Carbapenemase-Producing *Enterobacteriaceae* in Wastewater-Associated Aquatic Environments

Dedicated to my parents, my wife, my siblings, and last but not least, my daughter Ilsa.

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ABSTRACT

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The emergence of carbapenem resistance due to the carbapenem-hydrolyzing enzymes (carbapenemases) in *Enterobacteriaceae* has led to limited therapeutic options. The increased resistance to these "last-resort" antibiotics is fueled by overuse and misuse of antibiotics in human medicine and agriculture. According to the One-Health concept, the microbiomes of humans, animals and natural environments are interconnected reservoirs of antibiotic resistance genes (ARGs) and changes in one compartment will affect the other compartments. Thus, the environmental waters exposed to the pathogens, ARGs and other contaminants of human origin can play a significant role in the spread of resistance. The study aimed to characterize carbapenemase-producing *Enterobacteriaceae* (CPE) and ARGs in wastewaters and associated river and lake waters in Örebro, Sweden. The study also analyzed *de novo* development of resistance in *Klebsiella oxytoca* during long-term growth in river water and the effect of temperature on the emergence of resistance. OXA-48-producing *Escherichia coli* (ST131) and VIM-1-producing *K.oxytoca* (ST172) were repeatedly detected in the wastewaters and associated river, suggesting that these isolates were persistently present in these environments. Furthermore, *K. oxytoca* ST172 isolated from the river was genetically similar to two isolates previously recovered from patients in a local hospital, which shows the possibility of transmission of CPE from hospital to aquatic environments. A high diversity of ARGs was detected in these environments especially in hospital wastewater where ten different carbapenemase genes were detected. These results emphasized that the effective treatment of wastewaters must be ensured to reduce or eliminate the spread of antibiotic resistance. Increased resistance to meropenem (up to 8-fold) and ceftazidime (>10-fold) was observed in *K. oxytoca* after exposure to both river and tap water after 600 generations and resistance emerged earlier when the bacteria was grown at the higher temperature. The exposure to contaminants and increased environmental temperature may induce similar changes in the environmental microbiome, generating novel resistant variants at accelerated rates that may pose a significant threat to human health.

Keywords: Antibiotic resistance evolution, ESBL, *Klebsiella,* Multidrug, VIM

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PAPERS INCLUDED IN THESIS

Paper I

Faisal Ahmad Khan, Aminur Rahman, Bo Söderquist & Jana Jass. Carbapenemase-producing *Escherichia coli* ST38 and *Klebsiella oxytoca* ST172 are persistent in Swedish wastewaters and associated river. *(manuscript)*

Paper II

Faisal Ahmad Khan, Bengt Hellmark, Ralf Ehricht, Bo Söderquist & Jana Jass (2018). Related carbapenemase-producing *Klebsiella* isolates detected in both a hospital and associated aquatic environment in Sweden. *European Journal of Clinical Microbiology & Infectious Diseases* volume 37, pages 2241–2251. doi: 10.1007/s10096-018-3365-9

Paper III

Aminur Rahman*, Faisal Ahmad Khan*, Bo Söderquist, Neelu Nawani & Jana Jass. Diversity of antibiotic resistance genes in aquatic environments using culture-dependent and independent methods. *(manuscript)*
*Equal contribution

Paper IV

Faisal Ahmad Khan, Bo Söderquist & Jana Jass (2019). Prevalence and Diversity of Antibiotic Resistance Genes in Swedish Aquatic Environments Impacted by Household and Hospital Wastewater. *Frontiers in Microbiology*., 10. doi: 10.3389/fmicb.2019.00688

Paper V

Faisal Ahmad Khan, Aminur Rahman, Diarmaid Hughes, Bo Söderquist & Jana Jass. Environmental conditions promote genetic changes and increase resistance to β-lactam antibiotics in *Klebsiella oxytoca*. *(manuscript)*

PAPERS NOT INCLUDED IN THESIS

1. Anna Fagerström, Paula Mölling, Faisal Ahmad Khan, Martin Sundqvist, Jana Jass & Bo Söderquist (2019). Comparative distribution of extended-spectrum beta-lactamase-producing *Escherichia coli* from urine infections and environmental waters. *PLoS ONE* 14(11): e0224861. doi: 10.1371/journal.pone.0224861

2. Priti Prabhakar Yewale, Kiran Bharat Lokhande, Aishwarya Sridhar, Monika Vaishnav, Faisal Ahmad Khan, Abul Mandal, Kakumani Venkateswara Swamy, Jana Jass & Neelu Nawani (2019). Molecular profiling of multidrug-resistant river water isolates: insights into resistance mechanism and potential inhibitors. *Environmental Science and Pollution Research International*. doi: 10.1007/s11356-019-05738-2

3. Manish Goswami, Faisal Ahmad Khan, Admir Ibrisevic, P-E Olsson & Jana Jass (2018). Development of *Escherichia coli*-based gene expression profiling of sewage sludge leachates. *Journal of Applied Microbiology*, 125 (5), 1502-1517. doi: 10.1111/jam.14028

4. Ranjeet Kumar, Ajay Pradhan, Faisal Ahmad Khan, Pia Lindström, Daniel Ragnvaldsson, Per Ivarsson, Per-Erik Olsson & Jana Jass (2015). Comparative analysis of stress induced gene expression in *Caenorhabditis elegans* following exposure to environmental and lab reconstituted complex metal mixture. *PLoS ONE*, 10 (7). doi: 10.1371/journal.pone.0132896

ABBREVIATIONS

ANOVA	Analysis of Variance
ARB	Antibiotic-Resistant Bacteria
ARGs	Antibiotic Resistance Genes
bla	β-lactamase
cgMLST	Core-Genome Multi-Locus Sequence Typing
CPE	Carbapenemase-Producing *Enterobacteriaceae*
EARS-Net	European Antibiotic Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
ESBL	Extended-spectrum β-lactamase
Etest	Epsilometery Test
HGT	Horizontal Gene Transfer
IMP	Imipenemase
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization
MBL	Metallo-β-lactamase
MDR	Multi-drug Resistant
MGE	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
MLST	Multi-locus Sequence Typing
NDM	New-Delhi Metallo-β-lactamase
NMDS	Non-metric Multidimensional Scaling
OD	Optical Density
OMPs	Outer Membrane Proteins
OrthoANI	Orthologous Average Nucleotide Identity
OXA	Oxacillinase
PBP	Penicillin Binding Protein
PCA	Principal Component Analysis
PG	Peptidoglycan
RND	Resistance Nodulation Division
SNP	Single-nucleotide Polymorphism
ST	Sequence Type
T_d	Doubling Time
UV	Ultraviolet
VIM	Verona Imipenemase Metallo-β-lactamase
WWTP	Wastewater Treatment Plant

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INTRODUCTION

Therapeutic agents derived from secondary metabolites of living cells are called antibiotics. Antibiotics are organic compounds either natural or semi-synthetic that are used to treat bacterial infections. Since the discovery of penicillin by Sir Alexander Fleming in 1928, antibiotics have been extensively used against bacterial infections that were impossible to cure in the pre-antibiotic era. Since the introduction of antibiotics, the development of bacterial resistance has threatened their efficacy. Historically, the development and use of each new antibiotic have been followed by the emergence of resistant bacteria because the antimicrobial resistance was present long before the clinical use of antimicrobial compounds. In 1937, sulfonamides were introduced as first antimicrobials to treat bacterial infections and sulfonamide resistance was reported soon after the introduction.

Antibiotics act by inhibiting or interfering with cellular processes vital for bacterial replication and survival. Depending on the importance of the target process and the net effect on the bacterial cell, antibiotics are classified as either bactericidal or bacteriostatic. Bactericidal antibiotics kill the bacteria while bacteriostatic antibiotics inhibit their growth or reproduction. The example of bactericidal antibiotics is those that inhibit bacterial cell wall syntheses such as β-lactams and vancomycin. Bacteriostatic antibiotics interfere with cellular processes necessary for growth and proliferation such as nucleic acid synthesis, protein synthesis, and other metabolic processes. Examples of bactericidal antibiotics include tetracycline, fluoroquinolones, and sulfamethoxazole. The bactericidal antibiotics are preferred for the treatment of clinically significant human infection. However, in some cases, the bacteriostatic effect is sufficient for the immune system to eradicate the infection. Some antibiotics are effective against a wide range of bacterial species thus known as broad-spectrum antibiotics, while others have a limited spectrum of activity and regarded as narrow-spectrum antibiotics. Antibiotics are commonly categorized into groups, based on their molecular target within the bacterial cell: inhibitors of cell wall synthesis, cell membrane disruptors, inhibitors of nucleic acid synthesis, protein synthesis inhibitors, and inhibitors of other metabolic processes (Figure 1).

Microorganisms produce antibiotics as a competitive weapon against other bacterial species in the natural environment. Approximately 500 antibiotic drugs are microbially derived and ~200-220 are direct natural products while more than 250 are modified natural products (semi-synthetic) (Bérdy, 2012). The first known antibiotic was penicillin which was isolated

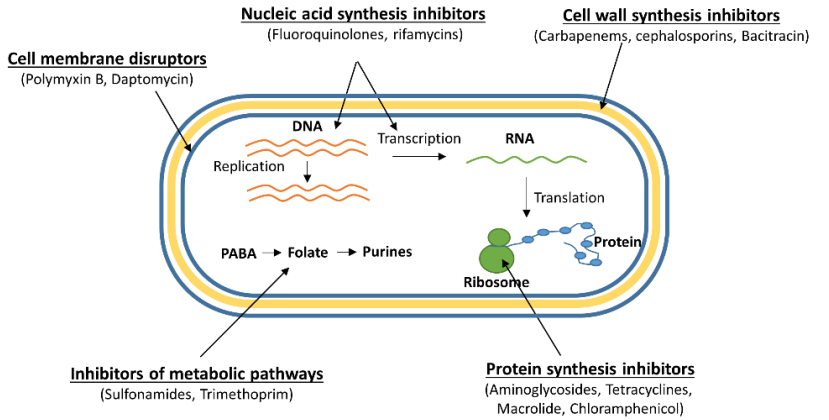

Figure 1. *Mechanism of action of different classes of antibiotics against Gram-negative bacteria. Among these include cell wall synthesis inhibition by antibiotics, disruption of cell membrane function, inhibition of protein synthesis by targeting 50S and 30S subunits of ribosomal RNA, inhibition of both DNA and RNA synthesis, and inhibition of bacterial metabolic pathways by antibiotics such as sulfonamides and trimethoprim. Adopted from (Bbosa et al., 2014).*

from a fungus, *Penicillium rubens* (Houbraken et al., 2011). Like penicillin, cephalosporins were also derived from a fungus *Acremonium chrysogenum* (previously *Cephalosporium acremonium*) (Liu et al., 2018). Members of *streptomyces* genus secrete a variety of antibiotics not only in the presence of other species but also during stress conditions like DNA damage and nutrient starvation (Cornforth & Foster, 2015).

The antibiotic resistome

The antibiotic resistome is the large reservoir of all the antibiotic resistance genes (ARGs) including those found in pathogenic bacteria in the clinics and natural environments, non-pathogenic antibiotic producers, and all other genes associated with antibiotic resistance. In antibiotic producer microbe, genes for self-protection (resistance) are either pre-acquired or coevolved with antibiotic biosynthesis and regulation genes. These resistance genes are usually located close to the antibiotic biosynthesis and regulation gene cluster and are expressed together (Hopwood, 2007). Often, some genes modify the antibiotic target protein or ribosomal RNA in the producer bacteria to

evade the deleterious effect of produced antibiotics. Almost always, there are genes for efflux pumps to transport the produced antibiotic out of the cell (Dantas et al., 2008; Hopwood, 2007). Similarly, the *Streptomycete cattleya* that produces a carbapenem thienamycin, carries ThnS β-lactamase that hydrolyzes any accumulated carbapenem inside the producer cell (Walsh & Wencewicz, 2016). Another example of a self-defense mechanism is the reprogramming of peptidoglycan (PG) assembly pathway in vancomycin producers that replaces D-Ala$_4$-D-Ala$_5$ of pentapeptide with D-Ala$_4$-D-Lac$_5$ termini, which is insensitive to vancomycin. Vancomycin producers may have acquired the genes to reprogram PG assembly from *Lactobacillus* and *Leuconostoc* species, well known for their vancomycin resistance (Handwerger et al., 1994).

Many of these genes are cryptic that are only expressed when a particular antibiotic is present in the microenvironment. These genes represent a large reservoir of ARGs, however, the majority of these genes have not been selected or mobilized under recent exposure to antibiotics (Wright, 2007) (Figure 2). Some of these genes have been acquired by multidrug-resistant (MDR) pathogens and antibiotic-producing species. Studies have shown that antibiotic genes have evolved from precursor genes encoding proteins for other metabolic functions (Wright, 2007). These genes were possibly the housekeeping genes involved in the primary metabolism and have evolved to confer resistance to antibiotics. The classic example is that subclasses of penicillin-binding proteins (PBPs) and β-lactamases have evolved from a common precursor PBP (Massova & Mobashery, 1998). Similarly, D-Ala-D-Lac ligase which is important for vancomycin resistance may have evolved from D-Ala-D-Ala ligase involved in classical PG assembly pathways.

Over time, the genes for self-protection (resistance) were incorporated onto mobile genetic elements (MGE) such as plasmids and transposons and were transferred from antibiotic producers (resistant) to sensitive bacteria. This transfer of resistance genes in known as horizontal gene transfer (HGT) and occurs not only between the bacteria of the same species but also between distant species (Skippington & Ragan, 2011). The HGT is suggested to have played a major role in the global dissemination of antibiotic resistance. Thus, antibiotic resistance has become one of the greatest threats to human health in recent times and antibiotic-resistant bacteria (ARB) have been recently found in virtually all the natural environments, including the

environments with no anthropogenic impact (Khan et al., 2018; Mills & Lee, 2019; Segawa et al., 2013).

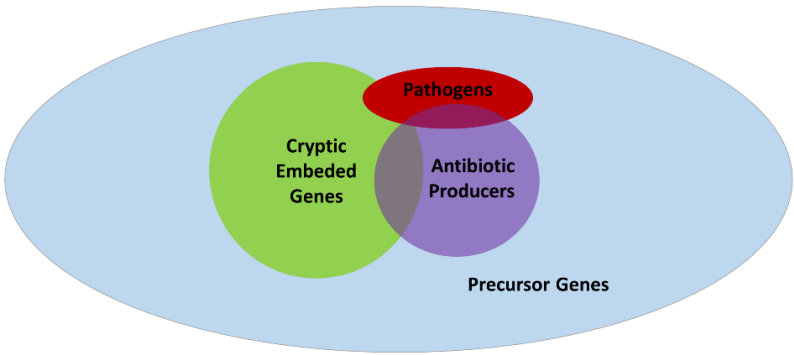

Figure 2. *The players that contribute to antibiotic resistome. In a global pool of precursor genes, there are several subsets: cryptic genes that confer resistance and ready to be mobilized, genes for self-defense (resistance) in antibiotic producers, and mobilized ARGs captured by bacterial pathogens. Adapted from (Wright, 2007).*

Antibiotic resistance as a global healthcare crisis

The remarkable efficacy of antibiotics against bacterial infections has led to their overuse not only in human healthcare but also in livestock and agriculture. According to the data from 71 countries collected during the last decade, a 36% increase in antibiotic consumption to treat human infections was observed (Hawkey & Jones, 2009). Global antibiotic consumption has increased between 2000 and 2015, from 21.1 to 34.8 billion defined daily doses (Klein et al., 2018). In 2010, more than 63,200 tons of antibiotics were used to treat and protect animals from bacterial infections, and as growth promoters (Van Boeckel et al., 2015).

This extensive production and usage have resulted in the distribution of antibiotics in natural environments and the emergence of novel antibiotic resistance. Recent data from European Centre for Disease Prevention and Control (ECDC, 2018) suggest that most countries with higher consumption of antibiotics (both at the hospital and in the community) have higher

proportions of antibiotic-resistant bacteria isolated from patients (Figure 3). However, there are countries such as Spain and Germany where higher resistance was observed even though the antibiotic consumption remained low (ECDC, 2018). It has been suggested that reducing antibiotic prescription may not reduce antibiotic resistance and emphasis should be given on the antibiotics that are proven to be less prone to select resistance (Livermore, 2005).

The efficacy of commonly used antibiotics is threatened due to the emergence of antibiotic resistance among pathogens; for example, *Escherichia coli, Klebsiella pneumoniae, Acinetobacter* spp., *Pseudomonas* spp., *Salmonella enterica, Staphylococcus aureus,* and *Streptococcus pneumoniae* have become resistant to key antimicrobials (Tacconelli et al., 2018; WHO, 2017). Recently, resistance to carbapenem antibiotics commonly used as a treatment of last resort for *K. pneumoniae* infections has spread worldwide (WHO, 2018). Similarly, the failure of 3rd generation cephalosporins against *Neisseria gonorrhoeae* infections has been reported in several countries. Moreover, several other bacterial pathogens such as *Mycobacterium tuberculosis* have acquired multidrug-resistance (WHO, 2018).

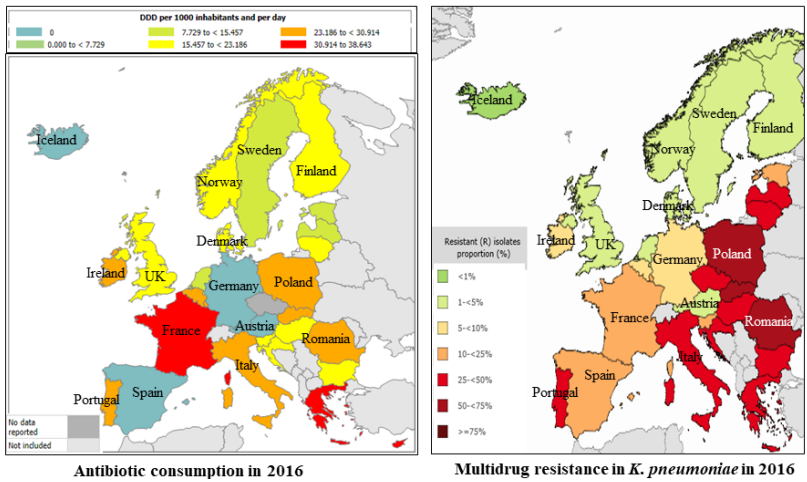

Figure 3. *Comparison of antibiotic use and the proportion of antibiotic resistance to aminoglycosides, macrolides, and cephalosporins in clinical K. pneumoniae observed in European countries in 2016. Most countries with high consumption of antibiotics show a high proportion of multi-drug resistance (MDR) in clinical K. pneumoniae. Graphs generated from ECDC antibiotic consumption and antibiotic resistance surveillance atlas (www.ecdc.europa.eu).*

Carbapenemase-producing *Enterobacteriaceae*

Carbapenem antibiotics

The first carbapenem thienamycin was discovered in 1976, produced by *Streptomyces cattleya* (Papp-Wallace et al., 2011). Although thienamycin was highly potent against a wide spectrum of bacterial species, due to instability in aqueous solutions, it was replaced by the next-generation carbapenems. These include imipenem, meropenem, ertapenem, doripenem and panipenem. Carbapenems are active against most Gram-negative and Gram-positive bacteria and possess the most broad-spectrum of activity among β-lactam antibiotics (Papp-Wallace et al., 2011). Due to their broad-spectrum activity, they must be used as "last-resort" antibiotics for critical infections by MDR bacterial isolates. However, ongoing dissemination of MDR pathogens seriously threatens their efficacy (Nordmann & Poirel, 2014).

Carbapenem antibiotics enter the bacterial periplasmic space via outer membrane proteins (OMP) and bind to the transpeptidase (TPase) enzymes (also referred to as PBPs) responsible for the final cross-linking step of PG synthesis (Papp-Wallace et al., 2011). During the cell wall synthesis in most Gram-negative bacteria, the formation of 3,4-interpeptide cross-link by D, D-transpeptidase results in acyl-enzyme intermediates with a half-life in milliseconds (Figure 4) (Edoo et al., 2017). This quick release of TPases is necessary to keep up with the demand for new PG strands in a growing bacterial cell wall. However, the inhibition of TPases by carbapenems (and most β-lactams) results in penicilinoyl-enzyme intermediates that are stable for hours. When TPase activity is blocked, the transglycosylases (TGases) work normally and continue to link the glycan molecules of new PG unit to the existing PG strand. This results in the production of flexible regions of uncross-linked PG chain. These uncross-linked PG strands recruit the lytic TGases that hydrolyze the flexible regions of PG chain, releasing free anhydrous-disaccharide pentapeptide units. This activity of TGases and lytic TGases comprise a futile cycle with no end-product (Cho et al., 2014). The increased metabolic burden results in a weakened cell wall that leads to lysis and cell death.

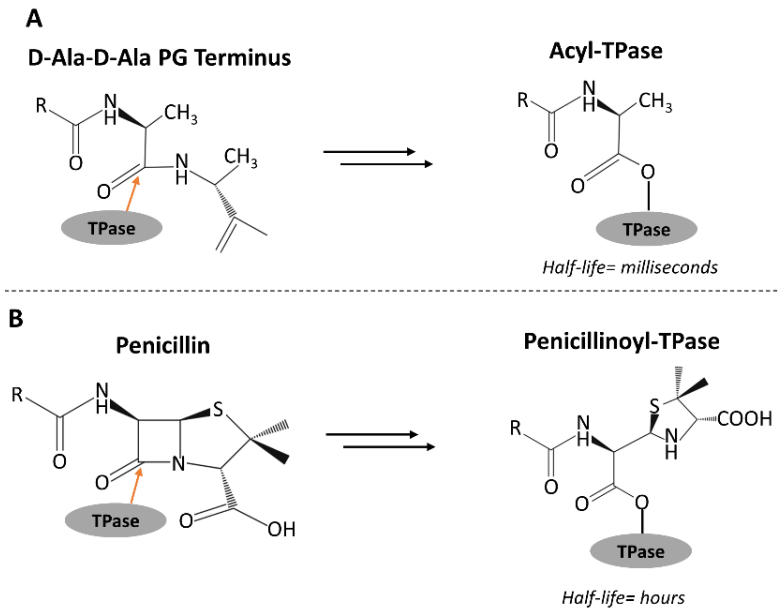

Figure 4. *The processing of D-Ala-D-Ala peptidoglycan (PG) substrate (A), and Penicillin by the transpeptidase (TPase) (B). The half-life of the Acyl-Tpase is in milliseconds, while Penicillinoyl-TPase intermediate is stable for hours. The lifetimes of these intermediates determine the fate of hydrolytic processing.*

Mechanisms of carbapenem resistance in *Enterobacteriaceae*

The *Enterobacteriaceae* are rod-shaped Gram-negative bacteria that are part of the normal intestinal flora of humans and other animals. *Enterobacteriaceae* family is one of the most common causative agents of nosocomial, as well as community-acquired infections (Nordmann & Poirel, 2014; Rupp & Fey, 2003). The resistance to carbapenems is increasing in many clinical *Enterobacteriaceae* such as *Klebsiella* spp., *E. coli*, and *Serratia* spp., and non-fermentative Gram-negative bacilli such as *Acinetobacter* and *Pseudomonas* species. The main mechanism of resistance to carbapenems in *Enterobacteriaceae* is the production of carbapenemases (Giske et al., 2009). However, other mechanisms such as porin loss/modification in association with upregulation of efflux pumps and overproduction of β-lactamases with

weak carbapenemase activity are also responsible for high-level carbapenem resistance in some isolates (Hammami et al., 2009).

Carbapenemases

Carbapenemases are versatile enzymes that can hydrolyze almost all β-lactam antibiotics including carbapenems, which have been mainly detected in Gram-negative bacteria. Carbapenemases were considered as species-specific, chromosomally encoded β-lactamases until the early 1990s (Queenan & Bush, 2007a). Resistance to carbapenem antibiotics can be mediated by carbapenemase enzymes found on both the chromosome and plasmids of many *Enterobacteriaceae* (Queenan & Bush, 2007b). For example, *Serratia marcescens* produces a carbapenem-hydrolyzing enzyme SME-1 (*Serratia marcescens* carbapenemase), encoded on the chromosome (Naas et al., 1994). Similarly, intrinsic resistance of *Stenotrophomonas maltophilia* to several β-lactams including carbapenems is due to the chromosomally-encoded bla_{L1} and bla_{L2} genes (Yang et al., 2014). There are several plasmid-borne carbapenemase genes found in *Enterobacteriaceae,* such as bla_{KPC} (*Klebsiella pneumoniae* carbapenemase) that are frequently detected in *K. pneumoniae*, *Enterobacter* spp. and in *Salmonella* spp. (Queenan & Bush, 2007b). The identification of plasmid-encoded metallo-β-lactamase (MBL) gene bla_{IMP-1} in *P. aeruginosa* (Watanabe et al., 1991), serine carbapenemase genes bla_{OXA-23} in *A. baumanii* (Scaife et al., 1995), and bla_{KPC-1} in *K. pneumoniae* (Yigit et al., 2001), changed the perception of carbapenem resistance problem from local clonal spread to global interspecies dissemination. Since then, carbapenemase-producing isolates have been reported worldwide, and a large variety of carbapenemases have been described.

The β-lactamases are periplasmic enzymes that hydrolyze β-lactams before they can bind to TPases. Carbapenemases are a type of βlactamase enzymes with catalytic efficiencies for carbapenem hydrolysis. β-lactamases are divided into four classes (Class A, B, C, and D) based on amino acid homology and known as Ambler classification (Ambler et al., 1991). Class A, C, and D have similar chemical architecture, and they have serine as a nucleophile in their active site to attack βlactam ring in the first step of the catalytic reaction, resulting in the formation of a covalent acyl-enzyme intermediate (Fisher et al., 2005). Class B β-lactamases, also known as MBL, use Zn^{2+} in the active site, which is a more robust nucleophile for the hydrolysis of β-lactamases (Palzkill, 2013). Initially, Class A (e.g., KPC, GES, and IMI) and C β-lactamases (e.g., CMY-10 and PDC) were identified as

penicillinases and cephalosporinases, respectively (Fisher et al., 2005). However, they have evolved to become effective against an extended range of β-lactam antibiotics including carbapenems, cephalosporins, penicillin, and monobactams, while inhibited by clavulanate and tazobactam (Rupp & Fey, 2003). Class D β-lactamases (e.g., OXA-types) were initially described for the hydrolysis of oxacillin, but many class D enzymes such as OXA-48 and OXA-23 can also hydrolyze carbapenems.

Metallo-β-lactamases can hydrolyze almost all β-lactams except monobactams. They are inhibited by metal chelators such as EDTA (Ethylenediaminetetraacetic acid), however, there are no MBL inhibitors available for clinical use (Drawz & Bonomo, 2010). The serine β-lactamases belong to a large superfamily of acyltransferases (SxxxK superfamily) and are structurally related to the PBPs, while the MBLs are related to separate superfamily of proteins with multiple functions. Several MBL are structurally unrelated to serine-β-lactamases which indicate an independent evolutionary origin (Meini et al., 2014; Palzkill, 2013).

The options for treating infections with carbapenemase-producing *Enterobacteriaceae* (CPE) are limited. The carbapenemase genes are mostly found on plasmids co-harboring genes that confer resistance to several other antimicrobials such as penicillins, cephalosporins, fluoroquinolones, macrolides, aminoglycosides and trimethoprim-sulfamethoxazole (Nordmann et al., 2011; Tang et al., 2017). However, many carbapenemase-producing bacteria remain susceptible to tigecycline and colistin, not considered as the drugs of choice for treating infection due to efficacy and toxicity issues (Akajagbor et al., 2013; Kumarasamy et al., 2010). Therefore, to protect the efficacy of carbapenems, it is important to understand the factors involved in the dissemination of carbapenem resistance.

Non-enzymatic mechanisms of carbapenem resistance

Apart from carbapenemases, reduction of antibiotic influx through porins and increased efflux serve as complementary mechanisms of carbapenem resistance in *Enterobacteriaceae* (Fernández & Hancock, 2012; Kaczmarek et al., 2006). However, reduced antibiotic permeability by modification and/or downregulation of OMPs results in low-level antibiotic resistance and often combined with other mechanisms such as efflux-pumps and the production of β-lactamases to attain high-level antibiotic resistance in bacteria (Munita & Arias, 2016; Nikaido, 2003).

Porins are outer membrane proteins that act as a selective barrier to protect bacteria from harmful substances and function as hydrophilic channels for molecules including antibiotics and nutrients to diffuse into the cytoplasm. In *E. coli*, OmpC, OmpF and PhoE porins are the most studied trimeric porins and they control the inflow of metabolites, amino acids, and antibiotics (Delcour, 2009). Reduced expression of these OMPs reduces the influx of antibiotics, resulting in increased resistance to β-lactams including carbapenems (Kaczmarek et al., 2006).

In most *Enterobacteriaceae*, resistance to antibiotics is attained by downregulation or structural modification of OMPs, consequently, preventing the antibiotic molecule from entering the cell (Figure 5). The OMPs such as OmpC, OmpF, and PhoE in *E. coli,* OmpK35 and OmpK36 in *K. pneumoniae,* and OprD in *P. aeruginosa* are well-characterized for their role in β-lactam resistance including carbapenems (Munita & Arias, 2016; Tsai et al., 2011). Mutations in porins such as OmpK35 and OmpK36 alone do not generally result in carbapenem resistance in clinical isolates, however, enhanced resistance is achieved in isolates that overproduce AmpC or extended-spectrum β-lactamase (ESBL) enzymes having low carbapenemase activity (Eichenberger & Thaden, 2019). Similarly, loss of outer membrane protein OmpC in *E. coli* causes reduced susceptibility to carbapenems and fourth-generation cephalosporins (Liu et al., 2012). Reduced synthesis of

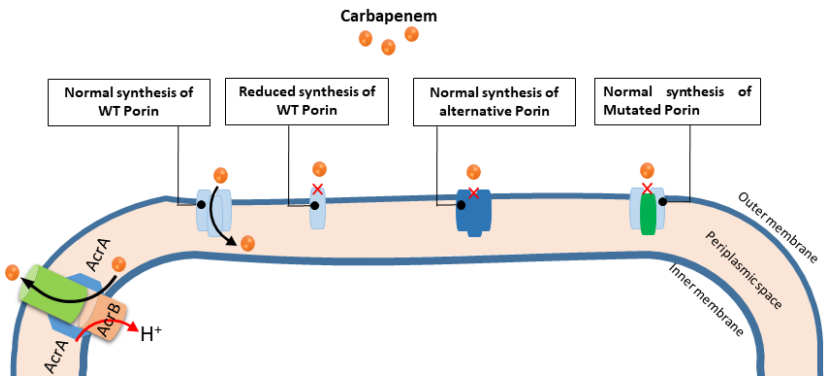

Figure 5. *An illustration of mechanisms of resistance to carbapenems other than carbapenemases in Gram-negative bacteria. Changes in the structure and expression of porins and increased efflux activity are associated with carbapenem resistance.*

OMPs in *K. pneumoniae* and *Enterobacter aerogenes* substantially increases their ability to resist antibiotics such as carbapenems and cephalosporins (Lavigne et al., 2013; Poulou et al., 2013; Tsai et al., 2011).

Efflux pumps in bacteria actively export antibiotics out of the cell and are the major determinant of antibiotic resistance. Some efflux systems are molecule specific such as the Tet efflux pump for tetracycline, while other efflux systems expel a wide range of antibiotics and are referred to as multidrug resistance efflux systems. In Gram-negative bacteria, the RND (resistance nodulation division) family of MDR efflux pumps transport a wide range of clinically relevant antibiotics. The efflux pumps belonging to the RND family are often found on the chromosomes of Gram-negative bacteria and are responsible for intrinsic resistance to several clinically important antibiotics. The RND efflux pumps in *E. coli* (AcrAB-TolC and AcrEF-TolC) and *P. aeruginosa* (MexAB-OprM) have three components spanning from the cytoplasm to the outer membrane of the bacterial cell (Figure 5). The RND efflux has a transporter protein in the inner membrane, an outer membrane protein channel, and a periplasmic accessory protein that connects the inner and outer membrane components (Piddock, 2006). The ligands can enter the efflux system either through the inner membrane protein or through periplasmic linker protein and can be actively exported. Overproduction of the efflux pump is linked to the decreased intracellular concentration of antibiotics. Combined with porin loss, increased drug efflux confers high-level resistance to clinically relevant antibiotics (Lavigne et al., 2013).

The global spread of carbapenemase-producing *Enterobacteriaceae*

The spread of carbapenemase-producing *Enterobacteriaceae* (CPE) has been identified worldwide. Several groups of enzymes having carbapenemase activity are spreading across all continents. The main drivers of carbapenem resistance spread are thought to be the clonal dissemination of successful carbapenemase-producing species that can maintain carbapenemase genes and HGT of plasmids carrying carbapenemase genes to other related bacterial species (Kitchel et al., 2009; Sheppard et al., 2016).

The KPC enzymes are one of the most important carbapenemases because they confer high-level resistance to carbapenems and other β-lactams such as third and fourth generation cephalosporins. After the first identification in *K. pneumoniae* in the USA in 1996, bla_{KPC} have been detected worldwide in many Gram-negative bacteria (Albiger et al., 2015; Kitchel et

al., 2009; Stoesser et al., 2017). In Europe, KPC has spread almost everywhere and is considered endemic in Greece and Italy (Albiger et al., 2015). The KPC- producers have been frequently reported in Asia, especially in China. According to a study, the majority (13 out of 16 isolates) of carbapenem-resistant *E. coli* from Shanghai harbored bla_{KPC} (Zhang et al., 2015). The IMP-1 ($bla_{\text{IMP-1}}$ gene) carbapenemase, the first transferable MBL was identified in a *P. aeruginosa* isolate in 1991 in Japan, while $bla_{\text{VIM-1}}$ was detected in 1997 in Verona, Italy (Lauretti et al., 1999; Watanabe et al., 1991). Since then, these carbapenemase genes are widespread across all the continents (Walsh et al., 2005). The recently identified MBL, $bla_{\text{NDM-1}}$ is of great concern in Southeast Asia. The $bla_{\text{NDM-1}}$ was first identified in *K. pneumoniae* in 2007 from a Swedish citizen who was hospitalized in India for the treatment of urinary tract infection (Yong et al., 2009). Later in 2009, a *K. pneumoniae* strain carrying $bla_{\text{NDM-1}}$ was recovered from a patient in Australia who had visited India (Kumarasamy et al., 2010). An epidemiological survey has shown carbapenem resistance mediated by $bla_{\text{NDM-1}}$ is widespread in India, Pakistan and Bangladesh (Kumarasamy et al., 2010).

In Europe, carbapenem-resistant *K. pneumoniae* is an emerging public health concern. High level of carbapenem resistance in clinical *K. pneumoniae* is always combined with resistance to several other groups of antibiotics such as fluoroquinolones, aminoglycosides, and third-generation cephalosporins thus treatment is limited to the use of colistin and combinational antibiotic therapy. The clinical data collected by European Antibiotic Resistance Surveillance Network (EARS-Net) shows an increase in the occurrence of carbapenem-resistant *K. pneumoniae* between 2013 and 2016, particularly in southern and eastern European countries (Figure 6). Since it is compulsory for the clinical laboratories in Sweden to report CPE to Folkhälsomyndigheten (Swedish Public Health Authority) there are statistics available for Sweden for each year since 2008. The data shows that the incidence of carbapenem resistance in clinical isolates of Sweden is still low (Folkhälsomyndigheten, 2018). However, the prevalence of CPE carriers in the community is unknown.

Increase in globalization, trade and international travels play a role in the dissemination of resistant bacteria across continents. A recent study of the gut microbiome of Swedish travelers that went to the Indian Peninsula demonstrated that 70% of travelers acquired ESBL-producing *E. coli* during their visit, without using any antibiotics (Bengtsson-Palme et al., 2015; Tangden et al., 2010). Another study reported that the acquisition of MDR ESBL-producing *Enterobacteriaceae* is common during international travel

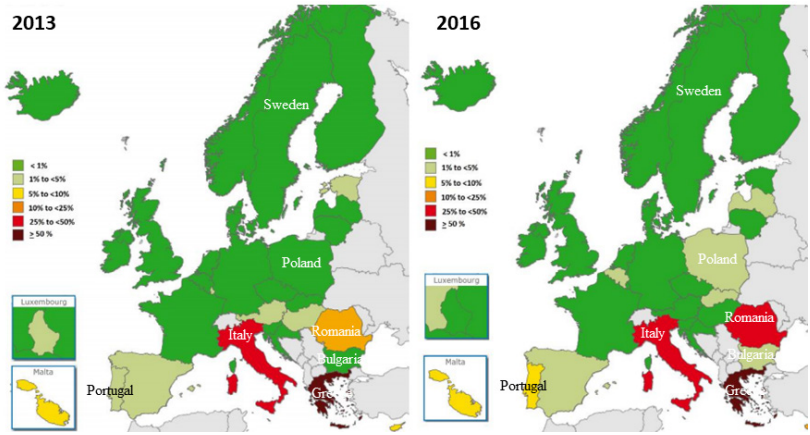

Figure 6. *The proportion of clinical K. pneumoniae isolates with carbapenem resistance in European countries in 2013 and 2016. An increase in K. pneumoniae with carbapenem resistance is observed primarily in eastern and southern Europe such as Portugal, Romania, Poland, and Bulgaria. Adapted from the EARS-Net annual report on Antibiotic resistance surveillance 2016 (www.ecdc.europa.eu).*

and the geographical area of the visit has the highest impact on acquisition (Östholm-Balkhed et al., 2013). Trade of food products between countries is also a possible route for the spread of ARB. Human consumption of foods contaminated with ARB may result in the transfer of resistant determinants to the normal gut flora. The resistance bacteria and genes from all these sources can further spread in the community and the environment.

Environmental factors that influence the development of antibiotic resistance

Dissemination of antibiotic resistance in the environment

Antibiotics are extensively used in clinical settings and resistant pathogens along with antibiotics from patients in the hospital are released as wastewater. Thus, hospital wastewaters can be reservoirs of ARGs related

to clinically important human pathogens and antibiotics used in clinical settings (Rodriguez-Mozaz et al., 2015; Varela et al., 2015). Resistant pathogens mainly spread between humans through direct or indirect contact, either in clinics or through the community setting. The most common indirect dispersal routes are contaminated aerosols, and food prepared by persons carrying the pathogen (Livermore, 2000). Apart from the spread between humans, different environmental compartments considered as potentially important routes for the spread of antibiotic resistance (Allen et al., 2010; Huijbers et al., 2015).

Antibiotics and ARB are also released into the wastewater by patients taking antibiotics as part of the outpatient antibiotic therapy. According to the European Centre for Disease Prevention and Control (ECDC, 2018), a major proportion of antibiotics consumed by humans is in the community rather than in hospitals. Eventually, a significant proportion of these antibiotics and ARB from all these sources reach wastewater treatment plants (WWTP). In many countries, wastewater from the hospital, household, and industries is processed in WWTPs, acting as "hot-spots" of ARB and ARGs (Rizzo et al., 2013). Contaminated water from WWTP is often used for agricultural and recreational purposes, and as drinking water after further treatment. Resistant bacteria may spread to humans via domestic animals that often drink contaminated surface waters. However, untreated sewage released into natural aquatic environments is a considerably larger threat than the treated effluents from WWTP, as wastewater treatment often reduce the relative abundance of resistance genes, and significantly lower the total bacterial abundance (Bengtsson-Palme et al., 2016; Khan et al., 2019).

Horizontal gene transfer

Horizontal gene transfer is an important driving force behind the spread of novel (and known) ARGs in the microbial population, as it allows resistance to transfer not only within related clones but also to unrelated bacterial species. This way, HGT makes ARGs available to a wider part of the bacterial community in a particular environment (Martinez, 2008). The ability of *Enterobacteriaceae* to acquire different resistance genes via HGT, mostly mediated by plasmids and transposons, is strongly associated with multidrug resistance (Leverstein-van Hall et al., 2003). Many of the antibiotic resistance determinants are found on MGE such as transposons, integrons, and plasmids, which can be acquired by human commensal and pathogenic bacteria by HGT (Aarestrup, 2005; Baquero et al., 2008). A classic example

of HGT is the presence of plasmid-encoded ESBL genes $bla_{CTX\text{-}M}$ in *Enterobacteriaceae* that potentially originated from the chromosome of environmental *Kluyvera* species (Canton & Coque, 2006). The genetic material can be transferred by three main mechanisms: conjugation, transduction, and transformation (Figure 7).

Conjugation occurs through cell to cell contact by sex-pili (Figure 7). These hair-like appendages allow the transfer of MGE such as plasmids, transposons, and integrons from donor to recipient cells (Skippington & Ragan, 2011). The conjugation is thought to be accountable for the majority of the antibiotic resistance spread due to the presence of multiple ARGs on a single MGE and their ability to transfer between genetically un-

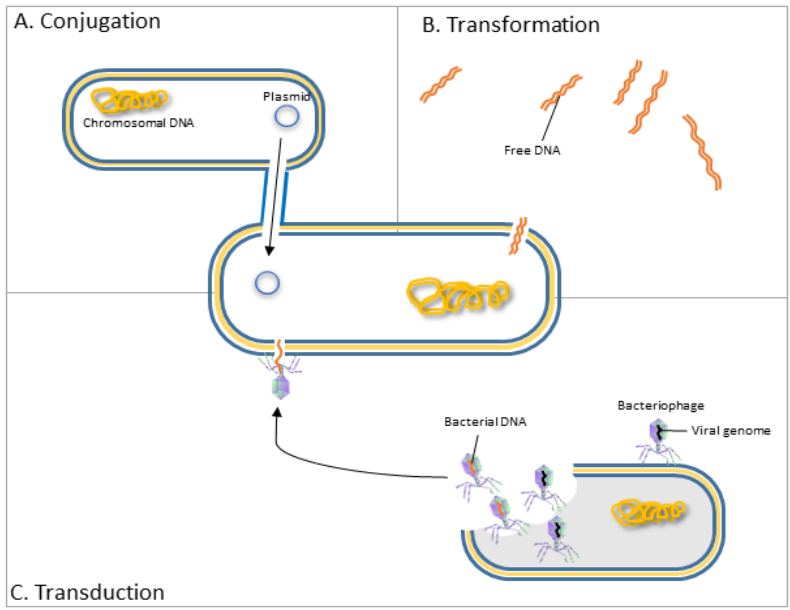

Figure 7. *Mechanisms of horizontal gene transfer in bacteria. Conjugation is the transfer of mobile genetic elements such as plasmid from a donor to recipient bacterium via direct contact (A). Transformation is the uptake and integration of extracellular DNA by competent bacteria (B). Transduction involves the transfer of genetic material from one cell to another through bacteriophages (C). The bacterial DNA fragments are excised and loaded onto the viral genome and are transferred to another bacterial cell. Adopted from (von Wintersdorff et al., 2016).*

related species (Klümper et al., 2014). For example, a study of Indian sewage and tap water found $bla_{\text{NDM-1}}$ (New-Delhi Metallo-β-lactamase) gene in over 20 different bacterial species, either on plasmid or chromosome, and most of them were able to transfer the gene by conjugation (Walsh et al., 2011).

Transformation involves the direct uptake of naked DNA by competent bacteria from its surroundings (Figure 7). Bacterial competence is the ability of a bacterium to acquire, integrate, and functionally express foreign DNA fragments. DNA is normally present in the surrounding environment resulting from lysed bacteria. This extracellular DNA often harbors genes for antibiotic resistance and it has been reported that several stress conditions such as antibiotic exposure stimulate the transformation of ARGs by enhancing bacterial competence (Prudhomme et al., 2006). It is suggested that transformation does not play a significant role in the spread of antibiotic resistance since there is a limited number of bacterial species that can naturally transform ARGs (Lopatkin et al., 2016).

Bacteriophages are viruses that play an important role in the horizontal transmission of ARGs among bacteria. Transduction is the process in which bacterial genes are incorporated into the viral genome and transferred to another bacterial cell in the next lytic cycle (Figure 7) (Casjens, 2003). The phages and phage-mediated transfer of ARGs are documented in several species as recent advances in whole-genome sequencing (WGS) have made it possible to identify phage-associated genes such as phage protease, integrase and structural proteins (Zhou et al., 2011). Several studies have detected ARGs harbored on bacteriophage genomes (Colomer-Lluch et al., 2011; Rolain et al., 2011). One study showed the transduction of ESBL gene $bla_{\text{CMY-2}}$ and tetracycline resistance genes *tetA* and *tetB* between *Salmonella* species (Zhang & LeJeune, 2008). A similar transfer of antibiotic resistance via bacteriophages is documented in *P. aeruginosa* (Blahová et al., 2001).

Stability and persistence of resistance in the environment

The problem of environmental antibiotic resistance is multidimensional and several factors influence the spread and persistence of resistance. The low concentration of antibiotics from anthropogenic sources exerts long-term selective pressure on the microbial population in the environment. Besides antibiotics, the ARB of human origin may proliferate and persist in environments with other anthropogenic contaminants such as heavy metals, de-

tergents, and household disinfectants. Moreover, factors other than anthropogenic contamination also contribute to the stability of antibiotic resistance (Figure 8).

Antibiotics-derived selection

The most evident factor is the presence of low levels of antibiotics from anthropogenic sources that might be enough to confer the selection and persistence of bacterial clones carrying resistance determinants. Bacteria in the environment are exposed to antibiotics of anthropogenic origin in concentrations far below the minimum inhibitory concentration (MIC) (Kummerer, 2009). It is well known that high concentrations of antibiotics (higher than MIC) select for the resistant isolates in a population (Wistrand-Yuen et al., 2018). Studies have shown that resistance evolves during low-level exposure and even without antibiotics (Andersson & Hughes, 2011, 2012; Knöppel et al., 2017). One study demonstrated the selective advantage of resistant mutant strains of *E. coli* against sensitive wild-type strain at 1/5 and 1/20 of the MIC for ciprofloxacin and tetracycline, respectively (Liu et al., 2011). Other studies have also shown that long-term exposure to low level (sub-MIC) antibiotics can select genetic changes that confer a high level of resistance (Chow et al., 2015; Wistrand-Yuen et al., 2018).

The presence of only one antibiotic can select and maintain resistance to multiple antibiotics, the process known as co-selection. The co-selection involves the presence of multiple resistance genes on the same genetic element such as plasmid and transposon. In an event of HGT, the closely located genes are more likely to be transferred together. Multiple ARGs are frequently detected on the same plasmid and therefore the presence of any of the antibiotics in the environment may select for all the ARGs present on the plasmid (Ridenhour et al., 2017; Tang et al., 2017).

Non-antibiotic selective pressures

In addition to antibiotics, non-antibiotic selective pressure may also maintain resistance to antibiotics (Figure 8). Bacterial resistance to metals selects for antibiotic resistance by physiological and genetic mechanisms. The physiological mechanism involves the resistance to multiple contaminants such as antibiotics and metals thus known as cross-resistance. Several multidrug efflux pumps are known for contributing to cross-resistance to both antibiotics and heavy metals by exporting them outside the bacterial cell

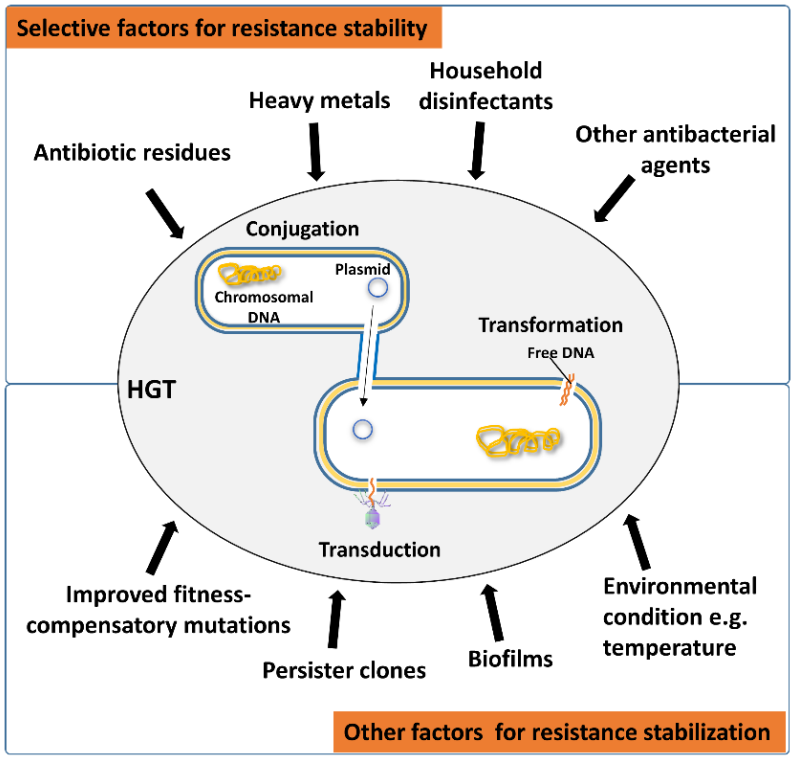

Figure 8. *The transfer and stability of antibiotic resistance determinants (antibiotic resistance genes and bacteria that carry these genes) in the environment. Resistance can be acquired through horizontal gene transfer. Several selective forces such as antibiotics and other contaminants of anthropogenic origin can contribute to the maintenance of the resistance. Other than selection, the development of resistant mutations and compensatory mutations, biofilms, and persister clones, and environmental conditions such and temperature can also play a role in the stabilization of resistance traits.*

(Martinez et al., 2009). Several plasmids have been identified as co-harboring genes for antibiotic and metals resistance (Osman et al., 2010; Rosewarne et al., 2010; Seiler & Berendonk, 2012). This suggests that not only antibiotics, heavy metal contamination may also select for antibiotic resistance in the environment.

The antimicrobial compounds in household disinfectants can also select for antibiotic resistance. Triclosan is commonly used antimicrobial agents in household cleaning products and consequently abundant in WWTP and associated natural environments (Carey & McNamara, 2015; Ricart et al., 2010). Triclosan has been shown to select for multidrug resistance, either by modification in target protein (Parikh et al., 2000). Many pharmaceuticals have weak antimicrobial properties that may contribute to the complexity of selection pressures in the environment (Backhaus et al., 2011). Thus, the environmental bacterial population is exposed to a complex mixture of contaminants and selective pressures, and the elimination of a particular selective pressure may not directly eliminate resistant bacteria.

The presence of high nutrient content in wastewater enhances bacterial growth and continuous exposure to low-levels of antibiotics may promote bacteria to acquire mutations that increase antibiotic resistance, which has been demonstrated by *in vitro* studies (Andersson, 2006; Andersson & Hughes, 2012). Harboring a resistance mechanism is often associated with a fitness cost as it involves important cellular processes. For example, resistance to aminoglycosides in *E. coli* due to a mutation in ribosomal protein interferes with normal cellular functions (Holberger & Hayes, 2009). In the absence of a selection pressure such as antibiotics, resistance determinant is an excessive burden to the bacterial cell. However, second-site mutations often compensate for the cost of the original mutation or carrying plasmids, thus stabilizing the resistance determinants (Vogwill & MacLean, 2015; Wistrand-Yuen et al., 2018).

Biofilms and persister cells

Bacteria attach to biotic and abiotic surfaces and form a complex matrix known as biofilms that enables them to evade environmental hazards such as exposure to antibiotics (Figure 9). Biofilms in the environment are frequently formed by a diverse community of microorganisms including bacteria, fungi, algae, and protozoa protected by a matrix of secreted polysaccharides (Balcázar et al., 2015). Biofilm production significantly increases the minimum biofilm eradicating concentration (MBEC) for bacterial cells in biofilms compared to the planktonic cells (Brady et al., 2017; Gilbert et al., 2002). Several factors could be involved in this increased resistance. Firstly, the sheath of the extracellular polysaccharide provides a physical barrier and results in poor penetration of antibiotics into the biofilm. In

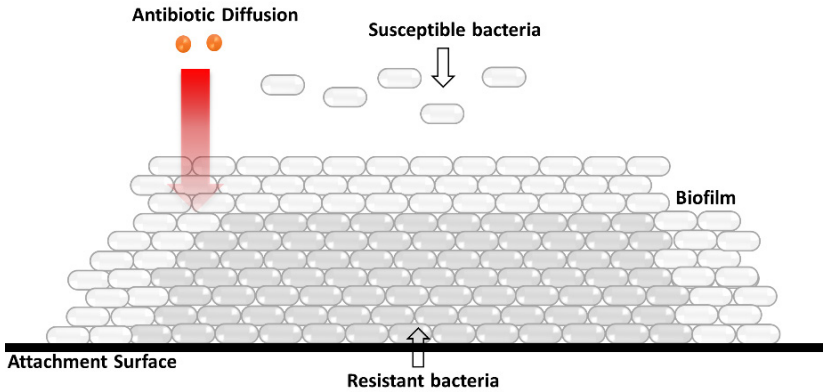

Figure 9. *Illustration of reduced susceptibility to antibiotics for bacterial cells buried deep in the biofilm, partly due to the decreased access to antibiotics.*

aquatic environments, biofilms develop in streambeds and sediments, and also on suspended macro– and microaggregates (Meinhard et al., 2002). Wastewater collection systems and treatment plants are important sections of the urban water cycle where complex microbial, chemical and physico-chemical processes take place. In densely populated environmental biofilms found in the wastewater collection system and WWTP, the conditions for HGT are optimal due to increased genetic competence and access to a large repertoire of resistance determinants (Balcázar et al., 2015; Fux et al., 2005). In these environments, biofilm formation improves the stability and persistence of bacteria carrying MDR plasmids (Ridenhour et al., 2017).

In many bacterial populations, there are cells with reduced metabolic activity and are much less susceptible to antibiotics as rapidly growing cells will be killed, while slow-growing and non-growing cells will evade the antibiotic treatment and persist (Tuomanen et al., 1986). This is true especially for β-lactams that act against bacterial cells that are actively multiplying. These persister clones may have similar (tolerant), or higher (resistant) MIC compared to the rest of the population. One major protective mechanism of reduced susceptibility of persister cells is the reduction in the rate of uptake of metabolites that also reduces the antibiotic uptake (Schmidt et al., 2014).

One-Health approach to antibiotic resistance

One-Health is a local, regional and global collaborative and multidisciplinary approach to achieve optimal health and well-being outcomes by recognizing the interconnections between people, animals, plants and their shared environment. Recent anthropogenic activities have accelerated the emergence and spread of antibiotic resistance and little is known about the processes involved in the emergence of resistance in natural environments (Hiltunen et al., 2017; Manaia et al., 2016). It is becoming increasingly important to apply a holistic approach (so-called One-Health approach) in tackling antibiotic resistance, which includes humans, animals, and natural environments at local and regional levels (Collignon, 2015; Ryu et al., 2017).

The microbiomes of human, aquatic environments, wastewaters, plants, animals, and soils are interconnected reservoirs of antibiotic resistance (Allen et al., 2010; Martinez, 2011). The resistance determinants, including ARB and ARGs, may flow between these reservoirs (Martinez, 2011). This transmission of ARB and ARG between geographically related ecosystems depicts the complex transmission network of antibiotic resistance between the three main domains inside the One-Health concept (Figure 10). Antibiotic residues from pharmaceuticals, agricultural farms and hospitals and community eventually reach natural environments such as rivers and lakes in urban areas (Andersson & Hughes, 2014). The natural environment is a major recipient of human-derived antibiotic resistance determinants and there are several routes of dissemination including hospital and community wastewaters. (Rizzo et al., 2013; Vaz-Moreira et al., 2014). Thus, the environment serves both as a sink and a reservoir of resistant human pathogens. Moreover, due to the recent extensive human travel and migration, infectious agents can spread readily and become pandemic. It is important to devise and maintain strategies to reduce the spread of antibiotic resistance not only in one geographical location but throughout the globe (Cabrera-Pardo et al., 2019).

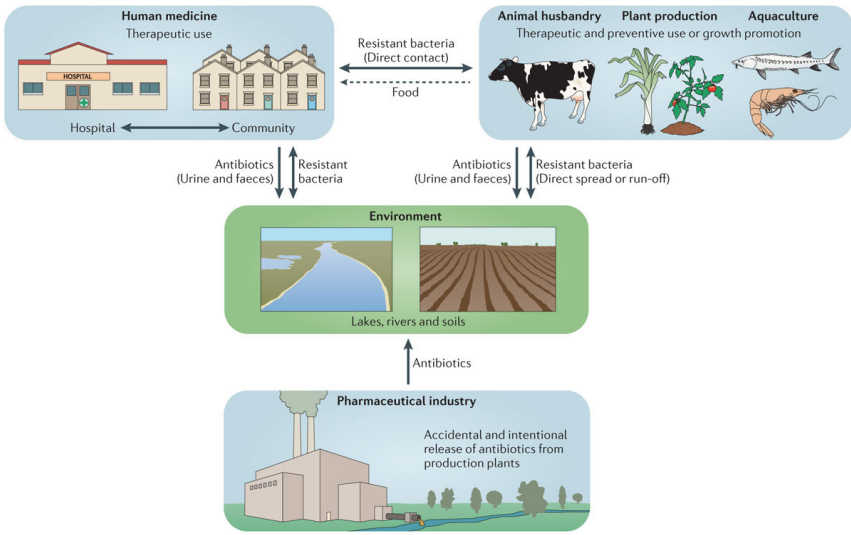

Figure 10. *An overview of how antibiotics and resistant bacteria flow between different environments, such as the clinical, agricultural, aquacultural, the pharmaceutical industry and the wider environments including soil and rivers. Reprinted with permission from (Andersson & Hughes, 2014).*

AIMS

The overall aim was to characterize the CPE in environmental waters such as wastewaters and associated river and lake waters in Örebro, Sweden and to study the *de novo* emergence of resistance in bacteria exposed to river water contaminated by wastewater effluent.

Specific objectives were,

1. To characterize CPE in Swedish wastewaters and associated surface water from a River and Lake to identify species that are potentially persistent reservoirs of carbapenemases and to investigate the genetic relationship of CPE from the environment and a nearby hospital. (**Paper I, II**)

2. To determine suitable methods to explore the diversity of ARGs in general and carbapenemase genes in particular in wastewaters and associated aquatic environments of Sweden, a country with a low prevalence of antibiotic resistance. (**Paper III, IV**)

3. To determine whether *de novo* resistance to β-lactam antibiotics develops in *Klebsiella oxytoca* after long-term growth in river water impacted by effluent water from a WWTP and to study the effect of environmental temperature on the emergence of resistance. (**Paper V**)

MATERIALS AND METHODS

Study location

The study was performed in Örebro city, located in central Sweden. The population of Örebro city is 155 696, seventh largest in Sweden (https://www.scb.se/en/, updated in November 2019). Örebro city is located near the western side of Hjälmaren Lake, which is connected to the Baltic Sea through Lake Mälaren. Svårtan is a 100-kilometer-long river starting from the Lake Ölen in Värmland and flows downstream through the city of Örebro before pouring into Hjälmaren Lake. The river flows through forests and small agricultural landscape before entering the city of Örebro. The untreated wastewater from the municipality, industries, and the hospital is transported to the WWTP which is located on the bank of Svartån River downstream of Örebro City. The treated wastewater is discharged into the river.

Sample collection

The environmental water samples were collected from directionally connected sites as illustrated in Figure 11; river upstream of Örebro City (UR), the river downstream of WWTP (RR), and lake downstream of WWTP (RL) (**Paper I, II, III & IV**). The WWTP samples were collected from incoming wastewater (IW) and effluent wastewater (EW) (**Paper I, II, IV**). Additionally, to detect the antibiotic resistance markers in aquatic environments and the contribution from hospital, samples were collected from the wastewater of Örebro University Hospital (H) (**Paper IV**). The wastewater from the hospital was collected from the outlet pipes of three main buildings (A-, B- and M-house) where the departments of infectious diseases, urology, pediatrics, surgery, and medicine are located. Triplicate samples were collected in one-liter sterile borosilicate glass bottles, transported and stored at 6°C and analyzed within 24 h.

Isolation of *Enterobacteriaceae*

The samples of appropriate volume were filtered through 0.45 µm polyethylene sulfonate membrane filters (Sartorius Stedim Biotech, Sweden). To isolate CPE, filters were placed onto chromID CARBA (bioMérieux, Marcy-l'Etoile, France) and chromID OXA-48 (bioMérieux) mSuperCARBA (CHROMagar, Paris, France) agar media and incubated for 18h at

37°C. The morphologically different colonies were picked randomly and streaked onto Chromocult Coliform agar (Merck, Darmstadt, Germany) to obtain single colonies. Species-level identification was performed using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry with Microflex LT system (Bruker Daltonik GmbH, Bremen, Germany).

To compare environmental and hospital isolates, all CPE from a pre-existing bacterial library of Örebro University Hospital were included in the analysis (**Paper II**). These CPE were isolated from patients with the gastrointestinal, urinary tract, and wound infections from the year 2008 to 2015.

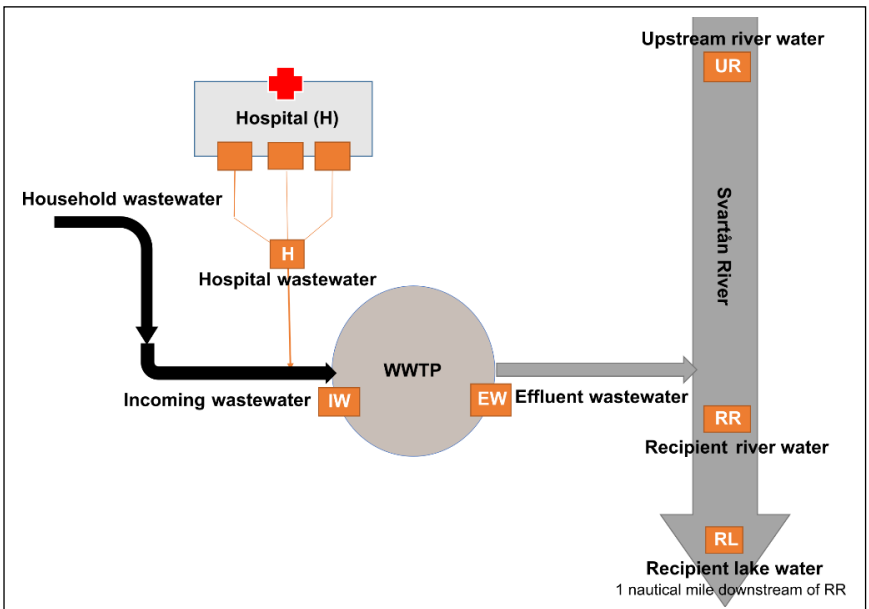

Figure 11. *Illustration of wastewater management in Örebro city and sample locations. Wastewater from households, industries, and the hospital are transported to the wastewater treatment plant that is located downstream of Örebro City, and treated wastewater is discharged into Svartån River. Svartån River flows through the city and pours into Hjälmaren Lake. Sampling sites: Hospital (H), incoming wastewater to WWTP (IW), and effluent wastewater (EW), Svartån River upstream of Örebro city (UR), Svartån River downstream of WWTP (RR) and Hjälmaren Lake (RL), which is one nautical mile downstream of RR. (Khan et al., 2019)*

Coliform counts

The *E. coli* and other coliforms were counted for each sample by filtering appropriate volumes through 0.45 µm polyethylene sulfonate membrane filters (Sartorius Stedim Biotech, Sweden) and cultivating on Chromocult Coliform agar. Colonies were counted after 24 h of growth at 37 °C and CFU/100 mL were calculated.

Detection of antibiotic resistance genes

For culture-dependent detection of ARGs, 100 mL of water samples from each location were filtered through 0.45 µm polyethylene sulfonate membrane filters (Sartorius Stedim Biotech, Sweden) and membranes were incubated on Chromocult Coliform agar at 37°C overnight (Figure 12). After incubation, the membrane filters were transferred to a sterile 50 mL falcon tube containing 10 mL phosphate-buffered saline (PBS), vortexed and centrifuged to collect the bacterial cultures from filter paper. The bacterial pellet was stored in -80°C for DNA extraction and subsequent microbial and antibiotic resistance genes analysis. For culture-independent detection of ARGs, 1 L water from each sample replicate was centrifuged at 8000 x *g* for 15 minutes at 4°C to collect the pellet. The pellet was stored in -80°C for DNA extraction.

Gram-negative bacteria from the wastewater of hospital, WWTP, and surface water samples were analyzed for the presence of ARGs belonging to different antibiotic classes; aminoglycosides, classes A, B, C and D β-lactamases, erythromycin, fluoroquinolones, macrolide-lincosamide-streptogramin B, tetracycline, vancomycin, and multi-drug resistance. High-throughput detection of ARGs was performed using Antibiotic Resistance Genes DNA qPCR Array (Qiagen, Sweden. BAID-1901ZRA) following the manufacturer's instructions. The DNA from the hospital, WWTP, and environmental waters were also analyzed for the presence of over one hundred bacterial species using the Qiagen bacterial identification DNA qPCR Arrays for Sepsis (Qiagen, Sweden. BAID-1903ZRA) and Water (Qiagen, Sweden. BAID-1405ZRA).

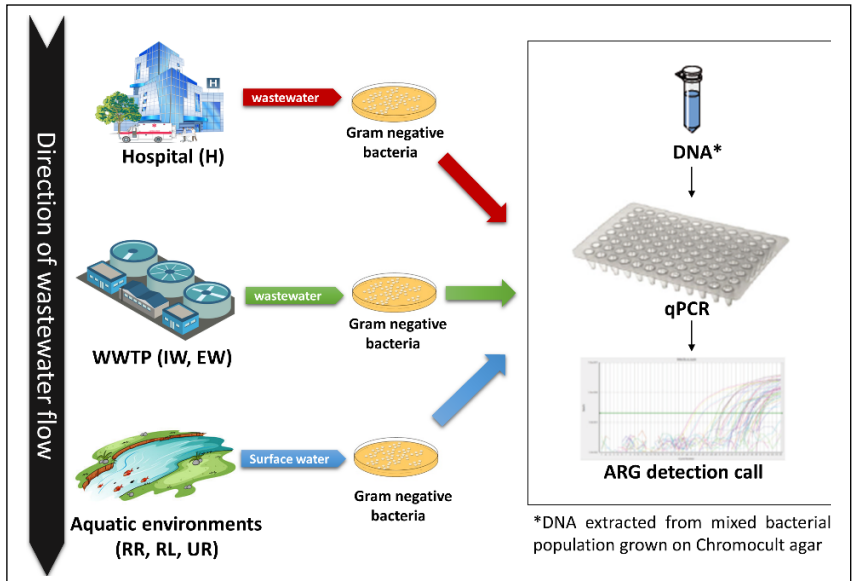

Figure 12. *Methodology for detecting antibiotic resistance genes (ARGs) from environmental waters. Surface water and wastewater were collected from central Sweden and Coliforms and Gram-negative bacteria were selectively cultured. The DNA from mixed bacterial population grown on selective media was extracted and qPCR array for ARGs was performed.*

Doubling time and relative fitness

To calculate doubling time, 0.5% overnight culture was added to 200 µL appropriate broth pre-dispensed into wells of the 96-well microtiter plate. Four biological replicates (n=4) were used to determine growth curves. The cultures were incubated at 37°C with shaking and optical density was measured every hour at 620 nm (OD_{620}) using an Infinite F50 microplate spectrophotometer (Tecan, Switzerland). Exponential growth rate and doubling time (T_d). The T_d of the clones was calculated and compared to the ancestral strain to determine their relative fitness. Relative T_d was determined by dividing the T_d of the evolved clones with T_d of ancestral strain.

Phenotypic analyses

Confirmation of carbapenemase production

To confirm the production of carbapenemase in isolates identified as *Enterobacteriaceae*, carbapenem hydrolysis assay using RAPIDEC® CARBA NP test (BioMérieux, Marcy-l'Etoile, France) was performed according to manufacturer's instructions (Poirel & Nordmann, 2015). The CARBA NP test is based on the direct detection of imipenem hydrolysis by CPE. The hydrolysis of imipenem results in the production of lactam acid and is observed via pH change indicated by phenol red indicator.

Antibiotic susceptibility tests

The antibiotic susceptibility was tested using the disc diffusion method and/or Etest (bioMérieux, Marcy-l'Etoile, France). The disc-diffusion test was performed following the recommendations from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). MIC was determined using Etest, following the manufacturer's recommendations. Clinical breakpoint values (Version 9.0) for zone diameters (mm) and the MIC values from EUCAST were used to categorize the isolates as susceptible (S), intermediate (I) and resistant (R).

Biofilm assay

The biofilm assay was performed in flat-bottom 96 well polystyrene microtiter plates in quadruplicate. The *P. aeruginosa* PAO1 was included as positive control and the LB broth medium was considered as the negative control. Briefly, a 5 µL of the bacterial suspension having 0.5 OD_{600} was inoculated in each well containing 95 µL Luria-Bertani (LB) broth. The microtiter plate was incubated at 37°C for 24 h. After incubation, planktonic cells were aspirated and washed twice with PBS. The biofilm was fixed with 99% methanol and stained with 100 µL of 0.1% crystal violet for 20 minutes. The excess crystal violet was drained, and biofilms were washed twice with tap water and air-dried. Finally, the cell-bound crystal violet was dissolved in 30% acetic acid and absorbance at OD_{595} was measured using a microplate spectrophotometer (Tecan). The cut-off OD (OD_{cut}) was defined as three standard deviations above the mean OD of the negative controls (OD_{cut} = standard deviation × 3 + average of OD negative control) (Rodríguez-Lázaro et al., 2018). Isolates were classified into four categories: non-biofilm formers (OD ≤ ODcut), weak biofilm formers (ODcut < OD ≤ (2 ×

ODcut)), moderate biofilm formers ((2 × ODcut) < OD ≤ (4 × ODcut)), or strong biofilm formers ((4 × ODcut) < OD).

Mass spectra analysis

To identify possible clonal dissemination of CPE between hospital and natural environment, phyloproteomic analysis was performed based on MALDI-TOF mass spectra. Briefly, a loop-full (approx. 1 µl) of fresh bacterial culture was suspended is 900 µl of 99 % ethanol. The suspension was centrifuged at 11,000 x g for 2 min and the pellet was air-dried at room temperature. The dried pellet was lysed in 20 µl of formic acid (70 %) for 3 minutes at room temperature and proteins were extracted with 20 µl acetonitrile. The mixture was vortexed and centrifuged at 11,000 x g for 2 min. Mass spectrometry was performed as explained previously. Two replicate spectra were obtained for each isolate. The mass spectra of the bacterial proteins were analyzed using BioNumerics version 7.5 by Applied Maths NV (http://www.applied-maths.com). Strict pre-processing of spectrum data using baseline subtraction, noise elimination, and curve smoothing was performed using default parameters. A similarity comparison was performed with the peak-based Pearson coefficient using default parameters and a phyloproteomic dendrogram was created.

Genetic analyses

DNA isolation and whole-genome sequencing

Isolation of DNA from individual bacterial cultures and selective metagenomic cultures was performed using the guanidinium thiocyanate-phenol-chloroform extraction method (Lemarchand et al., 2005). Briefly, bacterial pellet was incubated in lysis buffer (500 mM guanidium thiocyanate, 100 mM EDTA, and 0.5 % (w/v) n-lauryl-sarcosine) to lyse the cells. After lysis, DNA was separated from proteins and cell debris by adding ammonium acetate (4M, pH 5.2) and phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0). To separate the DNA from salts and other impurities, ice-cold isopropanol was added, and the precipitate was washed with 70% ethanol. After removing ethanol, DNA was dried in 40°C and dissolved in nuclease-free water.

DNA samples were sent to GATC Biotech (Konstanz, Germany) for WGS using Illumina HiSeq and 150 bp long paired-end reads were generated (> 5 million reads). Quality trimming and filtering of the paired-end reads was

performed with fastp version 0.20.0 (Chen et al., 2018). De novo genome assembly was performed using SPAdes Genome Assembler version 3.13 (Bankevich et al., 2012).

Multi-locus sequence typing

To identify the sequence types (STs) of CPE isolates, multi-locus sequence typing (MLST) was performed using MLST-1.8 Server provided by the Center for Genomic Epidemiology (Larsen et al., 2012).

Comparative genomic analyses and phylogenetics

The genomic contents of OXA-48-producing *E. coli* and VIM-1-producing *K. oxytoca* were assessed using BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011) (**Paper I**). Orthologous Average nucleotide identity (OrthoANI) and in silico genome-to-genome distance (GGD) between isolates were calculated with OAT (Orthologous Average Nucleotide Identity Tool) software e version 0.93 (Lee et al., 2016).

For phylogenetic analysis of CPE from hospital and environment (**Paper II**), core-genome single-nucleotide polymorphism (SNP) analysis was performed in Parsnp (Treangen et al., 2014) and core-genome multi-locus sequence typing (cgMLST) was performed in SeqSphere+ (Ridom Muenster, Germany).

Annotation of antibiotic resistance and virulence determinants

Antibiotic resistance gene profiles of the hospital and environmental CPE were identified Resfinder server 2.1 (Kleinheinz et al., 2014) and/or Resistance Gene Identifier (Jia et al., 2017) using the WGS data (**Paper I, II**). For **Paper II**, the presence of ARGs was also confirmed with oligonucleotide microarray-based assay developed by Alere Technologies (Germany) (Braun et al., 2016; Braun et al., 2014).

Virulence factors (VF) were detected using Pathosystems Resource Integration Center (PATRIC) using the PATRIC-VF database (Wattam et al., 2017). PathogenFinder was used to predict the probability of isolates as human pathogens (Cosentino et al., 2013).

Variant calling

For variant analysis, sequences of the clones evolved after 600 generations were aligned to the assembled genome of the parental strain using the Burrows-Wheeler Alignment tool (Li and Durbin, 2009). Variant-calling was performed with Freebayes version 2.0 (Garrison and Marth, 2012) and the variant effect was predicted with SnpEff version 4.3 (Cingolani et al., 2012).

Statistical analyses

Non-metric Multidimensional Scaling (NMDS) was applied to ARGs presence-absence data using the correlation matrix in the PAST software package (Version 3.14) by Palaeontologica Electronica. To visualize the co-occurrence of ARGs, a correlation matrix was constructed using pairwise Spearman's correlation between ARGs detected in more than 6 out of 18 samples (including the biological replicates). The co-occurrence network was constructed and visualized in Gephi using the Fruchterman Reingold layout. Student's *t*-test (unpaired, two-tailed) was used to compare coliform numbers from IW and EW from WWTP, while the one-way analysis of variance (ANOVA) was used to compare Coliforms from three environmental water samples (UR, RR, and RL) followed by Dunnett's posthoc test.

RESULTS AND DISCUSSION

Paper I

Background: It has been widely reported that WWTPs are considered as reservoirs of antibiotic resistance and is the major route of transmission of MDR bacteria from the community to the environment (Blaak et al., 2015; Rizzo et al., 2013). In countries with low antibiotic resistance profile, very little is known about the presence of CPE in wastewaters and aquatic environments.

Aim: This study aimed to characterize CPE in Swedish wastewaters and associated surface water from a River and Lake to identify species that are potentially persistent reservoirs of carbapenemases.

Methodology: The samples from WWTP (IW, EW) and surface water from both upstream (UR) and downstream (RR, RL) of Örebro City were collected and CPE were isolated during sampling sessions from 2015-2017 (Figure 11). Susceptibility of CPE isolates to 12 antimicrobials belonging to six different classes was tested using Etest. The ability of CPE to form biofilm was detected using the crystal violet assay. The CPE isolated during 2015-2017 were sequenced and comparative genomics methods were applied to determine species that are persistently detected during three years of sampling. The ARGs and virulence genes were annotated using RGI and PATRIC-VF, respectively. The probability of isolates as human pathogen was determined using PathogenFinder and MLST was performed using MLST-1.8 Server provided by the Center for Genomic Epidemiology. OrthoANI values were determined using OAT software.

Results and discussion: From a total of 269 isolates recovered from wastewater and environmental water samples during the three years of sampling, 19 (7%) were identified as CPE. The majority of CPE were *E. coli* (n=10), followed by *K. oxytoca* (n=6), and *Raoultella ornitholytica* (n=4). The CPE were recovered from wastewaters (n=13) and the river that receives effluent wastewater (n=6). No CPE was recovered from the upstream river and downstream lake water. All *K. oxytoca* isolates were resistant to at least three classes of antibiotics and were classified as MDR. The majority of the *E. coli* (7 out of 10) and one *R. ornitolytica* were classified as MDR.

The *Klebsiella* and *Raoultella* isolates produced strong biofilms while *E. coli* were non-biofilm producers. All 19 CPE were predicted as human pathogens.

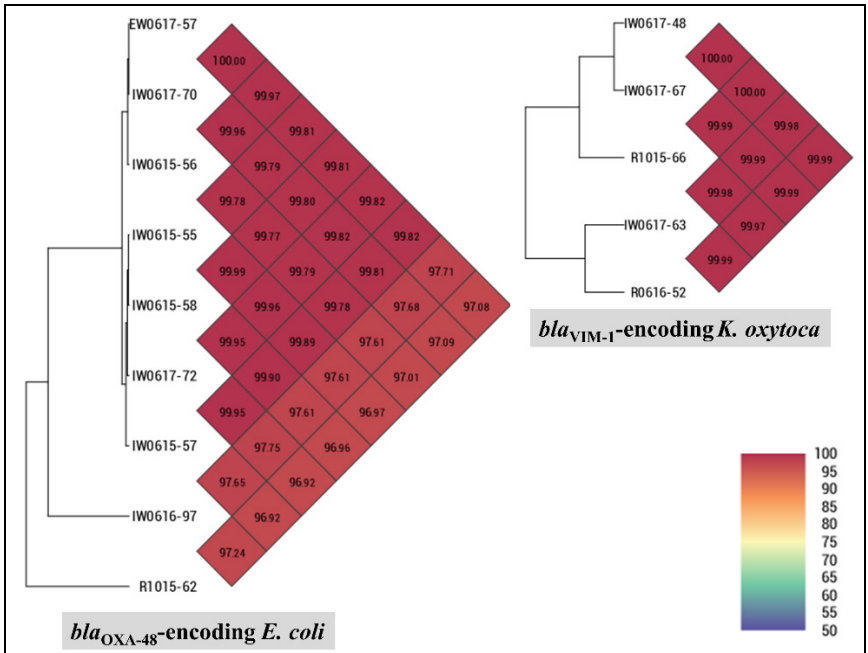

Figure 13. *Comparison of bla$_{OXA-48}$-encoding E. coli and bla$_{VIM-1}$-encoding K. oxytoca genomes recovered from wastewater and river water samples during 2015-2017 generated with OrthoANI values calculated from OAT (Orthologous Average Nucleotide Identity Tool) software.*

The *bla*$_{OXA-48}$ was the most commonly detected carbapenemase gene in CPE (9 *E. coli*, 3 *R. ornitholytica*), followed by *bla*$_{VIM-1}$ (5 *K. oxytoca*). High genetic similarity between isolates recovered from different sampling sessions was observed (Figure 13). For instance, the *bla*$_{OXA-48}$-encoding *E. coli* isolates EW0617-57 (isolated in 2017) and IW0615-56 (isolated in 2015) showed a 99.97% genetic similarity (Figure 13). Similarly, IW0615-56 and IW0617-70 exhibited 99.96% similarity. For *bla*$_{VIM-1}$-encoding *K. oxytoca*, the isolate recovered in 2015 (R1015-66) showed 100% genomic similarity with IW0617-48 isolate recovered in 2017 (Figure 13). Similarly, R0616-

52 isolate recovered in 2016 showed 99.99% similarity with IW0617-63, IW0617-67 and IW0617-48 isolated in 2017.

The OXA-48-producing *E. coli* were the most prevalent causative agents of CPE infections in Sweden since 2012 (Folkhälsomyndigheten, 2018), similar to the findings of this study where the majority of isolates were OXA-48-producing *E. coli*. Only 19 CPE were identified during the three years and four sampling sessions, indicating a low prevalence in the environment, especially in the downstream river. However, the detection of genetically identical isolates during different sampling sessions is still an interesting observation. This implies two possibilities; either these isolates are successfully adapted to these niches and are persistently present, or these isolates are continually released into the WWTP via community and hospital wastewater. Waterborne bacteria such as *K. oxytoca* are better adapted to survive the conditions often found in natural environments (Ayrapetyan et al., 2015). Likewise, environmental stability is often linked to the formation of biofilms as biofilm-forming species are more likely to persist (Sib et al., 2019). The *K. oxytoca* and *R. ornitholytica* isolates demonstrated strong biofilm production that may play a role in their persistence in these environments.

Conclusion: Carbapenemase-producing *E. coli* ST38 and *K. oxytoca* ST172 were persistent in Swedish wastewater and associated river water. The results of this study reiterate the need to employ advanced wastewater treatment methods (ultraviolet (UV) rays and ozone treatments) that have previously shown to reduce the levels of ARGs and potentially pathogenic bacteria into the environment (Jäger et al., 2018).

Paper II

Background: Wastewater from hospitals often release MDR human pathogens, which increase the risk of dissemination of CPE into the environment (Cahill et al., 2019; Paulus et al., 2019). The situation of antibiotic resistance in Sweden is satisfactory, however, the reports of CPE infections are increasing and in the majority of cases, patients have contracted CPE from carbapenemase endemic countries (Folkhälsomyndigheten, 2018). Very little is known about the situation of CPE in Swedish natural environments and with increasing reports of CPE infections, there is a concern that CPE are present in the environment.

Aims: This study aimed to investigate the genetic relationship of carbapenemase-producing *Klebsiella* spp. isolated from Swedish environments and patients in a local hospital.

Methodology: The wastewater samples were collected from influent (IW) and effluent (EW) wastewater from WWTP in Örebro City. The surface water was collected from the upstream river (UR), downstream river (RR) receiving effluent wastewater from WWTP, and downstream lake (RL) in 2015 and CPE were isolated (Figure 11). The genotypic and phenotypic comparison of environmental CPE to a pre-existing CPE collection at Örebro University Hospital was performed. All hospital isolates were *Klebsiella* spp.; *K. pneumoniae* (n=4) and *K. oxytoca* (n=2) and were collected from patients during the years 2008 to 2015. Susceptibility of isolates to 12 antimicrobials belonging to six different classes was tested using Etest. Both environmental and hospital CPE were sequenced, and phylogenies were constructed using core-genome SNPs (Parsnp) and cgMLST (SeqSphere+). Antibiotic resistance genes were detected using oligonucleotide microarray-based assay and confirmed by annotating ARGs from genomes using Resfinder. To further compare related species, mass spectra of the bacterial proteins of 2000–20,000 mass-to-charge ratio (m/z) were analyzed using BioNumerics version 7.5 (Applied Maths NV).

Results and discussion: From a total of 69 putative carbapenem-resistant Gram-negative bacilli recovered from the environmental samples, 2 isolates were identified as carbapenemase-producing *K. oxytoca*, one each from downstream in the Svartån river and incoming wastewater at the WWTP.

Surprisingly, no *K. pneumoniae* were recovered from environmental samples. All *Klebsiella* isolates recovered from environmental and clinical sources were MDR. Based on the genotypic and phenotypic analysis, *K. oxytoca* from the river (E1) was similar to two *K. oxytoca* strains from the hospital (H4, H6) and all of them harbored $bla_{\text{VIM-1}}$ carbapenemase gene (Figure 14). To best of our knowledge, this study was first to report CPE in the natural aquatic environments of Sweden.

The whole-genome comparative analysis provided strong evidence of the epidemiological linkage between hospital and environmental CPE. To best of our knowledge, this was the first study to report the presence of CPE in the Swedish aquatic environment, albeit at low levels, regardless of the stringent regulations for antibiotic use. Although the study is based on the comparison of a small set of isolates, the presence of highly related CPE in both hospital and directionally associated river is an important observation. The hospital CPE was most probably acquired from abroad as 88% of patients with CPE infections were reported to be infected abroad (Folkhälsomyndigheten, 2018), suggesting that alternative strategies must be employed to avoid the further environmental contamination. The results support the possibility of transmission of CPE from hospital to aquatic environment since the untreated wastewater from the hospital is transported through the drainage system into the WWTP. Although WWTP plays a key role to minimize the organic pollution from the wastewater, it can be the ultimate route of dissemination for CPE into natural environments.

Conclusions: Overall, these observations indicate that $bla_{\text{VIM-1}}$ producing *K. oxytoca* might be widespread in the hospital and further in the environment and community. Although antibiotic use in Sweden is limited, CPE are present in the Swedish aquatic environment at low but emerging levels.

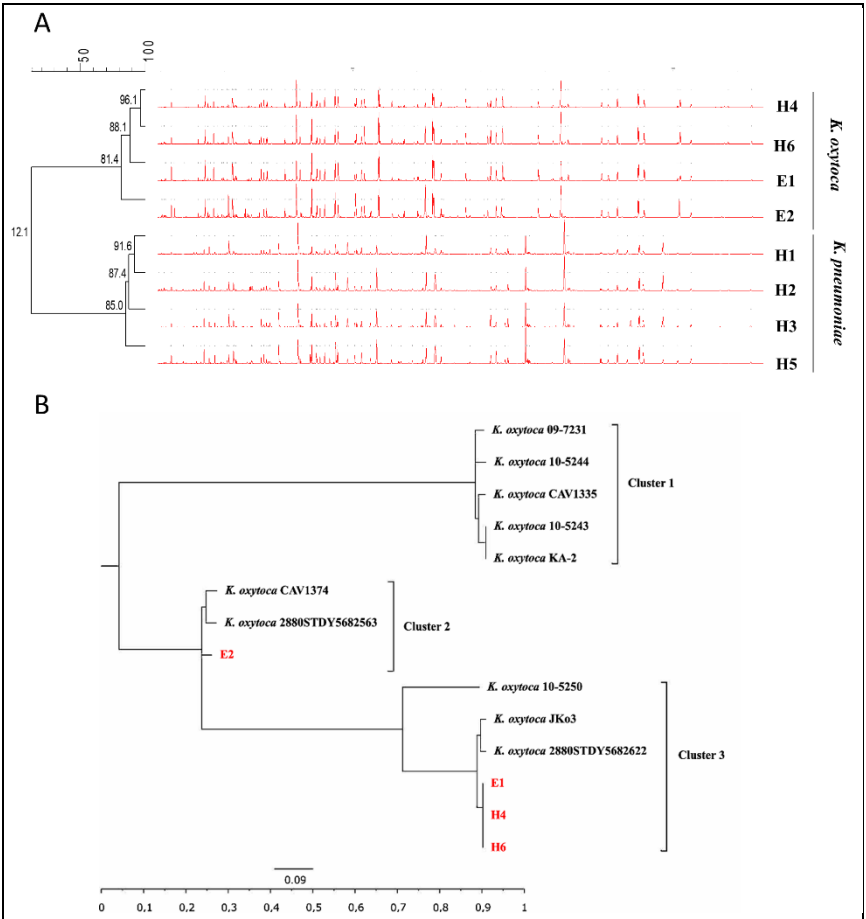

Figure 14. *Comparative analyses of bla*$_{VIM}$*-encoding K. oxytoca isolated from Swedish environments and patients in nearby hospitals. Environmental and hospital isolates are tagged as "E" and "H". A) Phyloproteomic dendrogram of MALDI-TOF mass spectra of K. pneumoniae and K. oxytoca isolated from Swedish environments and patients from Hospital. B) Phylogenetic relationship of the K. oxytoca isolates from this study compared to geographically unrelated strains. Phylogenetic maximum-likelihood tree constructed in FigTree based on core genome SNP analysis by using Parsnp. The isolates from the present study are highlighted in red. Scale bar represents genetic distances based on nucleotide substitutions per SNP site.*

Paper III

Background: Multidrug-resistant human pathogens and ARGs are considered as emerging environmental contaminants. There are various molecular methods to detect ARGs in the environment, including, next-generation sequencing and PCR based methods, as well as metagenomics. Both culture-dependent and independent methods combined with molecular techniques are commonly used for the detection of ARGs (Allen et al., 2010; Luby et al., 2016). Although culture-independent methods, especially metagenomics have revealed the microbial diversity and resistome of the natural environments (Bengtsson-Palme et al., 2014; Bengtsson-Palme et al., 2016), a major caveat of using these techniques is detecting very low levels of these genes, especially in countries with low antibiotic resistance prevalence. However, it is arguable if these methods are sensitive enough to measure the ARGs abundance at levels that may represent a risk for environmental and human health (Couto et al., 2018; Fortunato et al., 2018; Ni et al., 2013). The detection of ARGs using these may result in false-negative results for samples from low-prevalence areas for antimicrobial resistance, such as the aquatic environments of Nordic countries (Sundsfjord et al., 2004).

Aims: This study aimed to detect low levels of clinically relevant ARGs in Swedish aquatic environments using qPCR, and to compare their diversity after selective enrichment of Gram-negative bacteria on agar media. It was hypothesized that the detection of ARGs will improve after selective enrichment of clinically important group of bacteria.

Methodology: Surface water was collected from the river, both upstream (UR) and downstream (RR) of WWTP, and further downstream in the lake (RL) (Figure 11). The water was filtered through a 0.45 µm cellulose filter and placed on Gram-negative selective agar (Chromocult agar) to enrich the bacterial population. The agar plates incubated overnight at 37°C and DNA from the mixed population of culturable Gram-negative bacteria growing on Chromocult agar was isolated to detect an array of ARGs conferring resistance to several classes of antibiotics including beta-lactams, fluoroquinolones, tetracyclines, and aminoglycosides as well as MDR efflux pumps genes using qPCR. For comparison, ARGs were also detected from DNA extracted directly from surface waters without enrichment on selective media.

Results and discussion: From a total of 86 ARGs, 37 different ARGs were detected in all enriched samples, whereas only 23 were detected in all non-enriched samples. The highest numbers were detected in RR (38 ARGs), followed by the RL (23 ARGs) and UR (19 ARGs). In upstream river water, only 4 ARGs were detected in the non-enriched samples, while 18 ARGs were detected after enrichment (Figure 15. In the recipient river water, 33 ARGs were detected in the enriched samples compared to 23 ARGs in non-enriched samples. Interestingly, the enrichment of Gram-negative bacteria has significantly improved the detection from 1 ARG in non-enriched samples to 22 in enriched samples (Figure 15). The ESBL gene $bla_{\text{CTX-M-15}}$ and carbapenemase genes $bla_{\text{OXA-24}}$, $\text{bla}_{\text{OXA-50}}$ and $bla_{\text{OXA-51}}$ were exclusively detected in enriched samples.

Improved detection of ARGs in enriched samples indicates that the majority of the ARGs are present in the environment at levels below the detection limit of the culture-independent method. Some ARGs detected were unique in the non-enriched samples, such as *tetA* in the UR site and $bla_{\text{SFC-1}}$, $bla_{\text{SFO-1}}$, $bla_{\text{SHV(156G)}}$, $bla_{\text{IMP-5}}$ and *mefA* in RR site. The bacteria carrying these genes were likely unable to grow on the selective growth media for Gram-negative bacteria, or they were viable but non-culturable (Lange et al., 2013; Ramamurthy et al., 2014).

Conclusions: Selective enrichment for Gram-negative bacteria improved the detection of clinically relevant ARGs such as in the environment and culture-independent methods are less likely to detect ARGs of low abundance in less contaminated environmental samples. This work highlights the importance of culture-based detection of ARGs in environments with low-prevalence of antibiotic resistance.

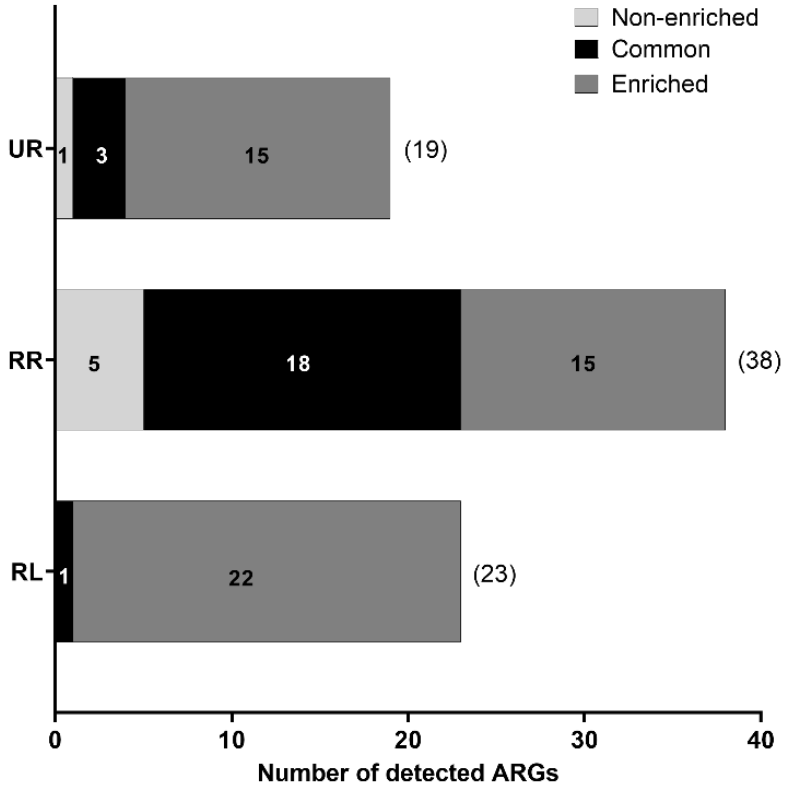

Figure 15. *Comparison of the total number of antibiotic resistance genes (ARGs) detected in the river waters directly (non-enriched) and after growth on Chromocult agar (enriched). The number of ARGs detected from enriched and non-enriched samples from the upstream river (UR), the recipient river (RR) near the effluent discharge from the WWTP, and the downstream lake water (RL). The number at the end of each bar represents the total number of ARGs detected in both enriched and non-enriched samples.*

Paper IV

Background: Human and animal gut microbiota are the major reservoirs of antibiotic-resistant Gram-negative bacteria, especially after exposure to antibiotics. Most ARGs are mobilized onto MGE, such as plasmids, transposons, and integrons that can be transferred between different bacterial species through HGT (Rizzo et al., 2013). As a result of the excessive use and misuse of antibiotics for human healthcare and agricultural purposes, ARB and ARGs have become ubiquitous in natural environments (Baquero et al., 2008). However, the emergence and primary sources of ARGs in natural environments of countries with strict regulations for antibiotics usage are not fully explored.

Aims: The present study aimed to evaluate the repertoire of ARGs including carbapenemase genes of culturable Gram-negative bacteria from directionally connected sites from the hospital to the WWTP, and downstream aquatic environments in central Sweden. It was hypothesized that diverse clinically relevant ARGs are already present in the environment but are not detected due to their low-level presence.

Methodology: The samples were collected from hospital wastewater (H), influent (IW) and effluent (EW) wastewater from WWTP, surface water from upstream (UR) and downstream (RR) river, and further downstream in the lake (RL) (Figure 11). By using qPCR, the presence of 84 ARGs was investigated in the genomic DNA isolated from a mixed population of coliform and Gram-negative bacteria selectively cultured on Chromocult agar. Non-metric Multidimensional Scaling (NMDS) was applied to ARGs presence-absence data in the PAST software package (Version 3.14). The co-occurrence of ARGs was visualized in Gephi using a pairwise Spearman's correlation matrix.

Results and discussion: A total of 55 ARGs were detected in all sites, and 36 were commonly present in all three environments (hospital wastewater, WWTP wastewater, and downstream aquatic environments) including $bla_{\text{OXA-50}}$, $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$ carbapenemase genes. The highest number of ARGs were detected in hospital wastewater (n=47) including ten carbapenemase genes (Figure 16). Although, wastewater treatment has reduced the number of ARGs from raw sewage, the river that receives treated

wastewater contained a high number of ARGs (n=43), similar to the untreated wastewater (n=44) (Figure 16). Several carbapenemase genes such as $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2}}$, and $bla_{\text{OXA-23}}$ were unique to the hospital wastewater and were not detected in any downstream environment. Moreover, β-lactamase genes such as $bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-8}}$, and $bla_{\text{SFC-1}}$, $bla_{\text{VIM-1}}$, and $bla_{\text{VIM-13}}$ were detected in downstream river water but not in the WWTP, suggesting the role sources other than WWTP in the environmental spread of ARGs.

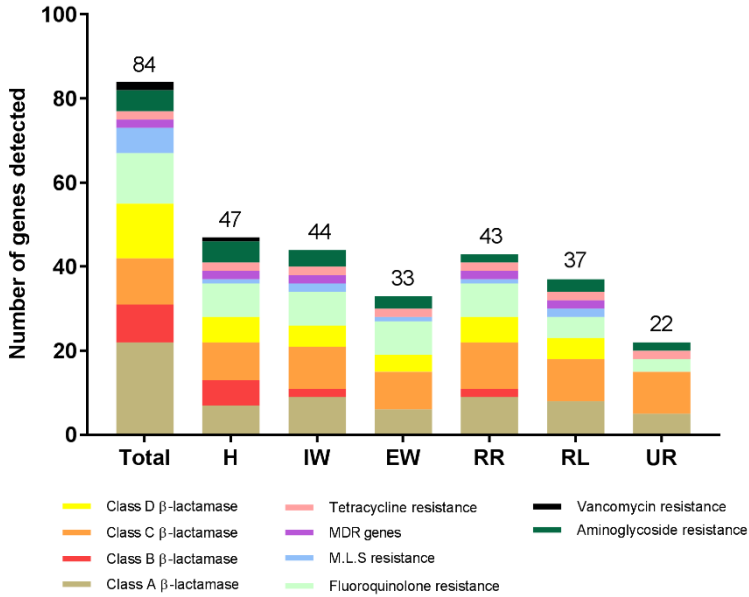

Figure 16. *The number of antibiotic resistance genes (ARGs) detected from wastewaters and environmental waters in Örebro Sweden. Wastewater samples were taken from hospital effluent (H), incoming wastewater to WWTP (IW), and effluent wastewater (EW). Environmental water samples were taken from Svartån River upstream of Örebro city (UR), Svartån River downstream of WWTP (RR) and Hjälmaren Lake (RL). The number at the top of each bar indicates the total number of detected ARGs in a particular sample. MDR indicates multi-drug resistance efflux pump genes. M.L.S indicates genes conferring resistance to either of the macrolide, lincosamide, and streptogramin B antibiotics.*

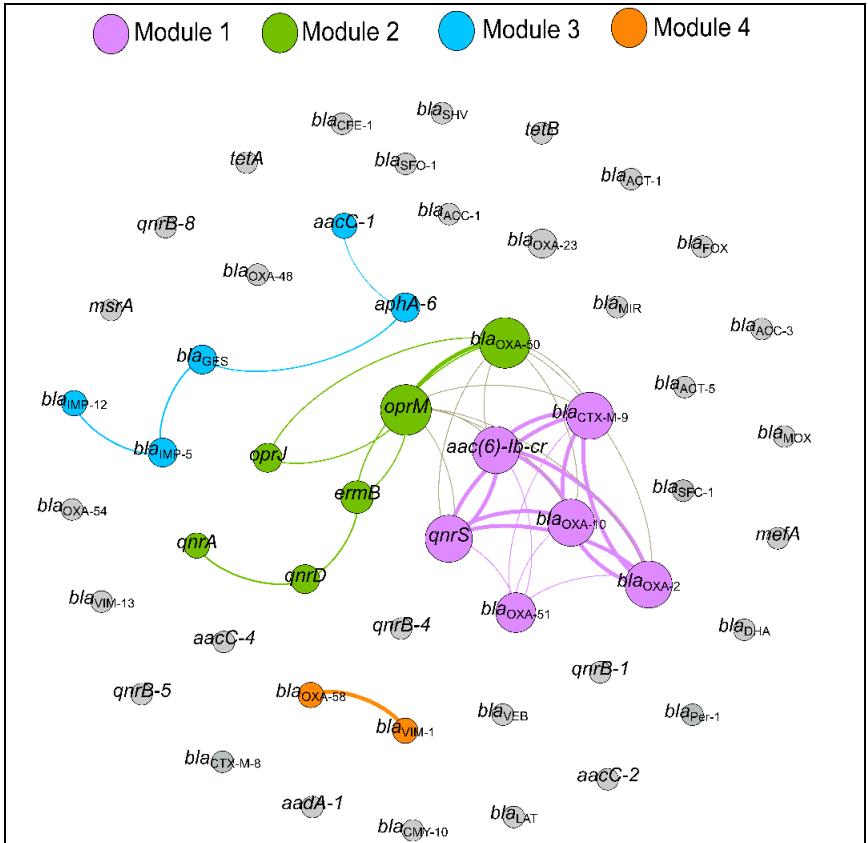

Figure 17. *Co-occurrence network of ARGs detected in wastewater and environmental water samples in Örebro, Sweden. The co-occurrence network is based on a correlation matrix among detected ARGs using Spearman's rank correlation. Only correlations with a high correlation coefficient (p > 0.8) and low P-value (P-value 0.01) are shown. The node (circle) size represents the number of connections with other ARGs. The thickness of the edge (line) is proportional to the Spearman's correlation coefficient (p) of the connection. The co-occurrence network is further divided into subnetworks (modules) based on modularity class (modularity index = 0.477). Genes in gray color do not show a significant correlation.*

The *bla*$_{\text{VIM-1}}$ gene was detected both in hospital wastewater and in the downstream river water, and not detected in the WWTP. Similar observations were reported in **paper II**, where genetically identical *bla*$_{\text{VIM-1}}$-encoding

K. oxytoca were recovered from patients in the hospital and downstream river water. The NMDS analysis divided the sites into five different clusters, however, samples from effluent wastewater (EW) and recipient river (RR) were clustered together. The co-occurrence network revealed that the clusters of ARGs conferring resistance to different classes of antibiotics co-exist in a particular environment. This suggests that multiple ARGs will be co-selected under one compatible selective pressure (Figure 17). It is therefore critical to formulating strategies targeting these groups to stop the spread of ARGs in the natural environments.

Conclusion: Hospital wastewater contained a high diversity of ARGs, including the clinically relevant carbapenemase genes, however, the low-level presence of these ARGs in aquatic environments of a low-endemic country is a concern. The study supports the proposal that wastewater effluent from WWTP contributes to the spread of antibiotic resistance in aquatic environments, however, there are other sources of dissemination. Therefore, the elimination of ARB and ARGs from the hospital and household wastewaters is important to maintain the low levels of resistance in natural aquatic environments.

Paper V

Background: It is well established that high concentrations of antibiotics can select and enrich antibiotic-resistant bacteria. It has been demonstrated that even the low levels of antibiotics can select resistant bacteria and drive the resistance evolution through mutation and horizontal gene transfer (Andersson & Hughes, 2012). Antibiotics used in human medicine (at the hospital or community) are generally present in high concentrations relative to wastewater environments, such as hospital wastewater with a strong antibiotic selection pressure. Once released into the aquatic environment through WWTP, antibiotic concentrations drop substantially due to dilution and exert a weaker selection pressure in these environments. Thus aquatic environments with a weaker selection pressure might be a nesting place for antibiotic resistance evolution, as bacteria have time to develop *de novo* mutations or acquire resistance by HGT (Hiltunen et al., 2017). In addition to antibiotics, other selective pressure such as heavy metals and household antimicrobials and disinfectants may also play a role.

Aim: This study aimed to investigate the development of resistance to β-lactam antibiotics in *K. oxytoca* after long-term exposure to river water contaminated by WWTP. We also aimed to study the effect of environmental temperature on the emergence of resistance.

Methodology: To study the emergence of resistance after long-term exposure of bacteria to river water impacted by wastewater, 8 independent lineages of *K. oxytoca* E1 (four lineages each in filter-sterilized river water (R) or tap water (T)) were grown for 600 generations (Figure 18). To avoid stress response due to starvation, 1% tryptone was added to both river and tap water as a carbon source. The lineages were started from independent overnight cultures in sterile 13 mL tubes with a ventilation cap. The cultures were incubated at 17°C; an approximate surface water temperature of Swedish aquatic environments during the summer. The cultures were serially passaged for 600 generations evolved lineages were preserved in -80°C after every 100 generations. (See Methods section in **Paper V** for detailed information). After 600 generations, randomly picked clone from each lineage was isolated and analyzed for their susceptibility to carbapenem (meropenem, imipenem) and cephalosporin (cefepime, ceftazidime, cefoxitin, cefotaxime) antibiotics relative to the ancestral strain. Doubling times (T_d)

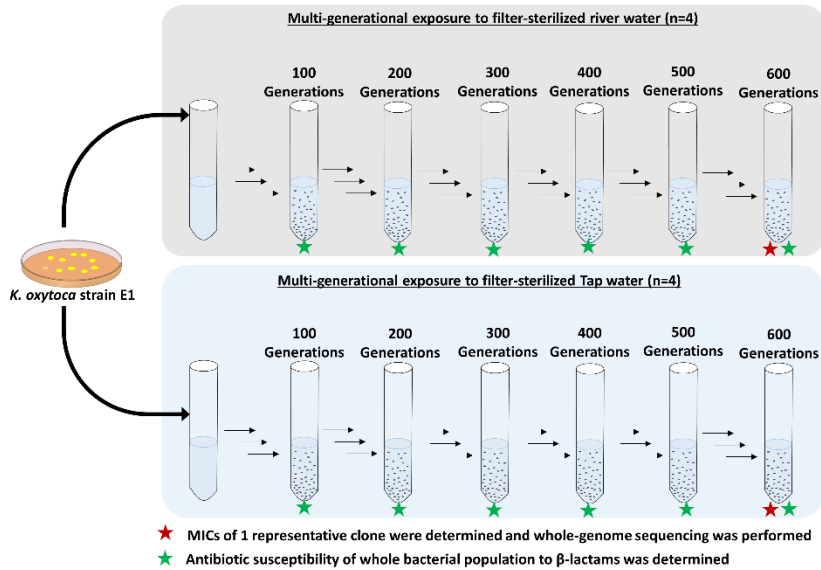

Figure 18. Experimental design to study changes in antibiotic resistance in Klebsiella oxytoca strain E1 after long-term exposure to river or tap water. *The K. oxytoca strain E1 was exposed to filter-sterilized river water (top half, grey-shaded) and tap water (bottom half, blue-shaded) at 17°C. Every 10th generation was serially transferred to a new tube containing fresh media. Antibiotic susceptibility of the whole bacterial population to β-lactams was tested after every 100 generations using the disk diffusion method (★). After 600 generations, a single clone was isolated from each replicate and MIC were determined for various β-lactam antibiotics (★). The same experimental design was used for long-term exposure to river water at 25°C, however, only susceptibility testing of whole populations was performed and compared with the cultures grown at 17°C.*

of the clones were also determined to assess the fitness cost. The clones were sequenced and mutations in evolved clones were detected with variant calling by following GATK best practices.

To study the effect of temperature on the emergence of resistance, a separate set of 8 lineages (four lineages each in river water and tap water) were grown at 25°C (Figure 18). The cultures were serially passaged for 600 generations and evolved lineages were preserved in -80°C after every 100 generations. The antibiotic susceptibility of the evolved populations (liquid culture) was compared to the populations evolved at 17°C.

Results and discussion: Among the clones identified in tap water, T4 showed the highest increase to tested antibiotics with >10-fold increase in MIC for ceftazidime while no substantial increase was observed for any of the other three clones relative to ancestral strain (Table 1). In contrast, 3 of the clones (R1, R2, and R4) grown in river water, showed a greatly increased MIC for most antibiotics relative to ancestral strain. For meropenem, the highest increase (8-fold) in MIC was observed in clone R1, followed by R2 with a 4-fold increase (Table 1). Of all the clones, R1 exhibited the highest increase in resistance to all tested antibiotics except cefotaxime (Table 1). A total of 30 mutations mapped to 14 genes were observed in clones evolved in river water, while 6 genes in tap water clones were found to have mutations. In general, clones with a higher number of mutated genes showed highly increased MICs for βlactam antibiotics.

The clone R1, that exhibited the largest increase in MIC for most antibiotics tested, carried a unique mutation at codon Leu84 (duplication of nucleotides 243-249) in the stationary phase sigma factor RpoS resulting in a frameshift that is predicted to inactivate RpoS. Studies have shown that *E. coli* with defective sigma factor S (rpoS::Tn10 mutation) exhibited reduced synthesis of OmpF (OmpK35) and OmpC (OmpK36), major porins responsible for the uptake of β-lactams inside the cell (Darcan, 2012; Knopp & Andersson, 2015; Tsai et al., 2011). The clone R1 also carried a mutated *rfbD* gene encoding an enzyme involved in the biosynthesis of a key component of Lipopolysaccharide (LPS). The resulting heterogeneity of LPS can lead to the failure of OmpK36 insertion into the outer membrane (Ried et al., 1990). The clone R1 had a significantly reduced growth rate (3-fold increase in doubling time). Mutation-derived resistance is often associated with a fitness cost which was observed for this clone with an increased doubling time (Adams-Sapper et al., 2018; Vogwill & MacLean, 2015).

Table 1. *Fold change in minimum inhibitory concentration of six antibiotics determined by Etest, relative to the ancestral strain, for K. oxytoca clones selected after growth in river water or tap water for 600 generations, and the identity of genes carrying non-synonymous mutations.*

Clones	Fold change in MIC						Non-synonymous mutations
	MEM	IMP	FOX	CAZ	FEP	CTX	
Clones evolved in tap water							
T1	1.5	1.3	2.7	>10	2	>2	*livK*, MEP1, MEP2
T2	1.5	1.3	1.3	2	1.3	0.75	*livK*, MEP1, MEP2
T3	1.5	1.3	2	2.6	1.3	1.5	*livK*, MEP1, MEP2
T4	1	1.3	1.3	1.3	1	1	*livK*, *rpoS*[1], MEP1, MEP2
Clones evolved in river water							
R1	8	>10	>10	>10	>10	>2	*rfbD*, *rpoS*[2], *livK*, MEP1, MEP2
R2	4	5.3	8	>10	4	>2	*livK*, *gst*, *lsp*, *czcD*, phage-tail monomer, hypothetical protein 1
R3	1	1	1	1	1	1	*livK*, *rpoS*[1], *ycgE*, MEP1, hypothetical protein 2
R4	3	4	2.7	>10	2	>2	*livK*, *rpoS*[1], *sbmA*, *cpxR*, MEP1, MEP2

livK (Leucine-specific ABC transporter), MEP (Mobile Element Protein), *rpoS* (Sigma factor S), *rfbD* (UDP-galactopyranose mutase), *gst* (Glycosyltransferase), *lsp* (Lipoprotein signal peptidase), *czcD* (cobalt-zinc-cadmium resistance protein), *ycgE* (Putative HTH-type transcriptional regulator), *sbmA* (Inner membrane transport protein), *cpxR* (Copper-sensing two-component system response regulator)

MEM meropenem; IMP imipenem; FOX cefoxitin; CAZ ceftazidime; FEP cefepime; CTX cefotaxime

[1]Stop-gain mutation, [2]Frameshift mutation

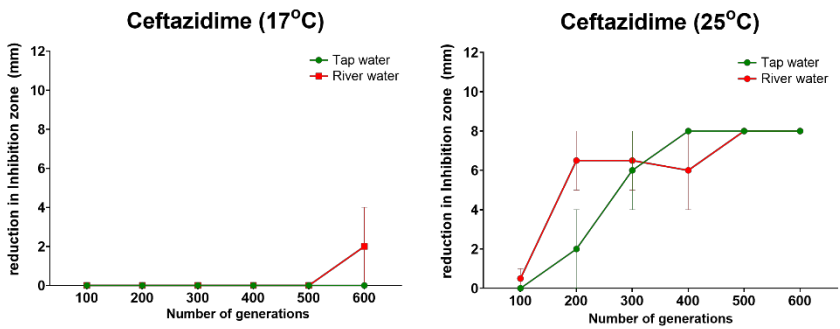

***Figure 19**. The relative change in inhibition zone diameters for ceftazidime of K. oxytoca population evolved after 600 generations of growth in river water or tap water at 17°C and 25°C. After every 100 generations, antibiotic susceptibility testing of evolved cultures was performed using disc-diffusion. The error bars represent the standard error of the mean of 4 clones.*

Results showed an acceleration in the emergence of resistance to certain antibiotics at a higher temperature. The resistance to ceftazidime evolved as early as 200 generations in river water at 25°C, while at 17°C, resistance evolved at 600 generations (Figure 19). Interestingly, no resistance evolved at 17°C after 600 generations in tap water, whereas all 4 lineages evolve an 8-fold increase in resistance by 400 generations. A similar trend was observed for carbapenem antibiotics. These findings suggest that current forecasts of the burden of antibiotic resistance could be a significant underestimate due to increasing climate temperatures (O'Neill, 2016).

Conclusion: Exposure to anthropogenic pollution and increased environmental temperature may induce similar changes in the environmental microbiome, generating novel resistant variants at accelerated rates that may pose a significant threat to human health.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The results in this thesis show that resistance determinants to carbapenems and bacteria that carry these determinants were persistent in Swedish wastewater and natural environments, contrary to the perception of low-endemic countries. This reiterates the opinion that resistance to these "last-resort" antibiotics is becoming common in the natural environments (Mills & Lee, 2019), even in countries with low-level of antibiotic resistance. Hospital wastewater harbored the majority of the tested ARGs including carbapenemases that were not reported in patients in the local hospital. There is a possibility that these persistent resistance determinants are common in the community. Thus, effective treatment of wastewater (both hospital and community wastewaters) must be ensured to reduce or eliminate the spread to natural environments. Before discharging into the environment, the wastewaters must be treated with advanced techniques such as UV and ozone treatments that have proven to significantly reduce the spread of ARB and ARGs (Jäger et al., 2018). Sources other than wastewaters may also contribute to the spread of CPE in the environment (Bouaziz et al., 2017; Ryu et al., 2017). To better understand the dissemination of CPE in the environments, including other sources such as international travelers and migratory birds in the studies, will provide a better overview.

The increased resistance to some β-lactams in *K. oxytoca* developed in conditions similar to the human-impacted aquatic environments. Surprisingly, resistance also emerged when grown in tap water for the long-term, however, not as much as in the river water that may contain anthropogenic contaminants. It is a proof of principle that resistance can develop in bacteria grown in the cleanest of environments (such as drinking water). With the signs of global warming already visible, it is alarming that increased temperature accelerated the emergence of resistance to some antibiotics.

The clone that exhibited increased resistance to most antibiotics tested had unique mutations. It will be interesting to further explore the mechanisms of resistance in the evolved clones. An interesting mutation was observed in *czcD,* which regulates the genes responsible for cadmium, zinc, and cobalt efflux-based resistance. A mutated *czcD* has been associated with the accumulation of Zn^{2+} inside the bacterial cell (Martin & Giedroc, 2016). This accumulated Zn^2 may play an important role in the resistance to β-lactams, as Zn^2 is required for carbapenemase activity of MBL such as bla_{VIM-1} (carried by ancestral and evolved clones). It will be interesting to study

the role *czcD* on the activity of MBLs. Studies on these novel mechanisms of resistance in bacteria may result in finding novel targets proteins for antibiotic therapy.

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