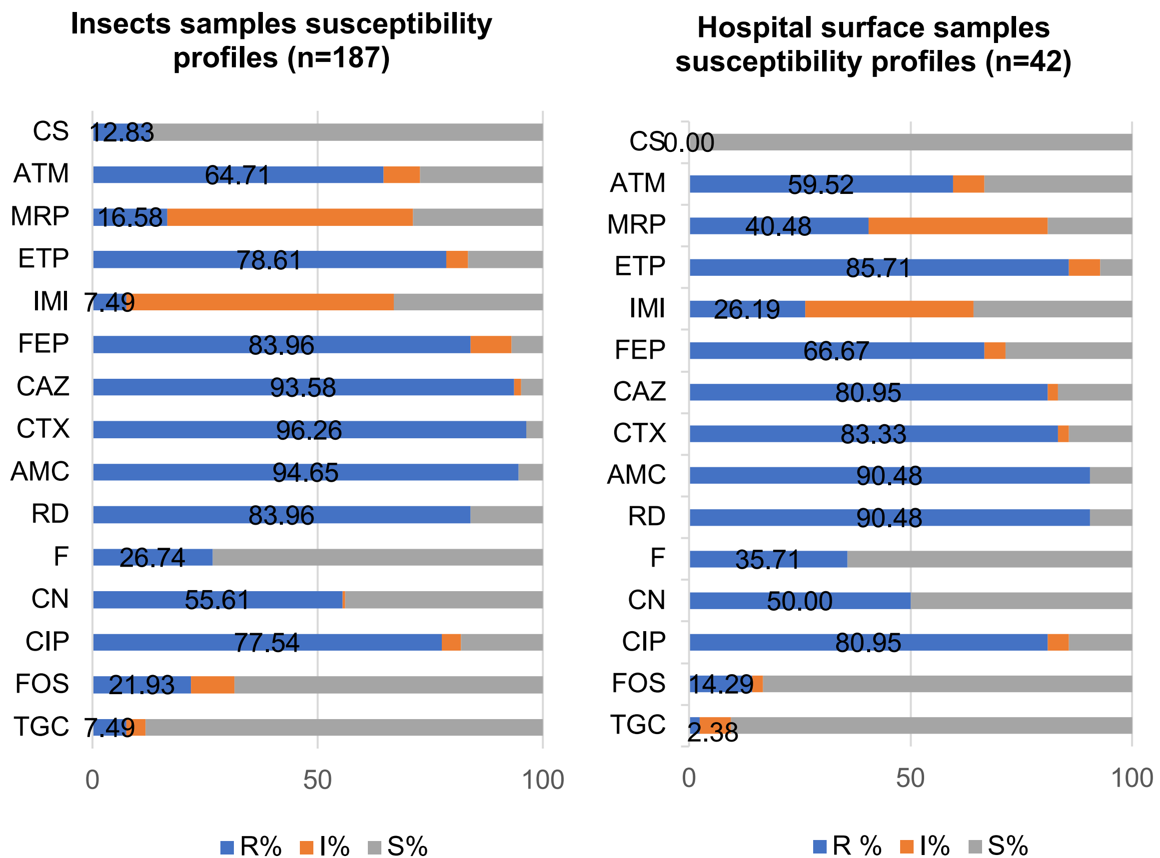


Figure 35. Antimicrobial susceptibility profiles of *bla*$_{NDM}$ and *bla*$_{OXA-48}$-like PCR-positives from insects and hospital surface samples. Tigecycline (TGC), Fosfomycin (FOS), Ciprofloxacin (CIP), Gentamicin (CN), Nitrofurantoin (F), Rifampicin (RD), Amoxicillin-clavulanic acid (AMC), Cefotaxime (CTX), Ceftazidime (CAZ), Cefepime (FEP), Imipenem (IMI), Ertapenem (ETP), Meropenem (MRP), Aztreonam (ATM), Colistin (CS). (Bar colour blue=resistant, Orange=intermediate and grey=sensitive).

5.2.4. Identification of bacteria carrying resistant genes from surface and insects, Peshawer.

In both surface and insect's samples, the maximum number of bacterial resistant isolates were identified to be *E. cloacae* and *K. pneumoniae*. The insects samples also had an increased number of *C. freundii, Pseudocitrobacter* spp and *E. coli.*

Additionally, a large variety of bacteria carrying resistant genes were isolated from samples of both origins of surface and insects (Figure 36 and 37).

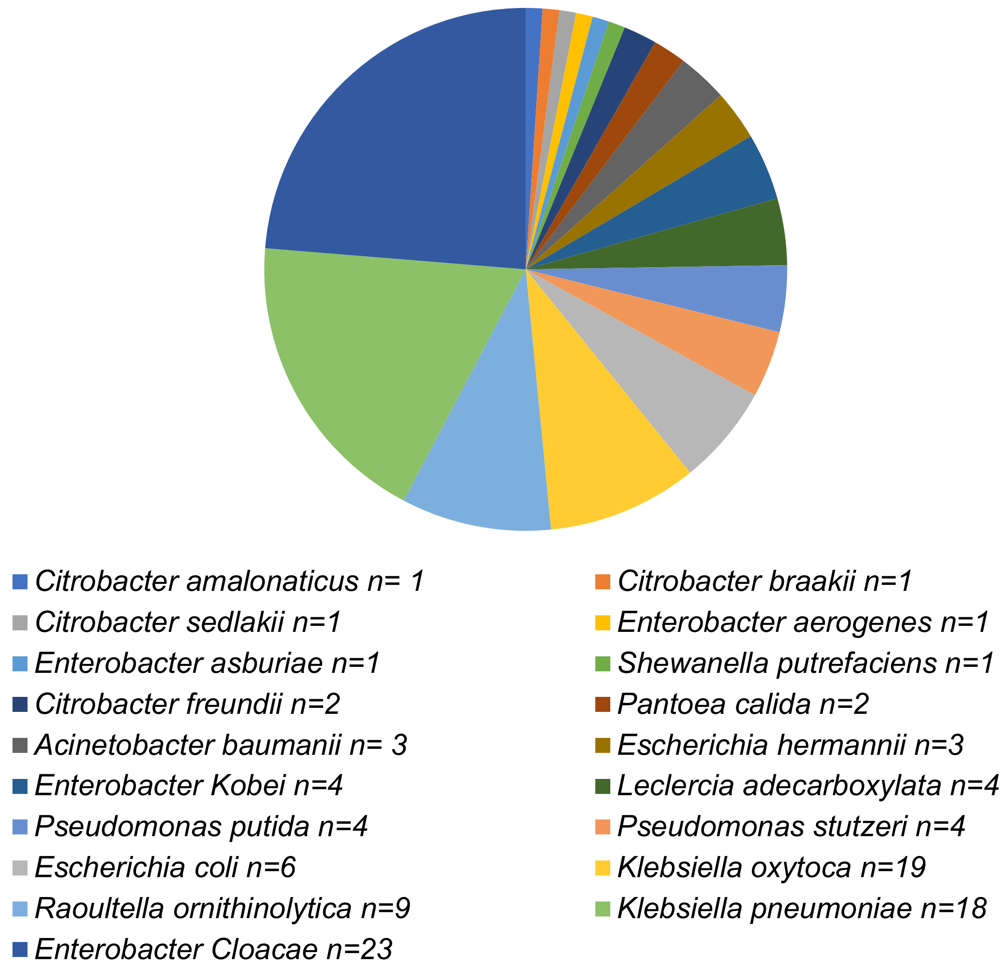


Figure 36. Bacterial species isolated from hospital surface samples, Peshawar, Pakistan (n=107). MALDI-TOFF results shown as species whereas 16s RNA identifications are shown at genus level.

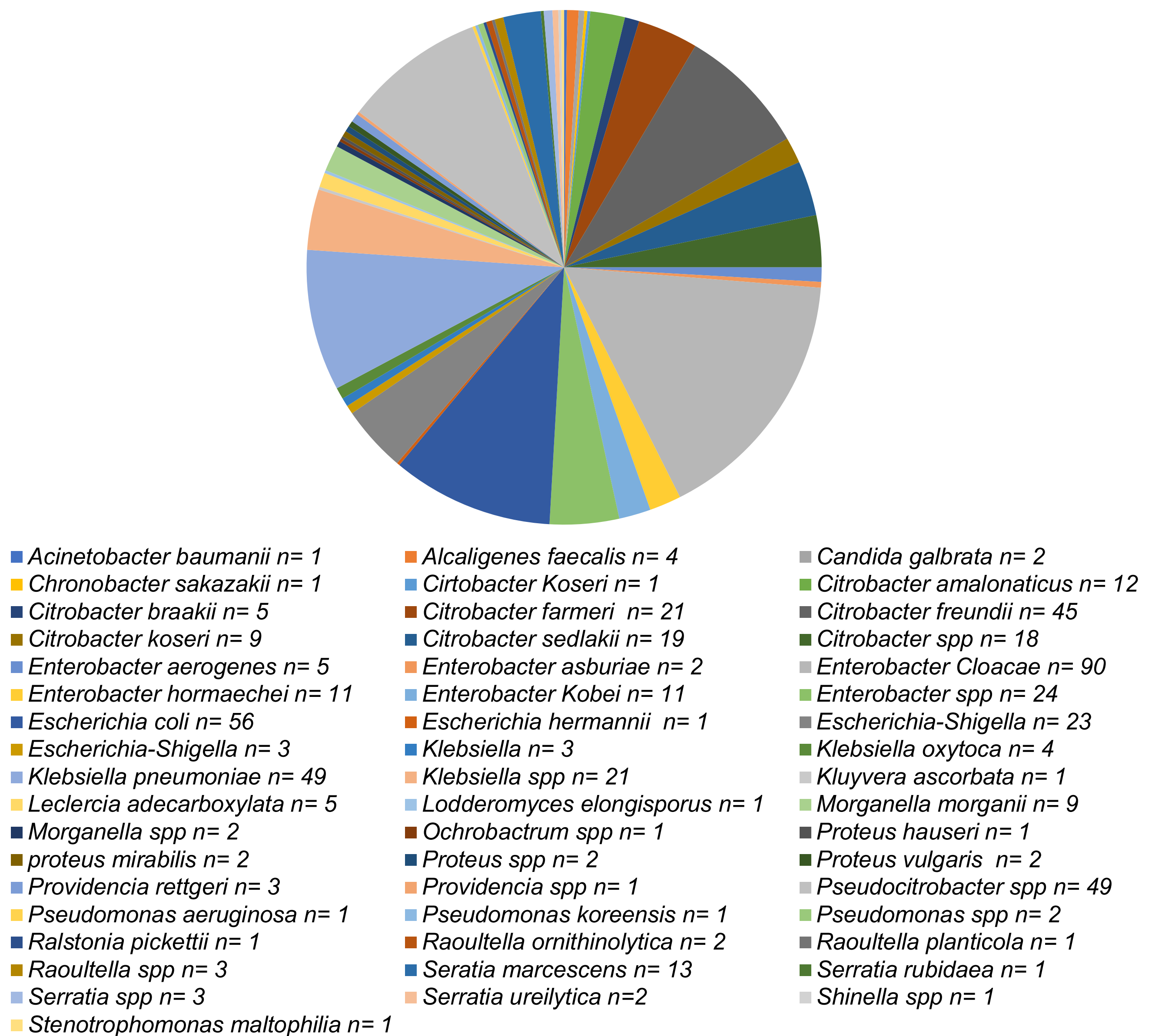


Figure 37. Bacterial species isolated from insects samples, Peshawar, Pakistan (n=552). MALDI-TOFF results shown as species whereas 16s RNA identifications are shown at genus level.

5.2.5. Sequence typing and phylogenetic analysis of hospital surface, insects and patients' wound samples by REP-PCR typing

REP-PCR results showed that the same or similar bacterial strains were isolated from insects, patients and hospital surface samples. This included seven sets of *K. pneumoniae*, 12 sets of *E. cloacae* and 12 sets of *E. coli* (Figure 38, 39 and 40). The minimum similarity coefficient for the two profiles being considered very similar, if not the same, was 84% for *E. coli*, 91% for *K. pneumoniae* and 87% for *E. cloacae*. Furthermore, similar ST groups were also shared between bacteria from samples of different environments (Figure 41, 42, 43, 44, 45 and 46). New ST groups and alleles were also identified and listed below (Table 23).

Table 23. New *K. pneumonia* ST and alleles.

Origin	*Isolate*	*gapA*	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*	*ST*
IW	206B	17	19	39	20	156	21	52	2660
IW	254B	4	7	2	1	9	4	25	2661
IW	454B	2	3	2	26	9	4	386	2662
IW	58G	17	19	28	39	51	21	385	2663
KB	CROW2	145	19	175	20	297	21	310	2664
IS	IS_1031G	2	1	145	1	1	1	6	2665
DW	JODIABAZAR	38	63	82	20	138	18	148	2666
PP	PP_231G	3	3	1	1	1	1	10	2667
PP	PP_287G	7	1	2	2	1	1	25	2668
PP	PP_422B	2	1	5	1	4	4	388	2669
PSS	PS_421G	4	1	5	1	7	11	24	2670
PSS	PS_465B	38	19	53	58	73	21	53	2671
PSS	PS_474G	17	19	28	20	117	18	148	2672
PSS	PS_556G2	18	22	26	22	94	20	51	2673
IS	IS_1103G	2	1	2	4	1	1	4	2674
IS	IS_823B	50	19	112	39	272	63	162	2675
IS	IS_1144B	17	55	96	20	138	18	277	2676
IW	609G	4	3	1	1	296	4	61	2677
IW	822G	50	19	122	39	272	63	162	2678
IS	IS_291B	154	1	11	1	298	1	13	2679
IS	IS_294B	2	1	2	37	8	1	387	2680
KS	S7_23	18	15	18	61	93	37	389	2681
PSS	PS_510G	17	19	39	20	299	18	52	2682
PSW	PS_253G2	4	7	2	1	9	4	25	2661
PSW	PS_310G	4	7	2	1	9	4	25	2661
IS	IS_809B	2	1	145	1	1	1	6	2665
IS	IS_463G	2	1	145	1	1	1	6	2665
IS	IS_1006G	50	19	112	39	272	63	162	2678

Table showing new alleles (Red font) and details about samples with new ST IW=Insects Winter, KB= Karachi Birds' droppings, DW= Drinking Water, PP= Peshawar Patients, PSS= Peshawar Surface Summer, PPW= Peshawar Surface Winter.

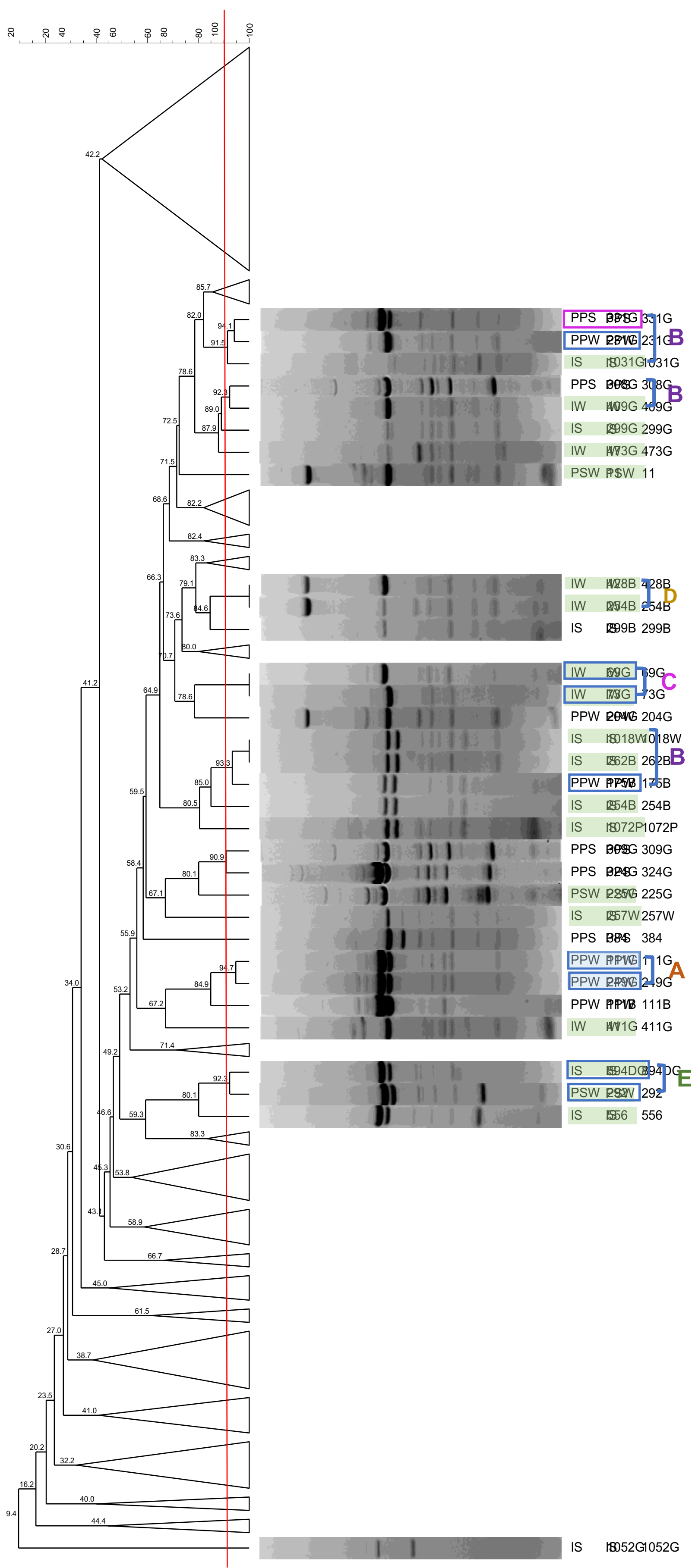


Figure 38. REP-profile of *K. pneumoniae* isolated from clinical and non-clinical samples of Peshawar. The minimum similarity coefficient for two profiles being considered very similar if not the same was 91%.

Legend: Green squares highlight non-clinical isolates. Blue boxes indicate bla_{NDM} Positive isolates where as $bla_{OXA\text{-}48}$ Like samples are shown in pink boxes. All other samples are $bla_{CTX\text{-}M\text{-}15}$ positives except the ones highlighted in blue.

A: Isolates with the same REP profile found in patients from same cleaned or un-cleaned wards within three weeks period.
B: Isolates with the same REP profile found in patients and insects from cleaned or un-cleaned wards > six months.

C: Isolates with the same REP profile found in Insects from same cleaned or un-cleaned wards within three weeks period.
D: Isolates with the same REP profile found in insects from different clean and un-cleaned wards within three weeks period.
E: Isolates with the same REP profile found in surface and insects from different cleaned or un-cleaned wards > six month.

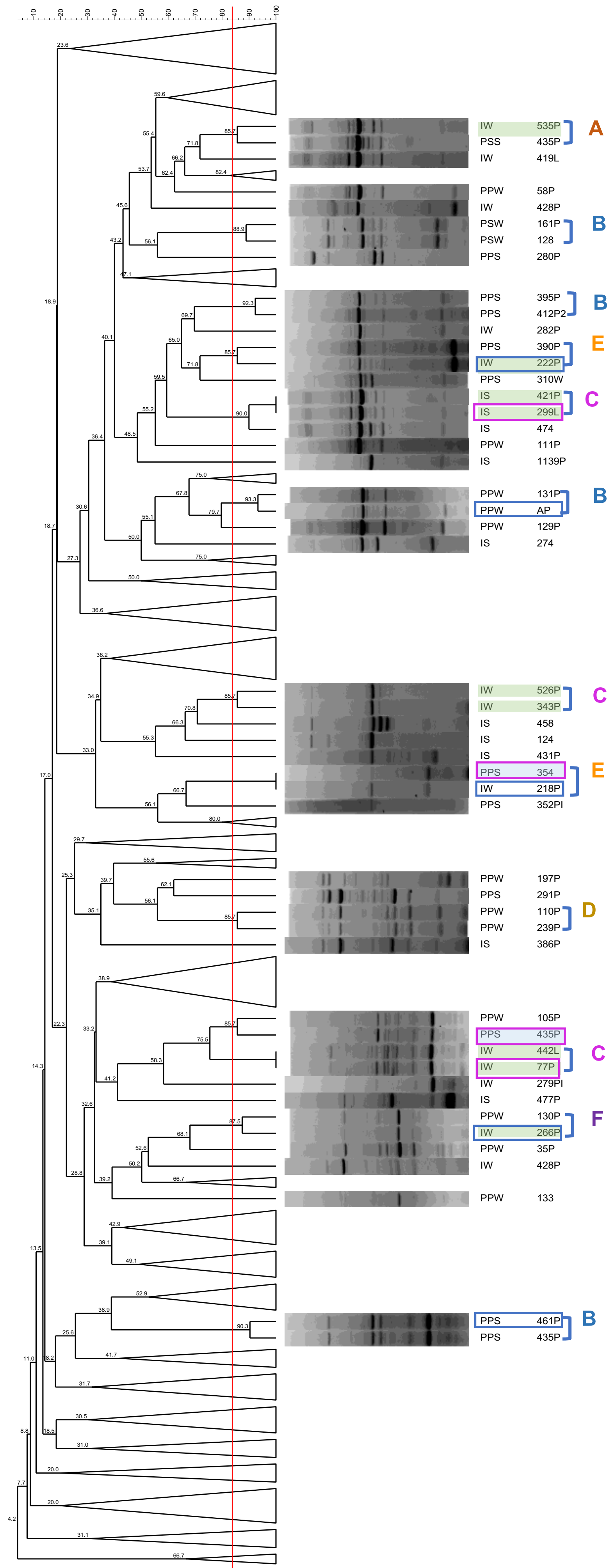


Figure 39. REP-profile of *E. coli* isolated from clinical and non-clinical samples of Peshawar. The minimum similarity coefficient for two profiles being considered very similar if not the same was 84%.

Legend: Green squares highlight non-clinical isolates. Blue boxes indicate *bla*$_{NDM}$ Positive isolates where as *bla*$_{OXA-48}$ Like samples are shown in pink boxes. All samples are *bla*$_{CTX-M-15}$ positives except the ones with blue colour.

A: Isolates with the same REP profile found in surface and insects from same cleaned or un-cleaned wards > six month.

B: Isolates with the same REP profile found in from distinct patients admitted to different clean and un-cleaned wards within three weeks period.

C: Isolates with the same REP profile found in Insects from cleaned or un-cleaned wards within three weeks period.

D: Isolates with the same REP profile found in distinct patients admitted to same clean and un-cleaned wards within three weeks period.

E: Isolates with the same REP profile found in surface and insects from different cleaned or un-cleaned wards > six month.

F: Isolates with the same REP profile found in Patients and insects from different cleaned or un-cleaned wards within 3 weeks period.

EnteroREP

EnteroREP

IS 1006G
IS 1065PG
IS 1006GW] D
IS 647GP
IS 501G
IS 275G
IW 473] F
IW 52PG
IW 445G
IW 472
IS 433W
IW 407] C
IW 412
IS 270B
IS 260P] C
IS 1155G
IS 223P
IS 355WG
IS 273PG
IS 650G] F
IW 472
IW 133G
IS 470P
IS 273G
IS 208P
IS 280L
IS 1137P
IS 257B
IS 576P] D
IS 471G
IS 489P] A
PSS 415DG
IS 663W
IS 259P] C
IS 655G
IS 292G
PSS 388
PSS 397DG] B
IS 459G
PSS 415] E
IW 555
IS 1078PG] C
IS 489G
IS 830G] D
IS 454G
IS 663G

Figure 40. REP-profile of *E. cloacae* isolated from clinical and non-clinical samples of Peshawar. The minimum similarity coefficient for two profiles being considered very similar if not the same was 87%.

Legend: Blue boxes indicate bla_{NDM} Positive isolates where as bla_{OXA-48} Like samples are shown in pink boxes. All other samples are $bla_{CTX-M-15}$ positives except the ones highlighted in blue.

A: Isolates with the same REP profile found in surface and insects from same cleaned or un-cleaned wards within three weeks period.

B: Isolates with the same REP profile found in surface and insects from different cleaned or un-cleaned wards within three weeks period.

C: Isolates with the same REP profile found in Insects from same cleaned or un-cleaned wards within three weeks period.

D: Isolates with the same REP profile found in insects from different clean and un-cleaned within three weeks period.

E: Isolates with the same REP profile found in surface and insects from different cleaned or un-cleaned wards > six month.

F: Isolates with the same REP profile found in insects from different cleaned or un-cleaned wards > six months period.

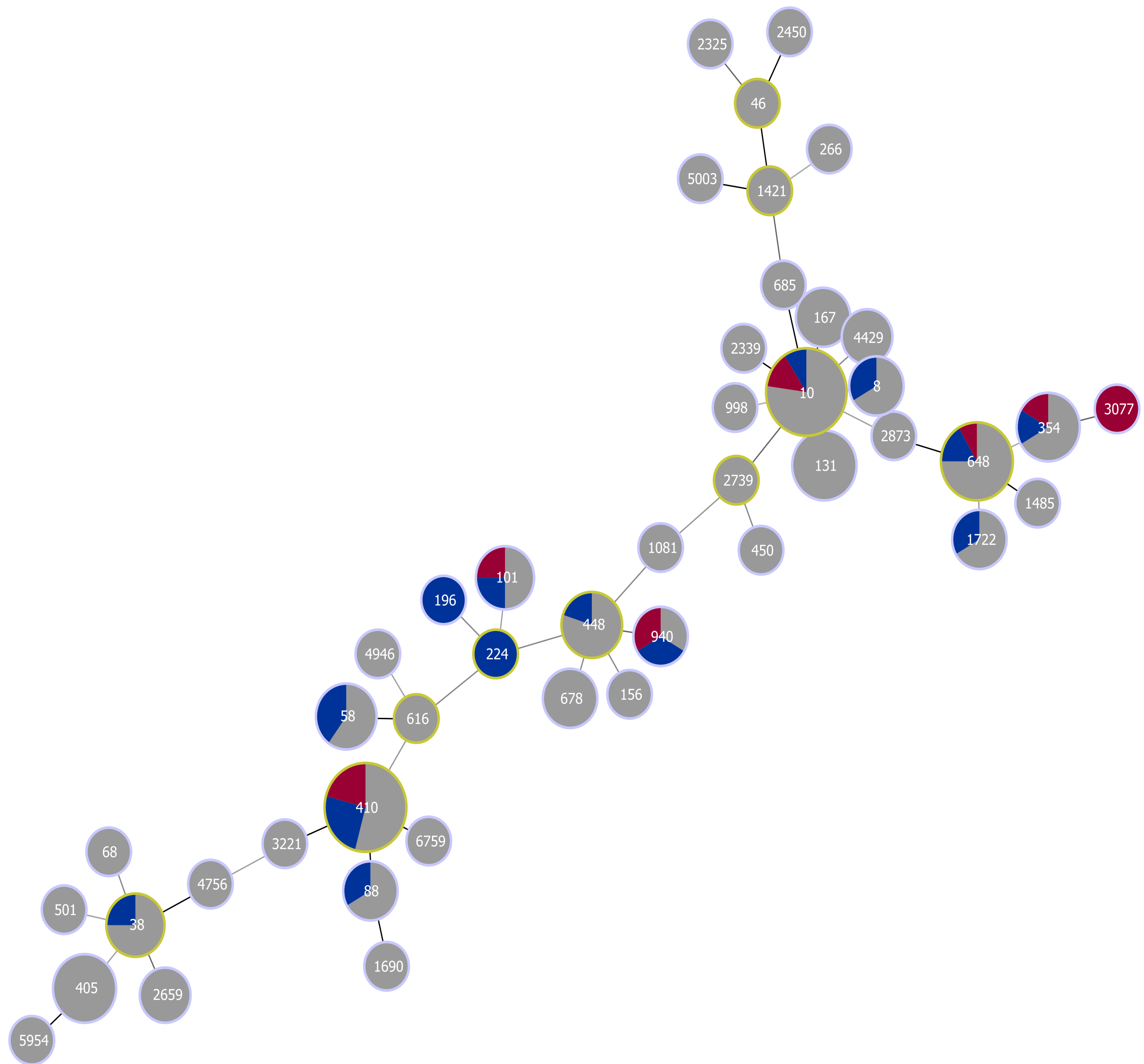


Figure 41. ***E. coli*** **ST groups among insects, patients and surface samples.** Colour is representing the types of resistance and the size of the circle is corresponding to the number of isolates (Blue: *bla*$_{NDM}$, Grey: *bla*$_{CTX\text{-}M\text{-}15}$ and Red: *bla*$_{OXA\text{-}48}$-like).

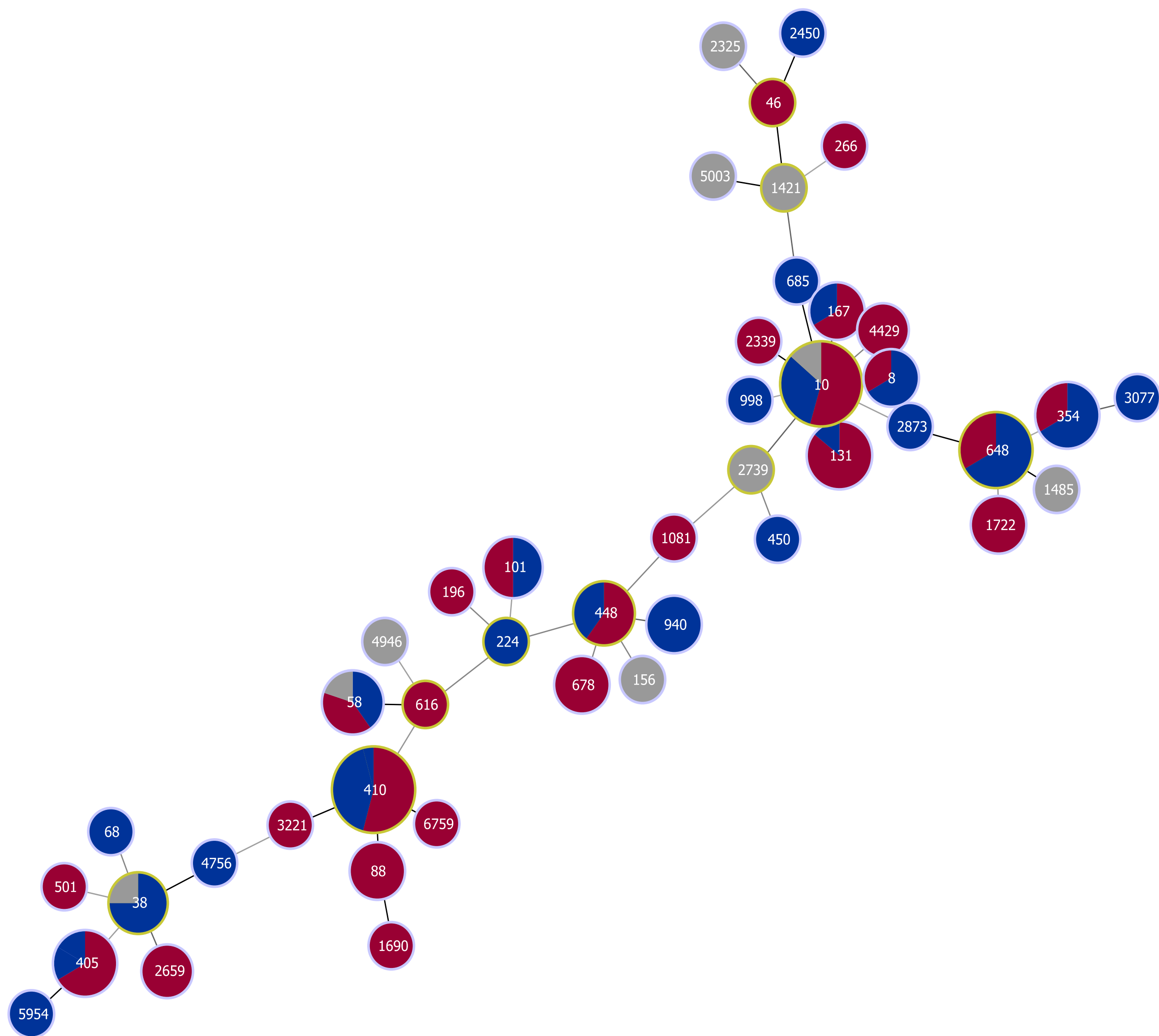


Figure 42. *E. coli* ST groups among insects, patients and surface samples. Colour is representing the ward of the sample and the size of the circle is corresponding to the number of isolates (Blue: clean wards, Red: uncleaned wards, Grey: not available).

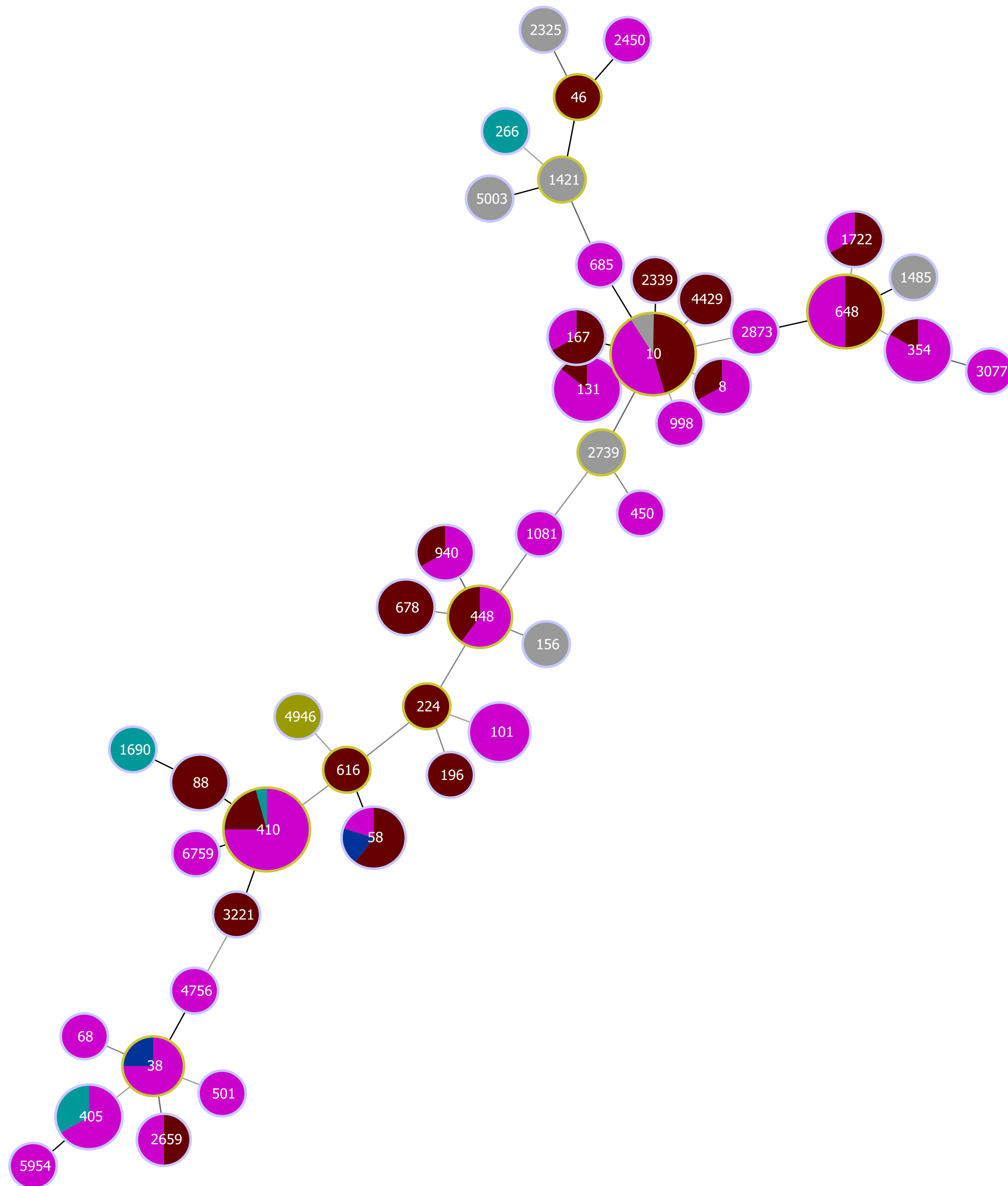


Figure 43. ***E. coli*** **ST groups among insects, patients and surface samples.** Colour is representing the origin of the sample and the size of the circle is corresponding to the number of isolates (Red: Peshawar insects, Sea-green: Peshawar hospital surface, Pink: Peshawar patients, Blue: Karachi hospital surface, Green: Karachi Insects, Grey: Karachi animal faeces).

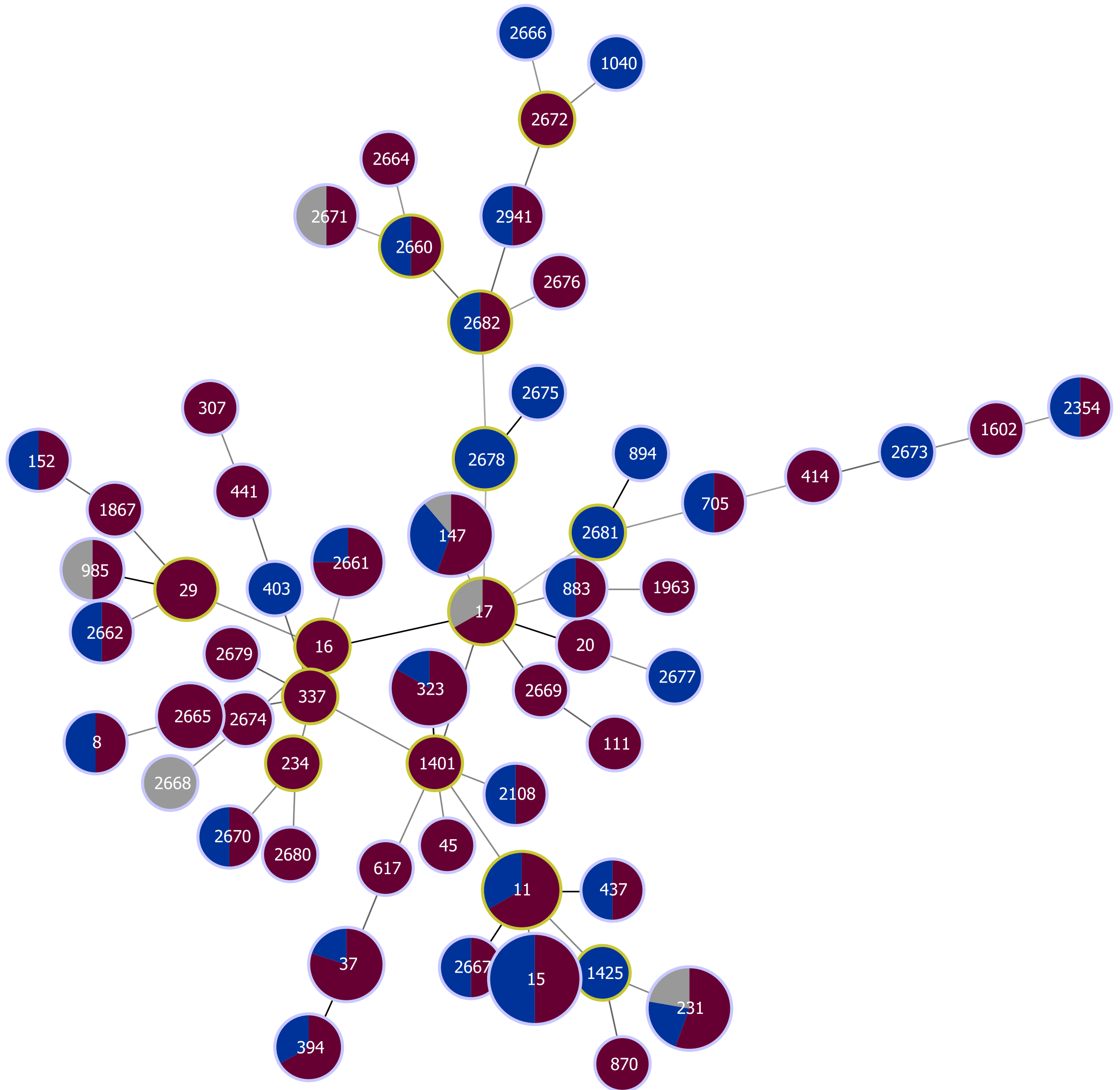


Figure 44. *K. pneumoniae* ST groups among insects, patients and surface samples. Colour is representing the types of resistance and the size of the circle is corresponding to the number of isolates (Blue: *bla*$_{NDM}$, Red: *bla*$_{CTX\text{-}M\text{-}15}$ and Grey: *bla*$_{OXA\text{-}48}$ like).

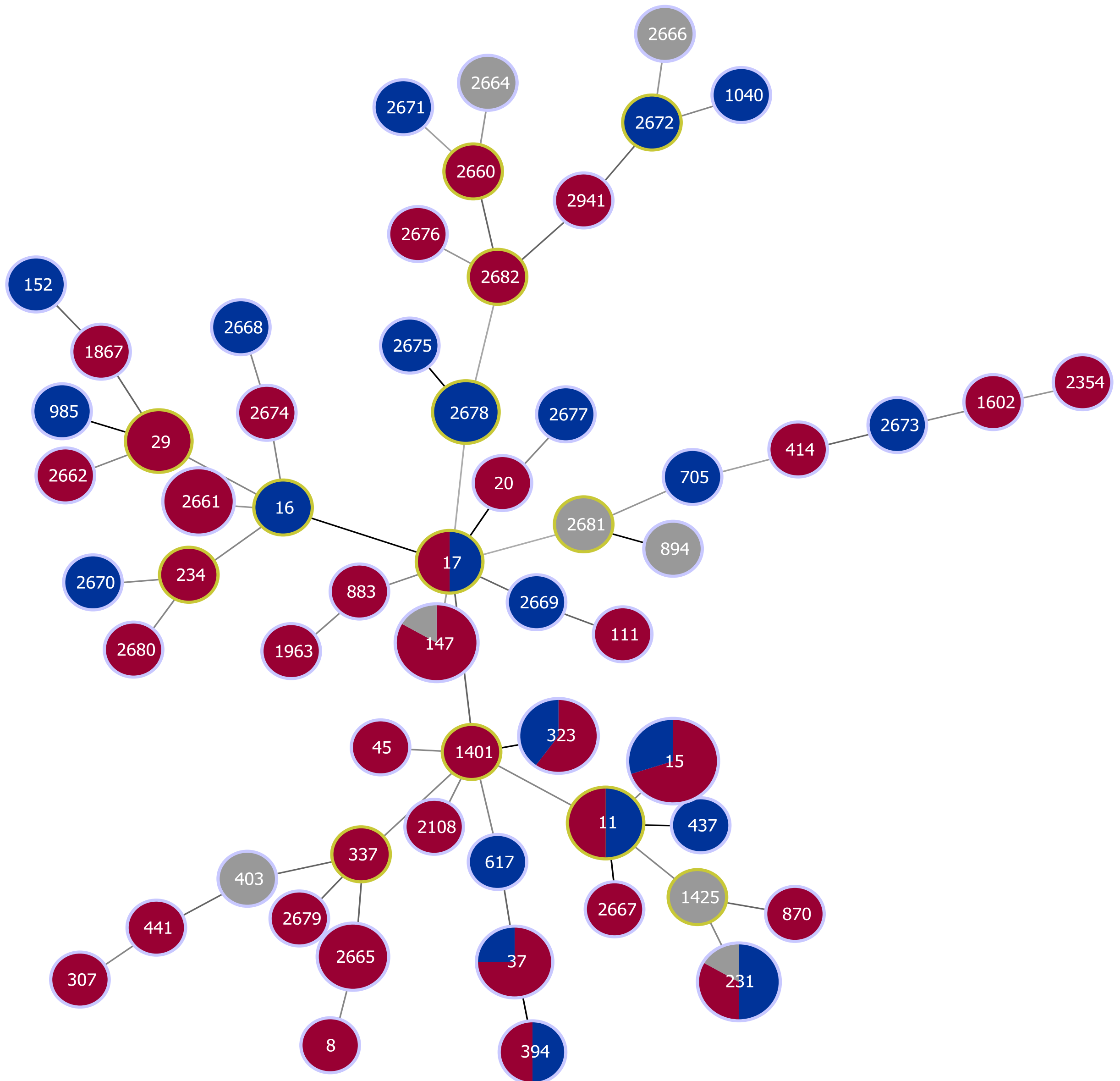


Figure 45. *K. pneumoniae* ST groups among insects, patients and surface samples. Colour is representing the ward of the sample and the size of the circle is corresponding to the number of isolates (Blue: clean wards, Red: uncleaned wards, Grey: not available)

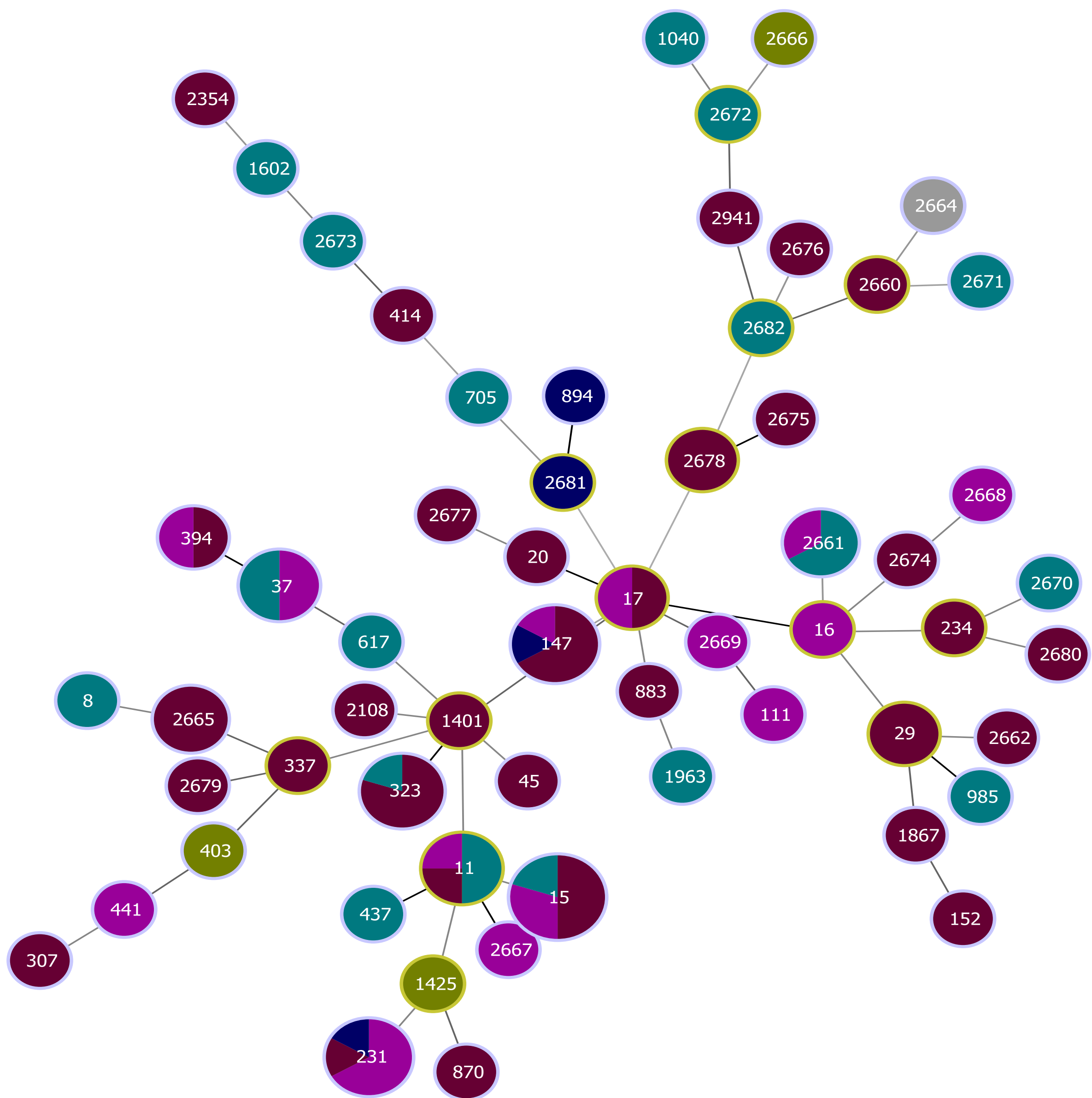


Figure 46. ***K. pneumoniae*** **ST groups among insects, patients and surface samples**. Colour is representing the origin of the sample and the size of the circle is corresponding to the number of isolates (Red: Peshawar Insects, Sea-green: Peshawar hospital surface, Pink: Peshawar patients, Blue: Karachi hospital surface, Green: Karachi drinking water, Grey: Karachi animal faeces).

5.3. Discussion

This study reports the high rate of carbapenem resistance reported in insects to date. The results indicate the spread of AMR to distinct environmental sectors previously ignored. Although there have been reports of $bla_{\mathrm{CTX\text{-}M}}$ resistance from flies, other insects and animals the carriage of bla_{NDM} has been rarely reported (Rahuma et al. 2005; Veldman et al. 2013; Woodford et al. 2014; Zurek and Ghosh 2014; Doosti et al. 2015; Moges et al. 2016).

Antibiotic resistant is more prevalent in South Asian countries where the unsatisfactory waste management and infection control, together with overcrowding and extensive use of antibiotics is believed to be the driving force (WHO 2014b; Quintela-Baluja et al. 2015; Laxminarayan and Chaudhury 2016). The hospital in this study is based in a congested area with broken roads, leakage prone sewage infrastructure and inadequate waste management. In fact, waste grounds can be seen just outside the hospital where used blood bags, syringes, plasters and other medical waste is disposed regularly. Hence, it is anticipated that the actual location of the hospital may contribute to the high resistance prevalence rate that is observed in this study (Figure 47, 48, 49 and 50).

Figure 47. The state of hospital waste management 1. A drinking water supply just outside the hospital

Figure 48. The state of hospital waste management 2. Dumping Ground Outside the Hospital

Figure 49. The state of hospital waste management 3. Birds can be seen (red circles) feeding on the waste

Figure 50. The state of hospital waste management 4. The pictures shows used glove, syringes, cast of plaster leg and other hospital waste in the dump nearby.

Peshawar also has a warmer climate which favour the growth of small insects. Various species of insects such as houseflies and moths are very common and found everywhere, especially in summers. Hot climate together with open infected waste can serve as reservoirs for insects where they can share and transmit different diseases including MDRB (Raka 2010; WHO 2014b).

In this study, proportionally more resistant bacteria were carried by cockroaches in winter and by flies in summer. Furthermore, *bla*$_{CTX-M-15}$ -positive bacteria were carried by flies and *bla*$_{NDM}$ and *bla*$_{OXA-4}$-like positive bacteria were mostly associate with cockroaches. Although, it was clear that the maximum number of insects collected in summer were flies and in winter were cockroaches but this doesn't align with the percentage of preferential carriage of one gene by one insect species over the another and should be subject to further investigation. Nevertheless, insects can become a vector for MDRB carriage and could disseminate to distinct environments or patients (Wang et al. 2017). In fact, it has been shown that bacteria can not only survive in the digestive tract of houseflies but also able to carry out genetic exchange between strains through frequent conjugation in the mouthparts and digestive tract of insects (Zurek and Ghosh 2014). In addition, the average annual temperature of Peshawar is approx. 30°C which is generally preferred for the growth of numerous key Gram-negative bacterial pathogens such as *E. coli, K. pneumoniae, Acinetobacter* spp., *Aeromonas* spp., *Burkholderia* spp., *Pseudomonas* spp. and *E. cloacae*. (Shah et al. 2013). Some studies also suggest higher conjugation transfer rates at conditions that are typical to this part of the world. Walsh et al. reported higher transfer rates by conjugation at 30°C rather than at 25 °C or 37°C (Boyce 2007; Timothy R. Walsh et al. 2011; Andremont and Walsh 2015). It has also been shown that HGT is possible on dry surfaces and MDRB can survive on contaminated

environmental surfaces for a long time ranging from days to months depending on the nature of the surface (Yang et al. 2013). For example, survival of more than 3 months is achieved on plastic surfaces and cloths, one week to two months on countertops, seven days to four months on dry polyvinyl chloride surfaces and more than a month on steel surface (Boyce 2007; Yang et al. 2013). Hence, it can be speculated that there is a greater possibility that the MDRB can persist in the environment and can be transferred to individuals and other animals via contamination route and to other bacteria via HGT. This is evident by the finding of this study where MDRB from insects, patients and surfaces shows distinct similarities including similar antibiotic sensitivity profiles, REP-PCR profiles and identical ST types for samples that were isolated from different environmental sectors (Boyce 2007). Regrettably, the plasmid of the resistant gene carrying bacteria were not analysed which would have provide more information about the possibility of HGT between different strains or species.

It is a common observation that *E. coli* is perhaps one of the most common producer of CTX-M-type ESBLs and carbapenemases whereas *K. pneumoniae* is more often associated with the carriage of carbapenemases (Ewers et al. 2012; Munoz-Price et al. 2013; Vasoo et al. 2015). Similarly, in this study *E. coli* and *K. pneumoniae* were found to be the major resistance gene carriers. Both MDR *E. coli* and *K. pneumoniae* are commonly associated with the development of infection (Ewers et al. 2012; Epson et al. 2014) and hence, their presence in high numbers in samples from patients, insects and surface is of concern. *E. coli* ST131 has been reported widely as the most dominated ST type carrying $bla_{\text{CTX-M-15}}$ not only limited to clinical but also isolated from multiple animal species (Woodford et al. 2011). However, in this study *E. coli* was dominated by ST410 and to a lesser extend ST10 and ST131 and only

one *bla*$_{CTX-M-15}$ expressing ST131 from an insect sample was identified. A recent study from Germany also had the similar findings when they analysed the ST of *E. coli* and found ST410 shared between wildlife, humans, companion animals and the environment (Schaufler et al. 2016). In contrast, ST10 is usually classed as low virulence strains but have also been associated with human infections occasionally (Day et al. 2016). However, the identification of *E. coli* ST10 in the other environment is not uncommon and have been isolated from poultry, retail meat and animal faeces (Day et al. 2016; Seni et al. 2016).

K. pneumoniae ST11 and ST147 and ST231 are known successful pathogenic strains of humans (Woodford et al. 2011) but have also been isolated from Peshawar insects and the hospital surface swabs from Peshawar and Karachi in this study. Another pathogen strain “ST15”, which was previously associated with human but has been recently identified as a dominant *K. pneumoniae* ST from animals, has also been recovered from several samples in this study carrying *bla*$_{NDM}$ and *bla*$_{CTX-M-15}$ from insects, patients and hospital surface in Peshawar (Damjanova et al. 2008; Ewers et al. 2014; Melegh et al. 2015). Nevertheless, it cannot be suggested that a certain ST was a dominant carrier or was responsible for the spread of MDR but rather a multi-clonal spread, including the acquisition of resistant genes in new ST, could be associated with the increased resistance rates. Multiple ST groups circulating in the environment with the ability to acquire any resistance genes or plasmids that are locally prevalent might be the one last push to widely distribute MDR across different environmental niches (Ewers et al. 2012).

6. PNA as an Alternative Therapy to Neutralize β-lactam Antibiotic Resistance

6.1. Introduction

Antisense PNA therapy is one of the revolutionary technologies with the potential to develop a novel antimicrobial agent. Advancement in genomic, synthetic chemistry and more recently the development of cell delivery system has opened further ventures that could be investigated for antimicrobial therapies (Lehto et al. 2016). PNA was constructed to mimic the behaviour of DNA and act as a ligand but has gained a lot of attention since the discovery of its gene editing capacity at an mRNA level (Patenge et al. 2013). Antisense PNA attached to CPP for delivery into the cell can bind to the complementary mRNA and inhibit gene expression leading to altering the activity of the genes of interest (Ghosal et al. 2013).

Traditionally, drug companies have mostly favoured broad-spectrum antibiotics as they advantageously provide rapid treatment of multiple pathogens without the need of time-consuming diagnostics (Casadevall, 2009). Antisense therapies tend to be more specific due to variations between different species' specific genomics. However, some may consider this as a drawback but it also provides a distinctive advantage over other therapies by inhibiting one target organism or gene and potentially avoiding the unnecessary off-target effects. Similarly, acquired resistance is usually encoded by one or few genes which can be easily transferred through HGT rendering antibiotics useless (Ramsay et al. 2016). Theoretically, antisense therapy could also be used to inhibit the expression of resistance genes and restore the sensitivity of resistant strains. Several studies have used this approach and successfully inhibited the expression of resistance genes including outer-membrane efflux protein (*oprM)* in *P. aeruginosa*, aminoglycoside N- acetyltransferase (aac(6')-Ib), chloromycetin acetyl transferase (*act*) and multiple antibiotic resistance operon

(*marORAB*) in *E. coli*, glycopeptide- resistant related protein (*vanA)* in *Enterococcus faecalis*, CmeABC multidrug efflux transporter (*cmeA*) in *Campylobacter jejun*, penicillin-binding protein (*mecA)* in *Staphylococcus aureus* and methionyl-tRNA synthetase /UDP-N-acetylenolpyruvoyl glucosamine reductase (*metS/murB)* in *Bacillus anthracis* (Bai and Luo 2012). In this chapter, antisense PNA will be designed to inhibit dominant β-lactamase genes such as bla_{NDM}, $bla_{\text{OXA-48}}$ *like*, bla_{KPC}, bla_{VIM} and class 1 integron. It is hypothesized that the treatment with anti-resistant PNA will result in initiating a synergetic response in combination with carbapenem antibiotics and result in conservation of the therapeutic potential of clinically important drugs.

6.2. Results

6.2.1. Minimum Inhibitory concentrations of anti-resistance PNA against *bla*$_{KPC}$, *bla*$_{OXA-48}$ *like, bla*$_{NDM}$, *bla*$_{VIM}$ and integrase of class 1 integron

All PNA's, except antisense-peptide PNA conjugate (AP-PNA-C) to *bla*$_{NDM}$, were ineffective in reducing the MICs of MDRB when tested up to 16μMol of highest concentration except PNA4476 against *bla*$_{NDM}$. Anti-NDM AP-PNA-C reduced MIC to a sensitive level by treatment of PNA at concentrations of 8μMol, 4μMol, 2μMol and 1μMol against *K. pneumoniae, A. baumannii, E. coli and P. aeruginosa*, respectively (Table 24).

Table 24. MIC of anti-NDM-PNA and meropenem against resistant strains carrying *bla*$_{NDM}$.

Strain	PNA (µMol)	Meropenem (µg/ml)							
		128	64	32	16	8	4	2	1
Klebsiella pneumoniae	16								
	8								
	4								
	2								
	1								
	0								
Acinetobacter baumannii	16								
	8								
	4								
	2								
	1								
	0								
Pseudomonas aeruginosa	16								
	8								
	4								
	2								
	1								
	0								
Escherichia coli	16								
	8								
	4								
	2								
	1								
	0								

K. pneumoniae (blue), *A. baumannii* (orange), *P. aeruginosa* (green) and *E. coli* (yellow).

Unfortunately, further testing of anti-NDM AP-PNA-C also revealed some off-target or toxic effects on the cells and resulted in the inhibition of *bla*$_{NDM}$ deficient cells at 16, 8 and 4 µMol PNA in all bacterial species tested (*K. pneumoniae, A. baumannii, E. coli and P. aeruginosa*). The second batch of AP-PNA-C against *bla*$_{NDM}$, *bla*$_{OXA-48}$ like, *bla*$_{KPC}$ and Class 1 integron precipitated upon dissolving in water presumably due to an error in the synthesis and the third and fourth batch of PNA's against *bla*$_{NDM}$, *bla*$_{OXA-48}$ like, *bla*$_{KPC}$ and Class 1 integron also didn't generate any inhibitory effects, except PNA 4703 against class 1 integron start-site which visibly inhibited the growth at concentration of 16µMol. Cloudiness was seen at higher concentrations (16 and 32µMol) of PNA however, this disappeared at lower concentrations (8, 4, 2, 1µMol) and hence suggested precipitation of the product rather than actual growth of bacteria. Therefore, a cut off OD measurement of 0.2 was considered as no growth. The growth of *P. aeruginosa* strain expressing *bla*$_{VIM-2}$ was reduced by 5-fold and the MIC was reduced from >64 to 1µg/ml in a *P. aeruginosa* strain with integron class 1 expressing *bla*$_{VIM-2}$ (Figure 51).

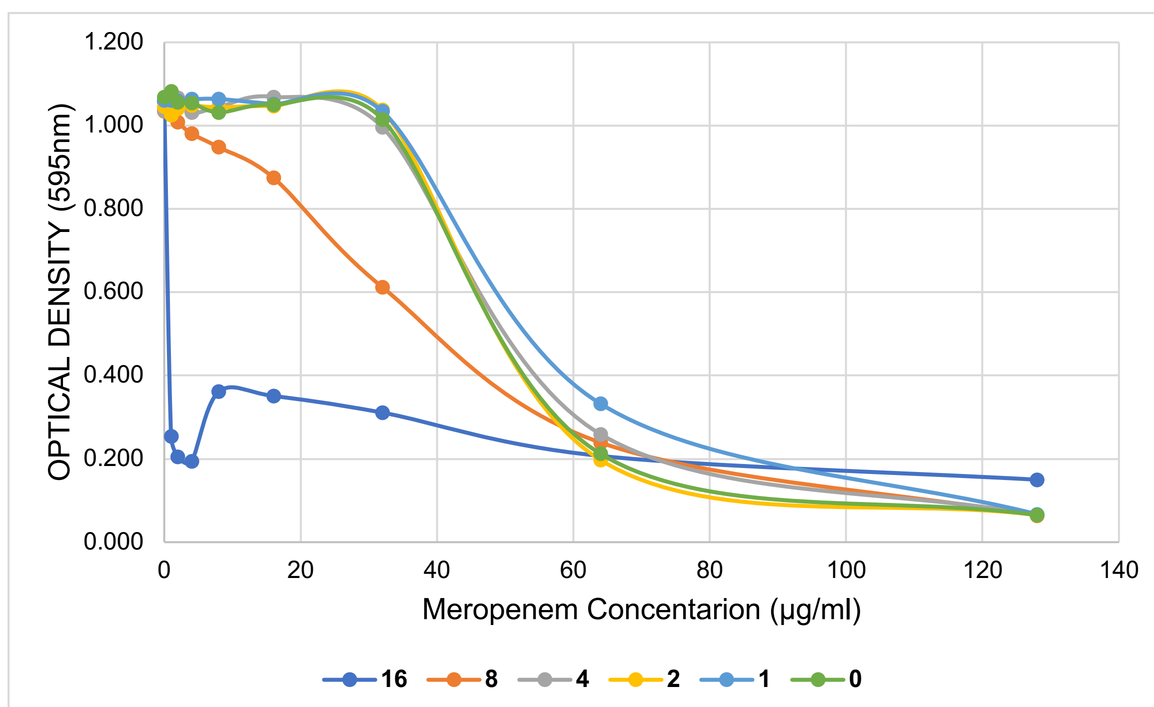


Figure 51. The effects of class 1 integron PNA 4703 on the growth of *P. aeruginosa* carrying *bla*$_{\mathbf{VIM-2}}$. The graph shows the effects of PNA4703 on the growth of *bla*$_{\text{VIM-2}}$ carrying *P. aeruginosa* strain RES-2074 by measuring the optical density of the culture. The concentration of PNA is displayed by coloured lines. The maximum growth reduction is seen by 16µMol PNA(light blue) where the meropenem MIC was reduced to 1µg/ml.

6.2.2. *AcpP* PNA target validation

Upon analysis of the *acpP* DH5alpha clones, it was revealed that the clone had almost double the MIC compared to the wildtype strains. The clone MIC to anti-*acp*-PNA EBL 366, 392 and 596 was increased by 1-fold and a 2-fold increase was seen by EBL183 and EBL264. All other antibiotics which were used as control included ciprofloxacin, streptomycin, nalidixic acid, tetracycline and ampicillin. Both the clone and wild-type showed similar MICs to those conventional antibiotics (Table 25).

Table 25*.* ***acpP*** **clone and wild-type MIC to anti-*****acpP*****-PNA and conventional antibiotics.**

Antibiotic/PNA	MIC Wildtype	MIC *acpP* clone
EBL366	32	64
EBL392	8	16
EBL506	64	128
EBL183	32	128
EBL264	16	64
Ciprofloxacin	0.12	0.12
Streptomicin	4	4
Nalidixic acid	>32	>32
Tetracyclin	1	1
Ampicillin	8-16	32

Data collected from three replicates and *E. coli ATCC25922* was used as control in all experiments.

Similarly, there was a significant difference (P-value <0.0001) between the expression levels of mRNA corresponding to *acpP* gene of the clone and the wildtype strain. The mRNA expression level of the clone increased by 3-fold when compared to the wildtype strain (Figure 52).

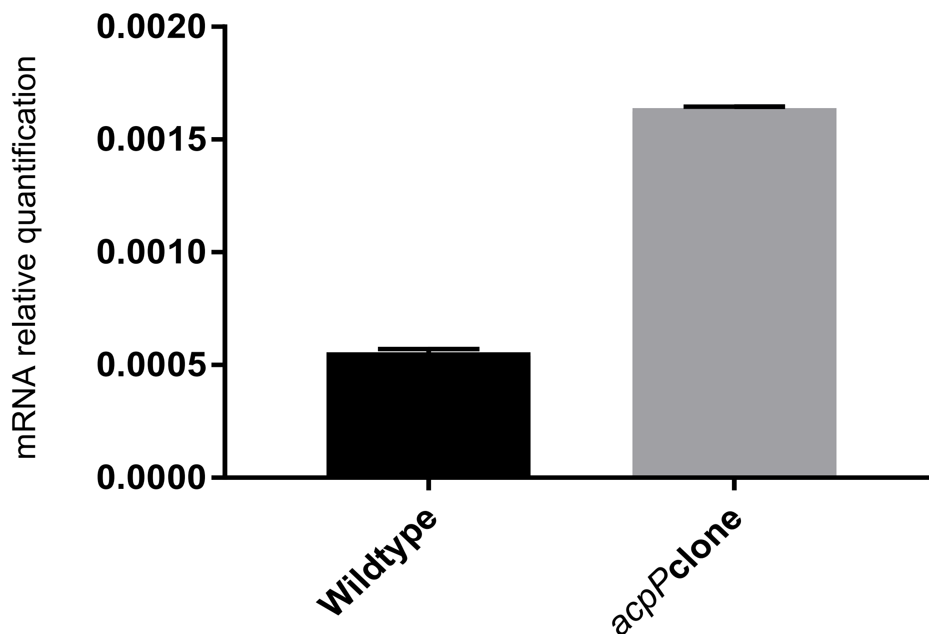


Figure 52. Expression analysis of *acpP.* The graphs show relative *acpP* mRNA quantification of the clone (grey) and wildtype (black) strain (P-value <0.0001- Statistical analysis was performed using Prism Software: Two-tailed unpaired T-Test.

6.2.3. Resistance analysis

The FoR assays to PNA EBL110, EBL111, EBL232, EBL237 were carried out to analyse the rate of mutant development. *K. pneumoniae ATCC13883* displayed a mutation rate of 1.38625 x 10^{-8} to PNA 110 and 5.8591 x 10^{-9} to PNA 111. However, no growth was observed in cells treated with anti-*acpP*-PNA EBL232 and EBL237. On the other hand, *E. coli* ATCC25922 did not show any growth when tested against four and eight times the MIC concentration of the PNAs and hence mutation rate was not established.

Selected strains were also processed through a series of serial passages to analyse the genetic makeup of the mutation that could arise against selected anti-*acpP*-PNAs (EBL183 and EBL264). Analysis of the whole genome sequencing results revealed that all the cultures resistant to the compound EBL183 had acquired either a frameshift mutation in the *sbmA* gene or a deletion of the region containing it. All the cultures with these deletions had a MIC of >128μg/mL compared to the MIC of the parental strains of 4 and 8μg/ml. Similarly, cultures selected for resistance to the compound EBL264 contained a single amino acid substitution in *sbmA* that resulted in an increased MIC to 64 μg/ml compared to the parental MIC of 4 and 2μg/ml.

MG1655 Mutants strains CH5095, CH5096 and CH5097 raised to PNA183 all revealed a 5-12Kb deletion in the genome. The changes were always observed in the same regions and were always identified with the deletion of all *sbmA* gene in all cases and differing nearby deletion by sizes. All these mutated strains adapted to a higher MIC range of 128μMol. CH5095 had a 12.7kb deletion whereas CH5096 and CH5097 exhibited a similar deletion of 5.4 kb (Figure 53 and 54). Another *ATCC25922* mutant raised to a different anti-*acpP*-PNA, EBL264, also had very similar 5kb *sbmA* deletion mutation which resulted in increasing MIC from 8 to 128 μMol.

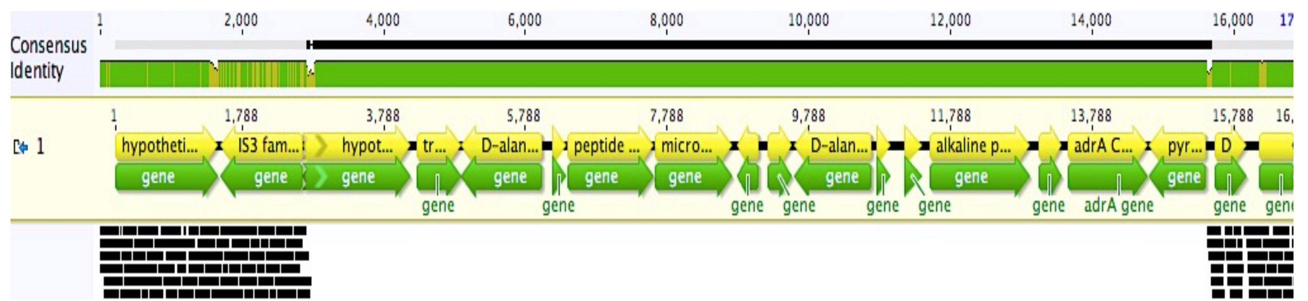


Figure 53. Mutation analysis of CH5095. The figure shows the coting alignment of sequenced mutant strain to the extracted reference region (NZ_CP009685). The 12.7kb deletion (2998-15,756bp) is highlighted in black on the consensus sequences.

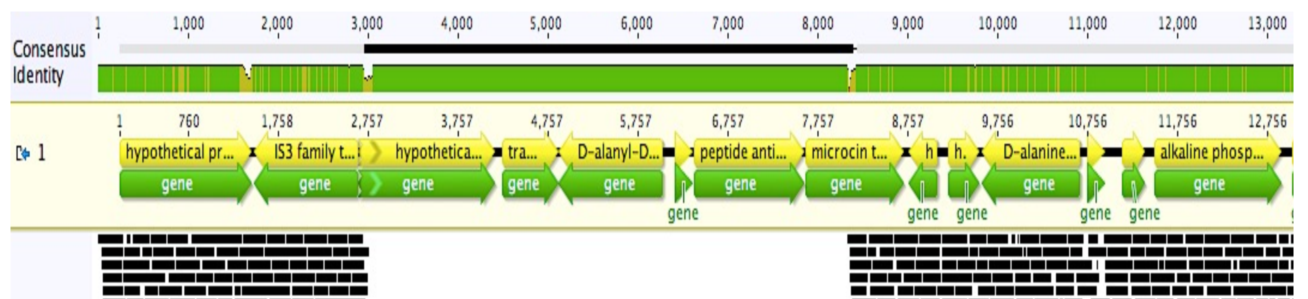


Figure 54. Mutation analysis of CH5096. The figure shows alignment of the sequences derived from mutant strain CH5096 in comparison to extraction of the reference strain (NZ_CP009685). The 5.4kb deletion (2962-8425) is highlighted in black on the consensus sequences. The same deletion was also seen in CH5097 (2954-8430) and Lineage #7 *ATCC25922* (1556-6675).

All other evolved strain of *MG1655* origin (CH5098, CH5099 and CH50100) raised to PNA 264 revealed a mismatch in *sbmA* gene (V106G) (Figure 55). For these strains, the mutant always had MIC of 64µM compared to the original MIC of 4 and 8µg/mL.

A multidrug resistant strain of *E. coli* (EN136:CH3490) which had MIC raised from 4 to 128µMol against PNA 264 also revealed the same mutation. However, the sequences of both evolved (mutant) and un-evolved strain (Control) revealed more than 6700 differences to the reference strain (*E. coli* IHE3034). Comparing the differences, the evolved strain has approximately 400 mutations that didn't appear in the un-evolved strain. Similarly, another MDR resistant strain of *K. pneumoniae* (EN141: CH3493) which had MIC increased from 2µMol to 64µMol tp PNA264 displayed 1,850 differences between evolved and un-evolved strains when compared to the reference genome (PittNDM01). The evolved strain had 69 mutations that didn't appear in the un-evolved strain and among them the same mutation in *sbmA* (sbmA V106G) was also present. The other resistances could be responsible for the observed resistance to PNA's. Though, it is difficult to determine this due to the MDR nature of the strains and hence those mutations were not further investigated.

Figure 55. Mutation analysis of CH5098. The figure shows a representation of sbmA gene mutation (V106G: T to G) by alimenting of CH5098 mutant's contig to the reference gene (*MG1655* strain NZ_CP009685). The mismatched in sbmA gene (V106G) is highlighted in black in the consensus sequences.

Another *E. coli* evolved strain (CH5101:EN1) of *ATCC25922* origin was selected for resistance to PNA183. In this strain, the MIC was raised to from 4 to >128μM and a frameshift mutation in *sbmA* region was identified which could have contributed to the elevated resistance (Figure 56).

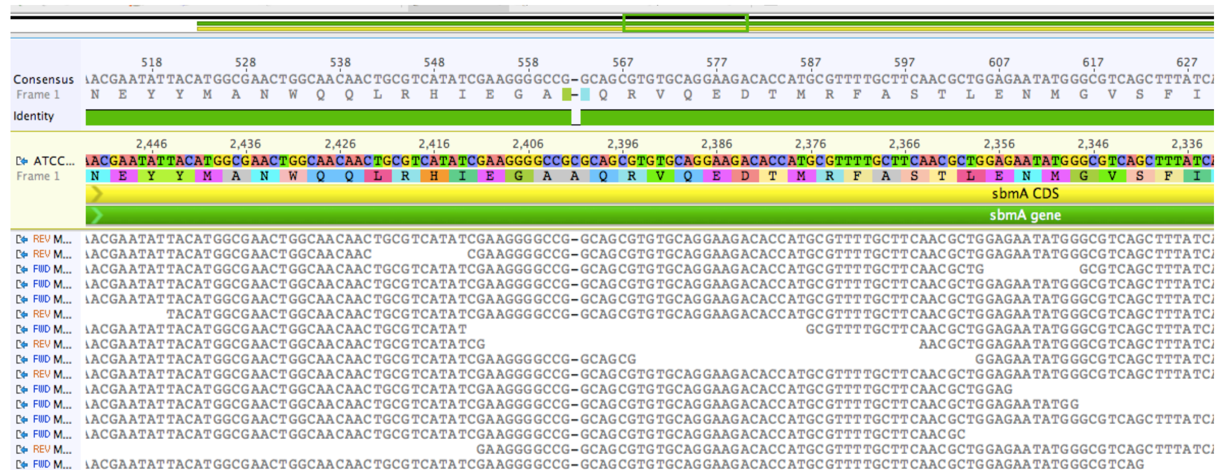


Figure 56. Mutation analysis of CH5098. The figure shows the frameshift mutation of *sbmA* (A188fs: GCG>Alanine to GGC>Glycine by deletion of amino acid C:63) in strain sequences of evolved strain CH501 compared to the reference gene (CP009072).

MDR *E. coli* strain (CH3493) evolved to PNA264 had MIC raised from 2 to 64 μMol. The un-evolved strain has 6,600 differences and the evolved displayed 7,400 differences from the closely matched reference sequence of *E. coli* ECONIH1. This discrepancy is probably due to low read depth in the un-evolved strain. Filtering the variants in the evolved strain with the variants in the un-evolved strain does not reduce the number of mutations down to a level that can reasonably be interpreted. However, a 3-nucleotide deletion in *sbmA* gene (ΔL407) seemed genuine and more likely a reaction to PNA treatment. (Figure 57).

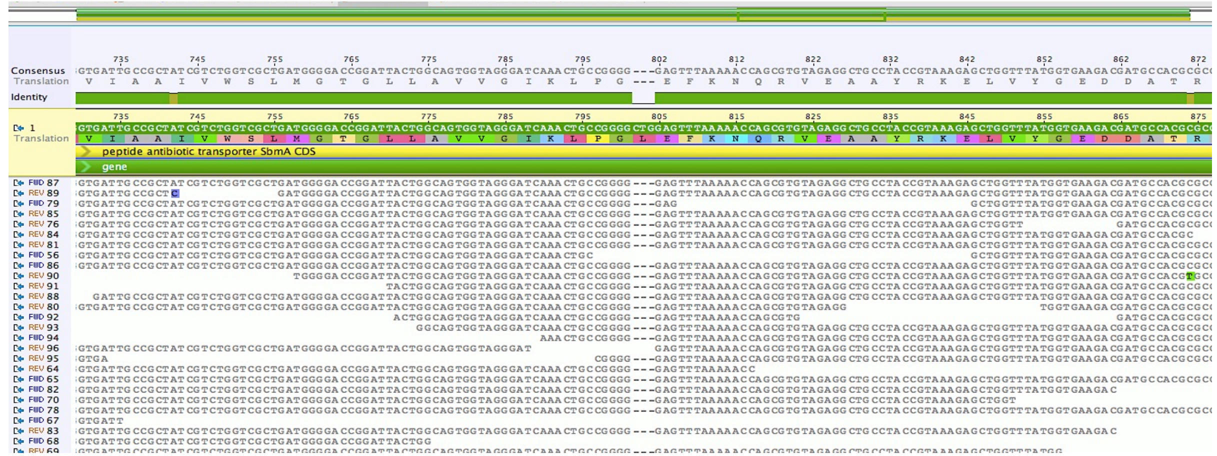


Figure 57. Mutation analysis of CH3493. The figure shows a 3 nt deletion in *sbmA* gene (∆L407).

Further MDR resistant strain of *E. coli* (EN137: CH3491) and *K. pneumonia*e (EN142: CH3493) had MIC raised from 2 and 4 to 64µMol against PNA 264. Analysis revealed an insertion in *sbmA* which looks like a transposon hopped into the gene. Other mutations included three silent mutations in EN142 and a conservative mutation (change amino acids to same properties) in EN137 but their role in the resistance is difficult to assume. (Figure 58).

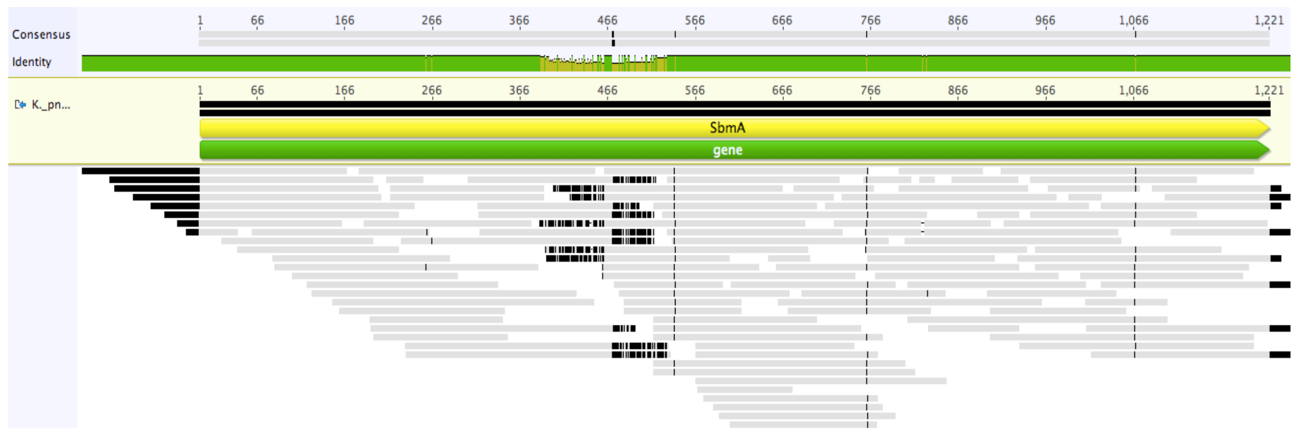


Figure 58. Mutation analysis of CH3493. Mutation in EN142: (L180G>T) Leucine CTG-CTT= Both Leucine: (G254G>T) Glycine GGG-GGT=Both Glycine: (T356T>C) Threonine ACT-ACC= Both Threonine. Mutation in EN137: (A293C>T Alanine GCG-GTG Valine. Reference genomes used were *K. pneumoniae* CG43 and *E. coli* MNCRE44.

6.3. Discussion

Most of the anti-resistant PNA's tested in this study were ineffective in generating a satisfactory response when tested in bacteria expressing carbapenem resistance. All AP-PNA-C tested in this study, except PNA4703 directed towards the start site of integron class 1, were found to be either toxic to the cells or have no inhibitory effects at all. No activity could have been caused by insufficient entry of AP-PNA-C in the cell or the ability to generate a stringent response even if the entry is achieved. However, the CPPs used in this study displayed good activity in the *acpP* PNA overexpressed mutant indicating no apparent issue with the capability of CPP to cargo PNA across the cell membrane. This is in accordance with several other published studies who have reported successful results with these CPPs (Patenge et al. 2013). Furthermore, they are also tolerated by bacterial cells and have shown no toxic activity at higher concentration of up to 60μMol (Good et al. 2001; Tan et al. 2005; Xue-Wen et al. 2007; Madani et al. 2011; Patenge et al. 2013). It is also unlikely that PNA sequence alone would have caused any toxicity as naked PNA or the conjugated peptide sequence used in this study was tested alone and have not revealed any toxicity on the bacterial cells (un-published results-Peter Nielson). Hence it is suggested that the combination of CPP and PNA sequence might have generated a toxic compound which could be responsible for the observed results.

Nevertheless, restricted cell uptake of oligomer-based antibiotics is an obvious difficulty in antisense therapy and would require further investigation (Good and Stach 2011). It can also be a weak link in relation to the development of resistance. The low or comparable to ciprofloxacin frequency of mutation rate indicated no apparent vulnerability to the development of resistance mechanisms in nature.

However, almost all mutated strains generated through resistant passages corresponded to a change in *sbmA* gene. *SbmA* is an inner-membrane transport protein associated with the transport of glycopeptides and antimicrobial peptides including AP-PNA-C across the cell membrane through electrochemical gradient (Runti et al. 2013). The deletion or mutation of *sbmA* is associated with altered transport and hence reduced sensitivity to AP-PNA-Cs (Ghosal et al. 2013). Similar findings were also reported when the deletion of *SbmA* gene resulted in generating an *E. coli* strain resistant to AP-*acpP*-C and the sensitivity was restored upon complementation with *sbmA* (Ghosal et al. 2013; Runti et al. 2013). However, it is important to note that no mutations were seen in the mRNA target region in this study or any published studies looking at the resistance mechanisms of AP-PNA-C. Therefore, it can be anticipated that resistance is more likely to arise due to the delivery system rather than mutation in the gene target sequence.

The findings report some of the challenges that could arise whilst trying to investigate potential new or alternative therapeutics. Such studies are essential in providing valuable information and generating a “lead” product for further investigation or establishing small scale “proof of concept” studies. AOs or PNA technology could generate ideal candidates for antimicrobial therapies, providing an appropriate delivery system is achieved. Therefore, further investigation into the development of an efficient delivery system is required.

7. General Discussion

7.1. Factors contributing to the environmental spread of AMR

The spread of AMR is one of the most important issues faced by humans in the 21st century (O 'Neill 2015; WHO 2015a). Resistance can arise due to the selection pressure exerted on bacteria by the overuse/abuse of antibiotics in several socio-sectors including human and veterinary medicine, livestock, aquaculture and agriculture (Henriques Normark and Normark 2002; Boyce 2007; WHO 2014b). They may transfer between environments, animals or humans through lack of hygiene, improper sanitation and waste management and could also spread to other bacteria by HGT (WHO 2014b). Contaminated environments may become reservoirs of resistance genes and further disseminate these to distinct socio-sectors such as farms and communities (Talon 1999). However, despite the obvious link of environmental contamination to increasing AMR rates, there, globally, appears to be little attention given by governments to rectify the situation.

MDRB are universally disseminated; however, the problem is augmented in LMICs of South Asia such as Pakistan and India. There is also a lack of research and no national surveillance system to monitor the changing prevalence rates of MDR (WHO 2013a; Albiger et al. 2015). The initial identification of bla_{NDM} in New Delhi environment succeed in highlighting this issue internationally. However, instead of encouraging such studies, the finding became a target for diplomatic debate and received unjustified criticism from Indian authorities and resulted in initiating a blockade for further studies. Although Indian authorities are slowly opening to collaborations, however, it is generally difficult to establish such studies with lengthy "set-up" periods and access to data and samples/strains. Nevertheless, since these countries have no national surveillance system, it becomes extremely difficult to determine the true burden of AMR in the environment (i.e. its impact on agriculture),

clinical settings and community or primary care patients (Toleman and Walsh, unpublished data). Currently, India, Pakistan and Bangladesh are regarded as hotspots for carbapenemase genes such as bla_{NDM} and $bla_{\text{OXA181/232}}$ (Dortet et al. 2014; Woodford et al. 2014). The environment in South Asia is rapidly becoming a gene pool for the acquisition and dissemination of AMR. Factors such as poor sanitation, waste management and overuse and misuse of antibiotics are regard as drivers of AMR; however, the hospital environment and its associated co-factors are rarely investigated. As explained in the general introduction, in countries like India and Pakistan there is no proper waste management. Household or even hospital waste is often discarded in some inappropriate places creating *ad-hoc* rubbish tips – often close to poor communities. Stray animals (particularly dogs), birds and insects are often seen feeding on these rubbish tips which might potentially contain un-degraded antibiotics and almost certainly MDRB. Furthermore, in South Asia, the sewage infrastructure is very old and gutters/pipes are often exposed; the water supply is also often contaminated by effluents from other sources (Picão et al. 2013). This was evident by our data when we recovered resistant bacteria from drinking water, insects and bird's droppings. All these factors contribute to the cycling of AMR in the community, patients and the environment (Figure 59).

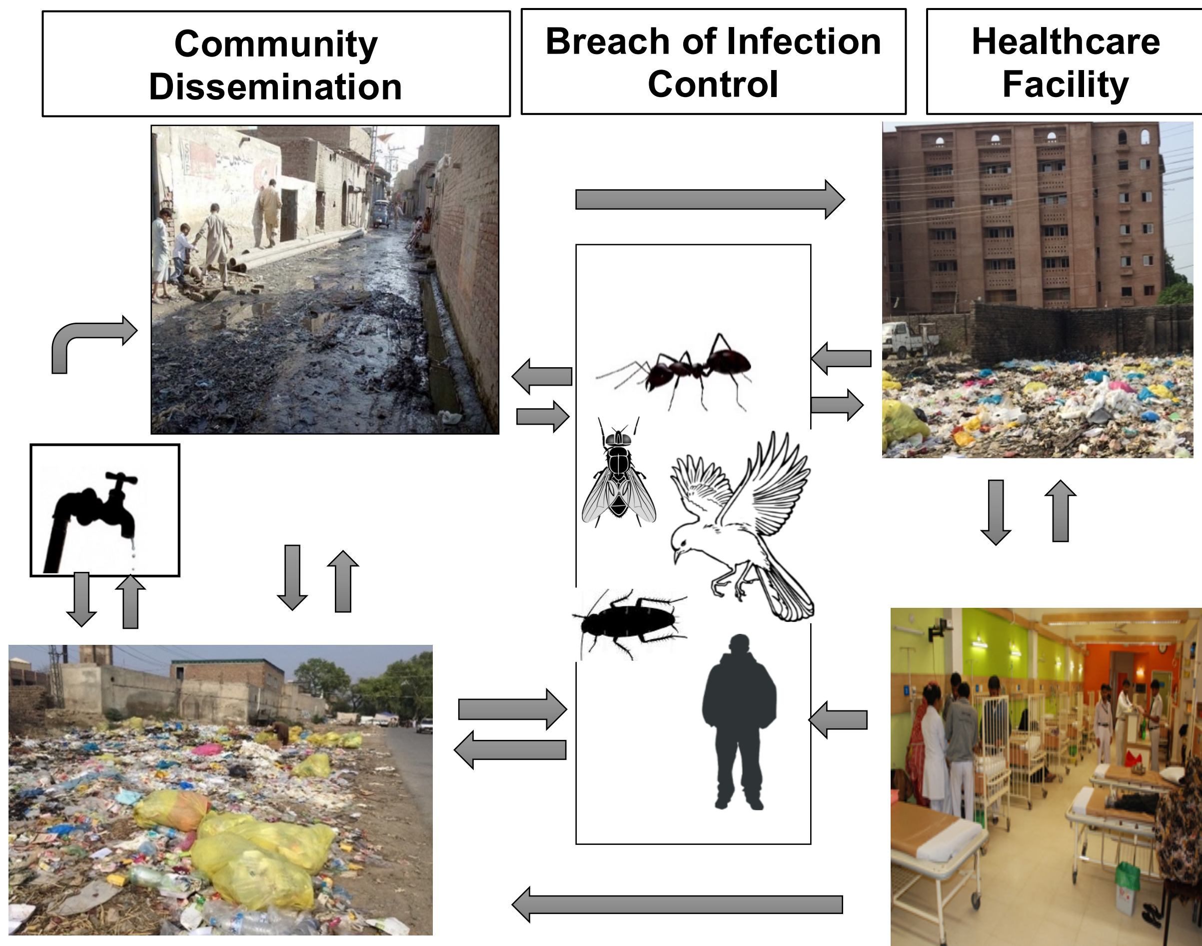


Figure 59. AMR transmission cycle. The figure shows a schematic representation of the possible transmission routes of AMR between patients, environment and the community.

7.2. The status of Healthcare in LIMCs

In addition to the detrimental situation outside the hospital, the conditions inside hospitals are generally suboptimal and lack basic commodities which may include essential lifesaving equipment and medication. The hospital infrastructure is often outdated, with limited or no maintenance and inadequate waste management (WHO 2014a; Quintela-Baluja et al. 2015). According to WHO, 58 % of hospitals in

developing countries have no adequate safe disposal system. There is also a lack of cleaning and hygiene and 67% of hospitals lack basic infection control policies (WHO 2014b). Consequently, contamination of immediate hospital environment could occur easily, which in turn can have detrimental effects on the patients' health, community and healthcare workers. Additionally, despite being heavily populated the expenditure on health-care systems is proportionately very low. Pakistan is the one of the emerging economies of South-East Asia but only spends a fraction, 0.5%, of its gross domestic product on health. Very limited, and below standard, facilities are provided by governmental health-care institutes and approximately 80% of the health-care expenditure is covered by individuals using own resources (WHO 2014b; WHO 2015b). The health care system is divided into private and public sectors with a striking difference in the quality and quantity of facilities. The public sector is run by the Pakistani government with very basic and inefficient amenities. In contrast, the private sector hospitals are comparable to that of developed countries; however, they are almost entirely restricted to the upper or upper/middle-class who make up the minority of the population. Consequently, most of the population is dependent on the governmental- run public hospitals with limited recourses and limited or no access to fundamental necessities (Raka 2010). These institutes struggle to provide essential care for the patients and have very limited financial resources for cleaning and disinfectants etc. Generally, the staff lack knowledge about infection control prevention strategies and effectiveness of cleaning and hygiene products, which can lead to the frequent use of contaminated equipment and utilities spreading MDR bacteria (Collins 2008; Carling and Polk 2011). Additionally, there is also a shortage of staff and basic facilities including beds, medications and equipment. It is not unusual to see patients or even babies sharing beds. Food in hospitals is generally

eaten in beds with hands, bedsheets and other hospital wear rarely washed and often found dirty (personal observations). Poor hygiene, infection control and sanitation lead to increased MDR contamination (Andremont and Walsh 2015), as is evident from our study where the resistance was frequently isolated from common-touch surfaces such as beds, windows, door handles and sink knobs to specialised equipment such as oxygen masks, IV, catheter and cannula lines. Moreover, it is a general observation that the majority of HAIs develop at surgical sites or invasive medical devices such as catheters, giving rise to central-line-associated bloodstream infections, ventilator-associated pneumonia and catheter-associated urinary tract infections (NHS 2010; Stone 2010). Findings of this study show that the conditions in LMIC hospitals may be facilitating the development of HAIs.

MDRB can be either transferred through direct or indirect contact. Direct transfer can occur between patients, healthcare workers or other hospital personnel. Indirect transfer can occur with the aid of an intermediate medium; for example, indirect transfer of a MDRB from one patient to another using a fly or insect as a medium, transfer of a MDRB from an infected patient to healthcare worker by contaminating a touch-surface or the transfer of a contaminant from one patient to another via contact with a healthcare worker (Collins 2008). However, this spread is not limited to the hospital setting and resistance genes can equally spread to the community due to close contact between visitors – and vice versa.

7.3. The global implication of increased MDR in LMIC

There is increasing evidence for the transfer of MDR from one location to another. MDRB can become part of normal microbiota which can open the opportunity for establishing dissemination on a global scale due to population dynamics. The spread of a resistance gene from its origin to distinct environments has been identified on several occasions: for example, in Europe, most of the initial bla_{NDM} cases, if not all, reported travel to South Asia or the Balkans (Walsh 2010; Livermore et al. 2011; Walsh and Toleman 2011; Dortet et al. 2014; Voulgari et al. 2014); bla_{KPC} may have been imported from USA as the first case in Europe was identified from a patient in France who had undergone medical treatment in New York (Munoz-Price et al. 2013; Nordmann 2014) and the introduction of $bla_{\text{VIM-4}}$ in Sweden, Hungry and Norway via patients previously hospitalised in Mediterranean hospitals (Walsh et al. 2005; Carmeli et al. 2010; Cornaglia et al. 2011; Giske et al. 2012). Similarly, the *P. aeruginosa* strain belonging to serotype O:12, which is generally associated with the carriage of bla_{VIM}, has shown very similar characteristics across all of Europe suggesting a common origin for the gene, at least in Europe (Pitt et al. 1989; Tsakris et al. 2000). Although MDR has dispersed across the globe, there are geographical hotspots for the carriage of resistance genes e.g. $bla_{\text{CTX-M}}$ and bla_{NDM} in India, Pakistan and Bangladesh. AMR is highly prevalent in the environment in South Asia including in drinking water and food, which is probably the most significant factor in influencing the composition of the gut microbiota. Hence, it's not surprising that in countries like Pakistan and India, more than 70% of the gut Enterbacteriaceae have $bla_{\text{CTX-M}}$ type ESβL and, represented with a massive reservoir and in India alone, more than 1.1 billion individuals are carry $bla_{\text{CTX-M}}$ (Woerther et al. 2013). Travelling to these regions has been acknowledged as an independent risk factor for antibiotic

resistance acquisition (Livermore 2012; Woerther et al. 2013). A study from the Netherlands found that 8·6% of individuals were colonized with ESβL-producing Enterobacteriaceae before travel to South and Southeast Asia but, after travel, the colonization rate increased to 30·5% (Paltansing et al. 2013). Predictably, this study confirms that MDR is not restricted to one region and the emergence of AMR in one region of the world will rapidly disseminated to other countries/continents. Therefore, a global collaborative approach is essential in tackling the spread of MDR. Surveillance programmes by organisations, such as CDC, WHO and food and agriculture organisation (FAO), provide the necessary information to keep updated with the current state of AMR across the world and take action by generating policies accordingly.

7.4. Preventative Strategies to Control the Spread Of MDRB

One possible solution is to eliminate the MDR reservoir which exists in the environment and/or clinical settings. Environmental reservoirs of MDR can enhance the spread of resistance to different ecological niches, whereas clinical contamination can often lead to the development of HAIs (Carling and Polk 2011; WHO 2013b). Appropriate infection prevention control strategies and decontamination of patient wards and the adjacent environment is advised (O'Neill 2016a), although the routine disinfection of surfaces is debatable and has initiated some arguments, especially for MRSA (Taneja et al. 2012; Munoz-Price and Quinn 2013; Zaman and Siddiqui 2015). Some researchers also dispute that surface disinfection is only beneficial in high risk areas and there is not enough evidence that a healthcare facility with increased cleanliness has reduced the risk of infections.

Furthermore, the increasing biocide associated selection pressure on bacteria and its potential toxic side-effects towards patients, healthcare workers and the environment may outweigh the benefits (Cookson 2005; Quintela-Baluja et al. 2015). However, it should be noted that providing evidence for the role of cleanliness and hygiene in a hospital setting is challenging as it is dependent on a lot of factors with limited control, most importantly: the behaviour and collaboration of staff, patients and visitors, the efficiency of locally used cleaning products and techniques and routine or frequency of cleaning. In this study, for example, the cleaning agent itself was contaminated with *P. aeruginosa*. Furthermore, it was also observed that there was a lack of routine cleaning and all individuals responsible for the cleaning, or the ones monitoring the process, had no knowledge about disinfection techniques. Nonetheless, there is a general agreement over the benefits of other infection control strategies for reducing HAI and the spread of MDR such as hand hygiene, contact precaution and appropriate contaminated waste disposal (Carling and Polk 2011; Timilshina et al. 2011a; WHO 2013b; O'Neill 2016a). Introduction of other interventions and strategies have also shown good results, for example antibiotic stewardship and educating staff and the general public on appropriate antibiotics use (Raka 2010; Laxminarayan et al. 2013). Although many European countries have introduced intervention platforms, the development in LMICs is tardy (Laxminarayan et al. 2013). AMR is universally recognized as a global threat by humanitarian organizations and efforts are being made to provide on-the-ground support to introduce surveillance, prevention control and education programs. An example is "National Action Plan" a WHO approach to encourage countries to produce a comprehensive document as to how each country will tackle AMR under the one-health approach. The NAP will dovetail with initiatives such as GLASS (a

comprehensive surveillance tool) proposed by the WHO (WHO 2015a). Another such program is the 'Global Antibiotic Resistance Partnership' which is focused on developing policies for antimicrobial resistance in selected low and middle income countries of Africa and Asia (Hellen 2015). In addition, a comprehensive national plan to tackle antibiotic resistance, with consideration to the local requirements, would be the way forward in reducing antibiotic resistance. An example of such programme is the Fleming fund which was proposed following Jim O'Neil's report in 2015 commissioned by UK prime minister David Cameron. The report analysed the economic and health burden of AMR and acknowledged the need for a global collaborative response to tackle MDR. The Fleming fund is increasing local collaborations and working with local health-care providers in LMICs to provide support with AMR surveillance and encourage rational antibiotic use by improving diagnosis capacity.

7.5. Alternative or New therapeutics to Control the Spread Of MDRB

The other solution to control the spread of MDR could be the development of new therapies. Irrefutably, the inevitable spread of antibiotic resistance means new therapies are absolutely essential. Small scale studies are required in providing the basis for further investigation into a test compound, for example CARB-X (Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator), JPI-AMR (Joint Programming Initiative on Antimicrobial Resistance) and ND4BB (New Drugs For Bad Bugs) (IMI 2015; JPIAMR 2015; CARB-X 2017). CARB-X is a global partnership aimed at bringing pharmaceutical industries and scientist together to accelerate the developing of therapeutics and diagnosis of AMR (CARB-X 2017).

Innovative medicine initiatives' "new drugs for bad bugs" are encouraging small scale studies in generating new drugs specifically for Gram-negative bacteria into the research pipeline (IMI 2015) and JPI-AMR is supporting research of new antibiotics in addition to stewardship of existing antibiotics (JPIAMR 2015).

Although the PNA's used in this study haven't generated satisfactory results in targeted antibiotic resistance inhibition, it provides an alternative approach to conventional antibiotics which could be investigated. The primary problem with AP-PNA-C therapy still seems to be entry into the cells or the conjugated carrier protein. However, new advancements in the delivery system might be able to overcome these hurdles (Sang and Blecha 2008; Woodford et al. 2009) though, it goes without saying that targeting a specific pathogen will eventually select for resistance (Coates and Hu 2007; Sang and Blecha 2008). However, the rise of MDR and the dearth in the development of new antibiotics require urgent attention. The strict approval regulations mean that any drug under-progress will take at least a decade before being approved for clinical use assuming timely progress to TC phase III and beyond (Projan and Shlaes 2004; Tillotson and Theriault 2013; Allen et al. 2014).

In retrospect, the decline in new drug development could be attributed to multiple reasons such as lack of profit, compliance with guidelines for clinical trials, increased competition in the marketplace and increased failure in the development of new drugs, leading to several large companies exiting the field (Projan and Shlaes 2004; Tillotson and Theriault 2013; Allen et al. 2014). Currently, there is an increased pressure on initiating new cutting-edge research in drug development technology aiming at their unmet medical needs and increasing commercial value (Tillotson and Theriault 2013). The therapeutic development of antisense technologies is still at

early investigation stage. A lot of research is still undergoing to understand the mechanisms and cellular pathways of different AO and explore their full potential as antimicrobial agents. Advancements in genomics and proteomics are providing different areas to explore and new potential target genes and pathways are identified. Further understandings into the intracellular trafficking of AO will significantly accelerate the development of an upgraded design with enhanced delivery systems for enhanced drug discovery (Peng et al. 2015).

Lastly, it should not be forgotten bacteria have shown incredibly amendable genomes which have been exposed to constant selection pressure from synthetic or natural antibiotics, biocides and other toxic substances and, subsequently, have evolved a very sophisticated bacterial machinery capable of overwriting all the antibiotics in use today. Furthermore, there is a discovery void and the finite changes to an existing antimicrobial compound is becoming exhausted. Novel antibiotics or alternative therapeutics strategies are essential to defeat bacteria in the “arm race” against time (Allen et al. 2014). Failure to do so will result in pushing humans back to pre-antibiotic era and, consequently, will bring an end to modern medicine.

8. Appendices

8.1. Tables and Figures

Table 26. Location of Drinking Water samples from Karachi, Pakistan

Bhains Colony	Abid Colony	Fb Area	Clifton
Bohrapir	Airport	Ghanchipara	Dastagir
Bottle Goli	Allahwala	Gulberg	Dharmala
Dha Phase 7	Bhitta	Gulshan E Hadeed	Hawkes Bay
Essa Nagri	Bin Qasim	Gulshan-E-Maymar	Hazara Colony
Garden	Buffer Zone	Hanifia Road	Hub Chowki
Gold Town	Golmar	Jodia Bazaar	Jinnah Abad
Haji Sheedi Goth	Gulshan-E-Jahan	Karsaz	Junaijo Town
Ibrahim Haidri	Jail Road	Kharadar	Khayaban-E-Jami
Kachi Colony	Jam Goth	Kumharwara	Kiamari
Kalakot	Jehangir Road	Millat Nagar	Malirpur Road
Kashmir Colony	Liaquat Abad	Paf Chata Malir	Manghopir
Kathiawar Society	Mianwali Colony	Razzaqabad	Mehmood Abad
Khuda Ki Basti	Nafees Colony	Sarafa Bazaar	Mehran Town
Korangi 5 No.	Narayan Pur	Sarjan Town	Memon Sociaty
Malir Cantt	Nawa Lane	Shah Baig Line	Nahak Wara
Miran Naka	Nusrat Bhutto Colony	Sharifabad	National Stadium
New Karachi	Ramswamy	Shershah Colony	Nice Town
Pakistan Chowk	Saadi Colony	Singo Cane	Parsi Compound
Pechs Society	Shah Faisal Colony	Sultanabad	Patel Road
Pib Colony	Azam Basti	Usmanabad	Punjab Colony
Railway Colony	Baghdadi	Zia Colony	Qayyam Abad
Shah Latif Town	Dehwan Goth	Akhtat Colony	Sorab Goth
Sherpao Basti	Dhobighat	Azam Town	Dhoraji Society
Bhangipara			

Table 27. Location of Surface and Equipment samples from Karachi, Pakistan

Location	n	Location	n	Location	n
Bed Handle	13	Dust bin	6	Lithotomy table	1
Air pump	1	Elevator	1	Lunch table	11
Bandage	2	Entrance door	8	Main entrance	2
Bed linen	32	Envelope CT scan	2	Mattress	4
Bedside table	5	Excercise Cycle	1	Medical supply table	1
Blood bag	1	Fire extinguisher	2	Medicine counter	3
Board for foot rest	1	Floor	13	Medicine trolley	3
BP cuff	1	Foley's catheter	2	Nebulizer	1
Cannula of patient	7	Food tray	2	Nasogastric tube	4
Waiting Chair	5	Fridge	1	Nursing counter	14
Corridor	2	Gel bottle	1	Nursing trolley	1
Cot Mattress	1	Gloves box	1	Oxygen cylinder	4
Nursing Cupboard	1	Grill railing	1	Oxygen mask	5
Curtain	3	Hand dispenser	4	Patient file	10
Cutter machine	1	Hand sanitizer	7	Phone	2
Doctor's table	2	Infusion tube	1	Pillar	1
Door	15	Instrument table	1	Pillow case	13
Drawer	5	IV line	2	Plaster board	1
Drip bag	18	Kettle	1	Patient's support plastic	1
Rods skeletal traction	1	Procedures Light	1	Potable water	1
Round wheel	1	Steel rod	9	Table	11
Skeletal traction stand	5	String to turn on the fan	1	Tape to secure cannula	3
Stair railing	5	Switch board	16	Tracheostome tube	1
Stairs	2	Syringe	1	Trolley	1
Ultrasound probe	1	Waiting area	1	Weighing machine	1
Visitor's chair	7	Wall	3	Wheel chair	3
Vitals monitor	1	Water purifier	1	Window	7
X-ray	4				

The numbers of samples are shown as (n).

Patients Data collection questionnaire

Patients Name:		Ward:
Personal Information:		
Age:	Sex:	Place of residence:
Clinical Information:		
Clinical symptoms/diagnosis:		Surgery Type:
Infection Developed: Yes / No		Sample site:
Antibiotic Therapy:		Length of hospital stay
Additional Information:		

Figure 60. An example questionnaire that was filled for every patient enrolled in Peshawar study

Table 28. Primers List

Primers pair	Target	Sequence (5'-3')	An °C	Size (bp)	Reference/ Source
NDM-F	bla_{NDM}	GGTTTGGCGATCTGGTTTTC	55	~700	Nordmann et al. 2011
NDM-R		CGGAATGGCTCATCACGATC			
CTX-M-15-F	$bla_{CTX-M-15}$	ATGCGCAAACGGCGGACGTA	55	~600	AWARE
CTX-M-15-R		CCCGTTGGCTGTCGCCCAAT			
KPC-F	bla_{KPC}	ATGTCACTGTATCGCCGTCT	60	~890	SACU
KPC-R		TTTTCAGAGCCTTACTGCCC			
OXA-48 LIKE-F	$bla_{OXA-48-}$ *like*	TTGGTGGCATCGATTATCGG	53	~744	SACU
OXA-48 LIKE-R		GAGCACTTCTTTTGTGATGGC			
VIM-F	bla_{VIM}	CCGACAGTCARCGAAATTCCG	55	~400	SACU
VIM-R		CTACTCRRCGACTGAGCGATT			
AcpP-R4	*acpP*	GACGCTTAGACACGTTTGTCC	54	~340	This Study
AcpP-F4		ATCGCGAAAGCGAGTTTTGA			
INTL1-QAC-F	*Class 1 integron*	ACAGCACCTTGCCGTAGAAG	54	Varies	This Study
INTL1-QAC-R		GCGATAACAAGAAAAAGCCAGC			
KPC-F-PNA	bla_{KPC}	AAGGAATATCGTTGATGTCACTG	54	~900	This Study/SACU
KPC-R		TTTTCAGAGCCTTACTGCCC			
OXA-48 F-PNA	$bla_{OXA-48-}$ *like*	GGGGACGTTATGCGTGTATT	54	~780	This Study/SACU
OXA-48 LIKE-R		GAGCACTTCTTTTGTGATGGC			
NDM-F-PNA	*blaNDM*	AAAAGGAAAACTTGATGGAATTG	54	~760	This Study/SACU
NDM-R		CGGAATGGCTCATCACGATC			
27F	*16S rRNA*	AGAGTTTGATCCTGGCTCAG	54	~1500	Lane, 1991
1492R		GGTTACCTTGTTACGACTT			
REP2I	*REP*	ICGICTTATCIGGCCTAC	40	Varies	Versalovic *et al.* 1991
REP1R		IIIICGICGICATCIGGC			
63F	*16S rRNA*	CAG GCC TAA CAC ATG CAA GTC		~1600	Marchesi et al. 1998
1387R		GGG CGG WGT GTA CAA GGC			
OXA-48 181-M-R	bla_{NDM}, bla_{KPC} *and* $bla_{OXA-48-}$ *like* Multiplex	AAGACTTGGTGTTCATCCTT	61	163	This Study
OXA-48 like48-181-M-F		GGCGTAGTTGTGCTCTG			
NDM-M-R		CTCAGTGTCGGCATCAC		655	
NDM-M-F		AGCTGAGCACCGCATT			
KPC-M-R		CCGTCATGCCTGTTGTC		333	
KPC-M-F		TAGTTCTGCTGTCTTGTCTC			

CTX-M-15-R	*bla*$_{CTX\text{-}M\text{-}15}$	CCGAGGTGAAGTGGTATC		~500	This Study
CTX-M-15-F		AAGTGTGCCGCTGTATG			
AcpP-3-F	*acpP qRT assay*	TGGTAATGGCTCTGGAAGAA	59	88	This Study
AcpP-3-R		TAATCAATGGCAGCCTGAAC			
AcpP-Probe-R		TCAGCTTCTTCGTCCGGAATCTCA	69		

Some primers were acquired from SACU (Specialist Antimicrobial Chemotherapy Unit) with permission. An °C (Annealing temperature).

Table 29. *K. pneumoniae* Isolates details with similar REP-PCR profiles.

K. pneumonia	*Sample*	*Pair*	*Gene 1*	*Gene 2*	*CD*	*Organism/Location*
IS	894	1	CTX-M-15	NDM	C	C
PSW	292	1	CTX-M-15	NDM	D	Dustbin/pillar base
IS	299G	2	CTX-M-15		D	F
IW	409G	2	CTX-M-15		D	F
IW	473G	2	CTX-M-15		D	C
IS	1018W	3	CTX-M-15		C	F
IS	262B	3	CTX-M-15		D	F
IW	428B	4	CTX-M-15		C	C
IW	254B	4	CTX-M-15		D	C
PPS	331G	5	CTX-M-15	OXA-48 LIKE	D	NA
PPW	231G	5	NDM	CTX-M-15	D	NA
IS	299B	5	CTX-M-15	OXA-48 LIKE	D	F
PPW	111G	6	NDM	CTX-M-15	D	NA
PPW	249G	6	NDM		D	NA
PPW	111B	6	NDM	CTX-M-15	D	NA
IS	1139B	7	CTX-M-15		D	F
PPS	356B	7	OXA-48 LIKE		C	NA

The minimum similarity coefficient for two profiles being considered very similar if not the same was 91%.

Table 30. *E. coli* Isolates details with similar REP-PCR profiles

E. coli	*Sample*	*Pair*	*Gene 1*	*Gene 2*	*CD*	*Organism/Location*
PSS	435	1	CTX-M-15		D	Dustbin/pillar base
IW	535	1	CTX-M-15		D	F
PSW	128	2	CTX-M-15		D	Washroom handle
PSW	161	2	CTX-M-15		C	Bed EX-7 drip hanger
PPS	395	3	CTX-M-15		C	NA
PPS	412	3	CTX-M-15		C	NA
IW	222	4	CTX-M-15	NDM	D	NA
PPS	390	4	CTX-M-15		C	NA
IS	299	5	CTX-M-15	OXA-48 LIKE	D	F
IS	421	5	CTX-M-15		D	F
IS	474	5	CTX-M-15		D	M
PPW	131	6	CTX-M-15		C	NA
PPW	A	6	CTX-M-15	NDM	D	NA
IW	343	7	CTX-M-15		D	NA
IW	526	7	CTX-M-15		D	NA
IW	218	8	CTX-M-15	NDM	D	C
PPS	354	8	NDM	OXA-48 LIKE	C	NA
PPW	110	9	CTX-M-15		D	NA
PPW	239	9	CTX-M-15		D	NA
PPW	105	10	CTX-M-15		D	NA
PPS	435	10	CTX-M-15		C	NA
IW	77	11	CTX-M-15	OXA-48 LIKE	D	C
IW	442	11	CTX-M-15		D	NA
PPW	130	12	CTX-M-15		C	NA
IW	266	12	CTX-M-15	NDM	D	NA
PPS	435	13	OXA-48 LIKE		C	NA
PPS	461	13	CTX-M-15	NDM	C	NA

The minimum similarity coefficient for two profiles being considered very similar if not the same was 84%.

Table 31. *E. cloacae* Isolates details with similar REP-PCR profiles

E. cloacae	*Sample*	*Pair*	*Gene 1*	*Gene 2*	*CD*	*Organism/Location*
IS	647	1	NDM		D	S
IS	1006	1	CTX-M-15	NDM	C	F
IS	260	2	CTX-M-15		D	F
IS	1155	2	NDM		D	F
IS	273	3	CTX-M-15		D	F
IS	273	3	NDM		D	F
IS	355	3	CTX-M-15		C	F
IS	470	3	NDM		D	F
IW	472	3	NDM	CTX-M-15	D	C
IS	650	3	CTX-M-15		D	F
IW	133G	3	CTX-M-15		C	C
IS	471	4	CTX-M-15		D	F
IS	576	4	CTX-M-15		C	F
PSS	415	5	NDM		C	Surface under dustbin
IS	489	5	NDM		C	F
IS	259	6	CTX-M-15		D	F
IS	655	6	CTX-M-15		D	C
PSS	388	7	NDM		C	Window surface
PSS	397	7	NDM		C	Window surface
IS	459	7	CTX-M-15		D	C
PSS	415	8	CTX-M-15		C	Surface under dustbin
IW	555	8	CTX-M-15		C	F
IS	489	9	NDM		C	F
IS	1078	9	CTX-M-15	NDM	C	F
IS	454	10	CTX-M-15		D	F
IS	830	10	CTX-M-15		C	F
IS	275	11	CTX-M-15		D	F
IW	473	11	NDM	CTX-M-15	D	C
IW	407	12	NDM	CTX-M-15	C	C
IW	412	12	NDM	CTX-M-15	C	C

The minimum similarity coefficient for two profiles being considered very similar if not the same was 87.5%.

8.2. Recipes for reagent and stock solutions prepared locally

All reagents purchased from one Thermo Fisher Scientific, Waltham, USA and Sigma Aldrich, St. Louis, USA. Buffers used in the study were prepared as following;

TBE Buffer (10×)

Tris (Fisher) Boric Acid (Sigma) Na2EDTA (Fisher) Made up to 1 L with sterile distilled water. Autoclaved before use.

TE Buffer (10×)

Tris (Fisher) Na2EDTA (Fisher) HCl (Fisher) Made up to 1 L with sterile distilled water. Autoclaved before use.

0.1M Tris HCl Buffer, pH 7.5

Tris (Fisher) HCl (Fisher) Made up to 100mls in sterile distilled water.

8.3. List of Culture Media

Columbia Blood Agar (CBA, E&O Laboratories Ltd, Bonnybridge, Scotland)

Used for primary culture and subculture of all isolates, and for plate mating assays.

Luria Bertani (LB) Agar, Miller (Thermo Fisher Scientific, Waltham, USA)

Supplemented with antimicrobials for subculture and passage experiment.

LB Broth, Miller (Thermo Fisher Scientific)

Used for subculture, mating supplemented with antimicrobials for passage experiment.

Mueller Hinton (MH) Agar (E&O Laboratories Ltd)

Used for disc and gradient strip susceptibility testing.

Brilliance™ UTI Clarity Agar (Oxoid Ltd, Basingstoke, UK)

Supplemented with antimicrobials for selective isolation in mating experiments.

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