The Role of the Aquatic Environment in the Spread of Antimicrobial Resistant Bacteria of Public Health Significance

A Thesis Presented to the National University of Ireland, Galway
for the Degree of Doctor of Philosophy

By

Bláthnaid Mahon

Discipline of Bacteriology,
School of Medicine,
National University of Ireland Galway.

Supervisors: Professor Dearbháile Morris, Professor Martin Cormican
Head of Department: Professor Dearbháile Morris

November 2019

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Amino	Aminoglycoside
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
ARO	Antimicrobial resistant organism
ATCC	American Type Culture Collection
AV	Avibactam
BIGSdb	Bacterial Isolate Genome Sequence Database
CA	Clavulanic acid
Cb	Carbapenem
CDC	Center for Disease Control and Prevention
CFU	Colony forming unit
CgMLST	Core gene multi-locus sequence typing
Cp	Cephalosporin
CPE	Carbapenemase-producing *Enterobacterales*
CRE	Carbapenem-resistant *Enterobacterales*
DDD	Defined daily dose
DNA	Deoxyribonucleic acid
E	Extended-spectrum cephalosporin
E. coli	*Escherichia coli*
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPA	Environmental Protection agency
ESBL	Extended-spectrum β-lactamase
ESBL-PE	Extended-spectrum β-lactamase-producing *Enterobacterales*
ESBL-PEc	Extended-spectrum β-lactamase-producing *E. coli*
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
Fluor	Fluoroquinolone

Fos	Fosfomycin
GAP	Global Action Plan
HGT	Horizontal gene transfer
HPRA	Health Products Regulatory Authority
IMP	IMP-type metallo-beta-lactamase
Inc	Incompatibility
IRUSE	Informatics Research Unit for Sustainable Engineering
IS	Insertion sequence
K. oxytoca	*Klebsiella oxytoca*
K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
LAMP	Loop mediated isothermal amplification
M	Monobactam
MALDI-TOF	Matrix-assisted laser desorption/ionisation time-of-flight
MBL	Metallo-β-lactamase
MGE	Mobile genetic element
MLS	Macrolide-lincosamide-streptograminB
MLST	Multi-locus sequence typing
MPF	Mating pair formation
NCPERLS	National Carbapenemase-Producing *Enterobacterales* Reference Laboratory Services
NCTC	National Collection of Type Culture
NDM	New Delhi metallo- β-lactamase
OIE	World Animal Health Organisation
ORF	Open reading frame
OXA	Oxacillinase
P	Penicillin
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PG	Phosphatidylglycerol
Phen	Phenicol
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction

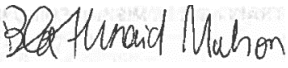
ST	Sequence type
Sul	Sulphonamide
SWWTP	Secondary wastewater treatment plant
Tet	Tetracycline
Tn	Transposon
Trim	Trimethoprim
UTI	Urinary tract infection
VIM	Verona integron-encoded metallo-β-lactamase
WGS	Whole-genome sequencing
WHO	World Health Organization
WWTP	Wastewater treatment plant

DECLARATION

This work is submitted to fulfil the requirements of the degree of Doctor of Philosophy at the National University of Ireland, Galway.

No part of this thesis has been previously submitted at this or any other university.

Apart from due acknowledgements, it is entirely my own work.

Signed:  Date: 26/01/2020

Bláthnaid Mahon

ACKNOWLEDGMENT

Firstly, I would like to express my sincere thanks to my supervisors Prof. Dearbháile Morris and Prof. Martin Cormican. I will be eternally grateful to you both for giving me this amazing opportunity. Dearbháile, you have always been there to offer guidance and support when I needed it. I really enjoyed our many trips together, both out West and further afield. Martin, thank you for all your great insights and guidance throughout the years.

I will be forever grateful to all the members of the Discipline of Bacteriology, both past and present. I would particularly like to thank Dr. Carina Brehony for being such a great friend during the past four years. Thanks for all the wisdom you imparted…I don't think I'd have made it through the bioinformatics without you! To Dr. Eoin McGillicuddy, James Killeen, Dr. Meera Tandan, Dr. Louise O'Connor, Niamh Cahill and Dr. Aoife Joyce (and everyone else). Thanks for making my time in the lab such an enjoyable experience and for always being there to offer a helping hand.

I am indebted to my Graduate Research Committee: Dr. Akke Vellinga, Dr. Florence Abram and Dr. Diarmuid O'Donovan for providing such great support, advice and lively discussion throughout my PhD.

To everyone in the Clinical Lab. Thank you for always making me feel welcome and for all your guidance and support during my PhD. I am particularly grateful to Elaine McGrath for all her amazing help and advice.

I am truly thankful to the Environmental Health Officers, and to those in Galway County Council, the Pasteur Institut and Oxford who provided assistance along the way (and anyone else that I might have forgotten to mention).

I would like to thank my family, in particular my parents, Madeleine and Gerard, who have always been there for me, and would drop everything in a heartbeat if they thought I needed something. To my sister Dearbhs, who has shared much of this journey with me...you can do it, you are over half way there! I would also like to thank my friends, especially Gally (I will miss our lunch time walks), Hazel and Caroline for being such good friends over the past four years, and always. Thank you.

DEDICATION

I dedicate this thesis to my parents, Madeleine and Gerard. Thank you for always being there.

ABSTRACT

Antimicrobial resistance (AMR) is one of the greatest threats to human health of the 21st Century, with certain antimicrobial resistant organisms classified as being a greater threat to public health than others. At the top of that list are carbapenemase-producing *Enterobacterales* (CPE) and extended-spectrum β-lactamase-producing *Enterobacterales* (ESBL-PE). It is well recognised that a 'One Health' approach is necessary when addressing the growing problem of AMR, as the health of humans, animals and the environment are interconnected. However, the environment remains the most under investigated of the three, and the role it may play in the emergence, persistence and dissemination of AMR is largely unknown. The aim of this thesis was therefore to add to current knowledge in this area, by assessing recreational waters and sewage for the presence of CPE and ESBL-PE and comparing the genomes of the isolates obtained to those originating from humans.

Two sites in the West of Ireland were examined in this study. Sampling points at the first site consisted of two beaches (seawater samples), a river and two streams (fresh water samples), a sewage collection system and a sewage outflow where sewage is released untreated into the ocean (at a point between the two beaches). Sampling points at the second site consisted of a beach (seawater samples) and a nearby secondary wastewater treatment plant (untreated influent and treated effluent sewage samples). Following collection, samples were examined for the presence of CPE and ESBL-PE. Preliminary characterisation was subsequently performed on the isolates obtained, which consisted of antimicrobial susceptibility testing and real-time PCR. The genomes of a subset of isolates were sequenced using an Illumina HiSeq platform and the genomes compared to collections of clinical CPE and ESBL-PE.

The same two strains of NDM-19-producing *Enterobacterales* (*Escherichia coli* ST167 and *Klebsiella pneumoniae* ST11) were detected repeatedly in recreational water samples taken at the first site. These findings represented the first identification of CPE in seawater in Europe. An untreated sewage discharge was identified as the probable source of CPE at this site. Findings indicated that three clinical NDM-19-producing *Enterobacterales* harboured $bla_{\text{NDM-19}}$ on a similar IncX3 plasmid, with two of those isolates (one *E. coli* and one *K. pneumoniae*) found to be closely related to the two environmental strains of NDM-19-producing *Enterobacterales*. ESBL-producing *E. coli* (ESBL-PEc) was commonly identified among seawater, fresh water and sewage samples at this site (in 31/53 of samples (58.5%)). Comparison of these genomes to a collection of clinical ESBL-PEc identified 12 of the same AMR-associated genes and five plasmid replicon types among ESBL-PEc originating from all four sample types (seawater, fresh water, sewage and clinical samples), with $bla_{\text{CTX-M-15,}}$ found to be the most common variant of $bla_{\text{CTX-M}}$ detected. ST131 was the most commonly identified sequence type among all sample types. OXA-48-like-producing *E. coli* ST131 and OXA-48-like-producing *K. pneumoniae* ST101 were detected in seawater samples collected at the second site. These isolates harboured $bla_{\text{OXA-48}}$ on similar mobile genetic elements to those identified in a clinical collection (pOXA-48 fragment in *E. coli* and IncL(pOXA-48) plasmid in *K. pneumoniae*). The source of the CPE at this site was not determined.

In conclusion, multiple similar strains of CPE and ESBL-PEc and/or their mobile genetic elements were identified among isolates originating from humans and the aquatic environment. The findings of this thesis highlight the need for the environment's role in the emergence, persistence and dissemination of antimicrobial resistant organisms to be further investigated.

CHAPTER 1

INTRODUCTION

1.1 Antimicrobials

Antimicrobials are natural or synthetic substances used to kill or inhibit the growth of bacteria (antibacterial), viruses (antivirals), or fungi (antifungals) (1). In this thesis the term antimicrobial will refer solely to antibacterial drugs, unless otherwise stated. Antimicrobials work by targeting and disrupting specific bacterial cell processes, which results in either cell death (bactericidal) or the inhibition of cell replication (bacteriostatic) (2). Any compound which exhibits toxicity towards microbial cells may be referred to as having antimicrobial activity (3).

1.1.1 A brief history

While antimicrobials have been used by humans in a variety of forms for 1000s of years (4), the most noteworthy breakthroughs relating to antimicrobials did not come until the early 20th century. In 1907, Paul Ehrlich's laboratory synthesised the drug Salvarsan or 'compound 606', which was subsequently used in the treatment of Syphilis (5). Undoubtedly, one of the most important milestones in modern medicine followed over a decade later, in 1928, when Alexander Fleming discovered that the mould *Penicillium notatum* prevented the growth of the Gram-positive organism *Staphlococci* (6). The antimicrobial penicillin was eventually purified from this mould in 1940, by Oxford's Howard Florey and Ernest Chain (5). Penicillin's release onto the market in 1943 coincided with World War II and it is credited with saving the lives of many soldiers during this time (6). In 1945, the Nobel Prize in Physiology or Medicine was jointly awarded to Fleming, Florey and Chain, for 'the discovery of penicillin and its curative effect in various infectious diseases' (7).

Encouraged by the success of this 'wonder drug', many research teams began identifying other forms of antimicrobial agents. This resulted in the 'golden age' of antimicrobial discovery between the 1950s and 1970s, with several new classes of antimicrobials identified (5). The availability of antimicrobials

changed human medicine in a profound way. Infections such as bacterial pneumonia and rheumatic fever that might once have proven fatal were now treatable (2). Medical interventions such as organ transplants, cancer chemotherapy and invasive surgeries such as joint replacements that leave patients vulnerable to infection were now possible (2).

1.1.2 Classification of antimicrobials

Antimicrobials can be classified based on a number of different schemes, the most common of which are based on their molecular structure and mechanism of action (8).

Classification based on molecular structure

Examples of classes of antimicrobials grouped together based on their molecular structure include aminoglycosides, β-lactams, quinolones and tetracyclines. Aminoglycosides are natural or semisynthetic antimicrobials that contain a common core structure consisting of 3-amino sugars connected by glycosidic bonds. Examples include streptomycin, neomycin, gentamicin, amikacin, and plazomicin (9). β-lactams are natural or semisynthetic antimicrobials that are grouped together due to the presence of a beta-lactam ring (4-membered 2-azetidinone ring) in their structure. Members include penicillins, cephalosporins, monobactams and carbapenems (Figure 1.1) (10). Quinolones are natural or synthetic antimicrobials that are structurally derived from the bicyclic compound quinoline and examples include nalidixic acid, norfloxacin, ciprofloxacin and levofloxacin (11). Tetracyclines are also natural or semisynthetic compounds and they share a core structure of four hydrocarbons. Examples include chlortetracycline, doxycycline, minocycline and tigecycline (which is a glycylcycline) (12).

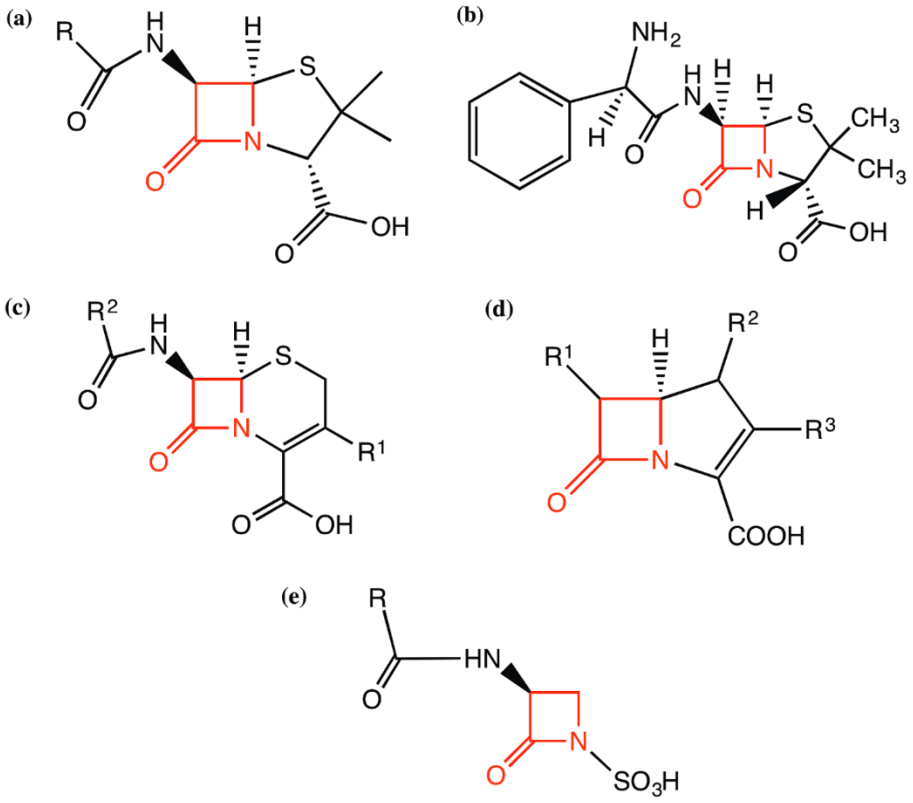


Figure 1.1 Chemical structures of β-lactam antimicrobials. *The β-lactam ring is highlighted in red in all the structures. (a) core structure of penicillins, (b) structure of ampicillin, a broad-spectrum antibiotic in the penicillin group of antimicrobials, (c) core structure of cephalosporins, (d) core structure of carbapenems and (e) core structure of monobactams* (13). *Copyright obtained from* (13) *via Copyright Clearance Centre, RightsLink® service.*

Classification based on mechanism of action

There are five main sites/processes of a bacterial cell that antimicrobials target: the cell wall, the cell membrane, nucleic acid synthesis (DNA and RNA), protein synthesis and folic acid metabolism (Figure 1.2). As these targets are either absent or structurally different in eukaryotic cells this ensures that antimicrobials are relatively non-toxic to humans and animals (2).

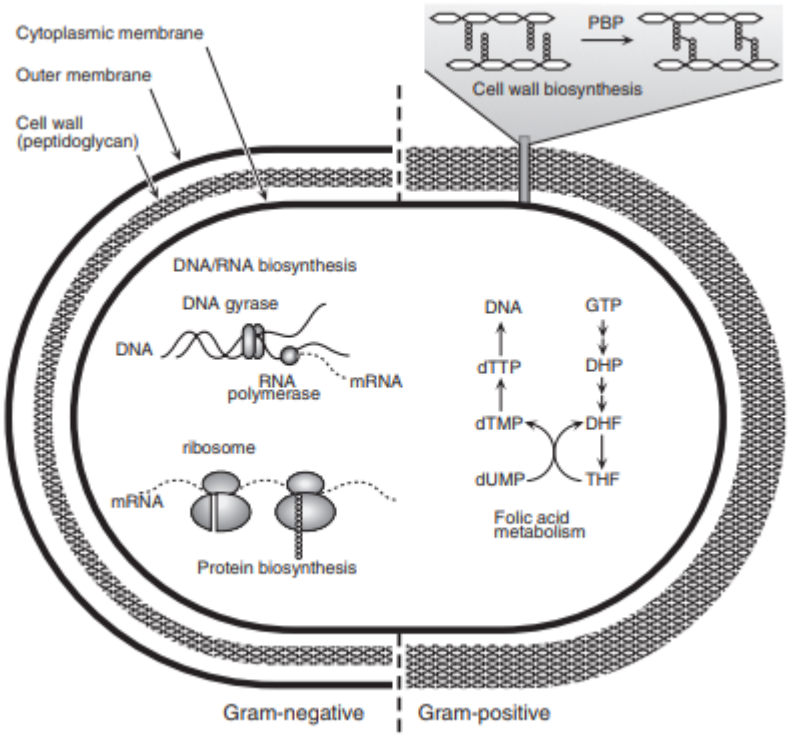


Figure 1.2 Major targets of antimicrobials. *PBP: penicillin binding protein, DHP: dihydropteroate, THF: tetrahydrofolate* (14)*. Copyright obtained from Taylor & Francis via Copyright Clearance Centre, RightsLink® service.*

Bacterial cell wall

Bacterial cells are encased in a cell wall which provides structural integrity and protects the bacterium against mechanical and osmotic stresses (15). Cell wall biosynthesis is a vital physiological process for bacterial survival. One of the main components of the cell wall is peptidoglycan, which is a cross-linked polymer matrix consisting of a peptide-linked β-(1-4)-*N*-acetyl hexosamine (16). The enzymes responsible for maintaining the peptidoglycan layer are known as transglycosylases and penicillin-binding proteins (PBPs). They maintain this vital layer by extending the glycan strands of existing peptidoglycan molecules with the addition of disaccharide pentapeptides and cross-linking adjacent peptide stands of peptidoglycan molecules (16). Antimicrobials including the β-lactams and the glycopeptides both target cell wall synthesis by inhibiting the cross-linking process. β-lactams are structurally similar to the PBP target site (D-Ala-D-Ala), resulting in the PBP covalently binding to the β-lactams instead of their intended target site,

hereby preventing the enzymes from carrying out their crosslinking duties (17). Glycopeptides act by binding to the acyl-D-Ala-D-Ala terminus of the growing peptidoglycan, inhibiting the PBP from binding to these subunits, again preventing cross-linking from taking place (18). These disruptions in cell wall synthesis result in cell lysis due to the turgor pressure from the cytoplasm (19).

Bacterial cell membrane

The cell membrane is composed of a phospholipid bilayer which acts as a permeability barrier that regulates molecules entering and leaving the cell. Phosphatidylglycerol (PG) makes up approximately 25% (this % varies according to species of bacteria) of the total lipid composition of the membrane and is essential for providing structural stability (8). Daptomycin is a lipopeptide, calcium-dependent antimicrobial which targets the cell membrane function of Gram-positive bacteria. It acts by interacting with PG, which disrupts the membrane function resulting in the leakage of ions, leading to cell death (20). While daptomycin is active against Gram-positive bacteria, polymyxin antimicrobials act against Gram-negative bacteria, through interacting with the lipid A domain of the lipopolysaccharide in the outer membrane of these cells (21). This interaction is thought to induce phospholipid exchange, leading to an imbalance in osmotic pressure, resulting in cell death (21).

Nucleic acid synthesis

Certain classes of antimicrobials act by disrupting the metabolic pathways responsible for the synthesis of the nucleic acids (DNA and RNA), which are essential processes for cell survival and replication. Quinolone antimicrobials are DNA synthesis inhibitors. These antimicrobials target DNA gyrase and topoisomerase IV, introducing a conformational change in the enzymes, resulting in the inhibition of DNA replication (22). Rifampicin is a very potent, broad spectrum antimicrobial which inhibits RNA transcription by binding to the DNA-dependent RNA polymerase (16) (23).

Protein synthesis

Translation of mRNA into proteins occurs through three steps: initiation, elongation and termination. This process involves ribosomes and multiple cytoplasmic accessory factors (16). Antimicrobials inhibit protein synthesis by targeting either the 30S or 50S ribonucleoprotein subunits of the bacterial ribosome (24). Aminoglycoside and tetracycline antimicrobials are 30S inhibitors. Aminoglycosides work by binding to the 16S component of the 30S subunit, which can promote tRNA misreading and lead to protein mistranslation, while tetracyclines act by obstructing aminoacyl-tRNAs from accessing the ribosome (16). 50S inhibitors generally work by either blocking the initiation step or by translocating the peptidyl-tRNAs, thereby inhibiting the elongation step of protein translation. Examples include macrolides and phenicols (16).

Folic acid metabolism

Folic acid is essential for the metabolism of both nucleic acids and amino acids. Antimicrobials including the sulphonamides and trimethoprim act to disrupt folic acid metabolism (14). When used in combination, these antimicrobials are bactericidal, as each class effects a separate step of folic acid metabolism, with the sulphonamide acting as a dihydropteroate synthase inhibitor and trimethoprim as a dihydrofolate reductase inhibitor (14).

1.2 Antimicrobial Resistance (AMR)

The majority of antimicrobials are naturally produced by microorganisms (e.g. environmental fungi) or are semisynthetic derivatives of them, with very few antimicrobials, such as the fluoroquinolones, being completely synthetic (25). Bacteria have therefore been exposed to these molecules in the natural environment for millions of years. Due to their impressive genetic plasticity, bacteria have continually evolved to withstand the harmful effects of this

exposure (26). As a result, the genetic instructions by which to resist these molecules existed within the bacterial population long before antimicrobials were discovered by humans (27). Resistance to penicillin was first observed in 1940, before it had even been brought to market (28). A similar pattern of emerging resistance was seen following the introduction of each new antimicrobial/modified antimicrobial onto the market, as illustrated in Figure 1.3.

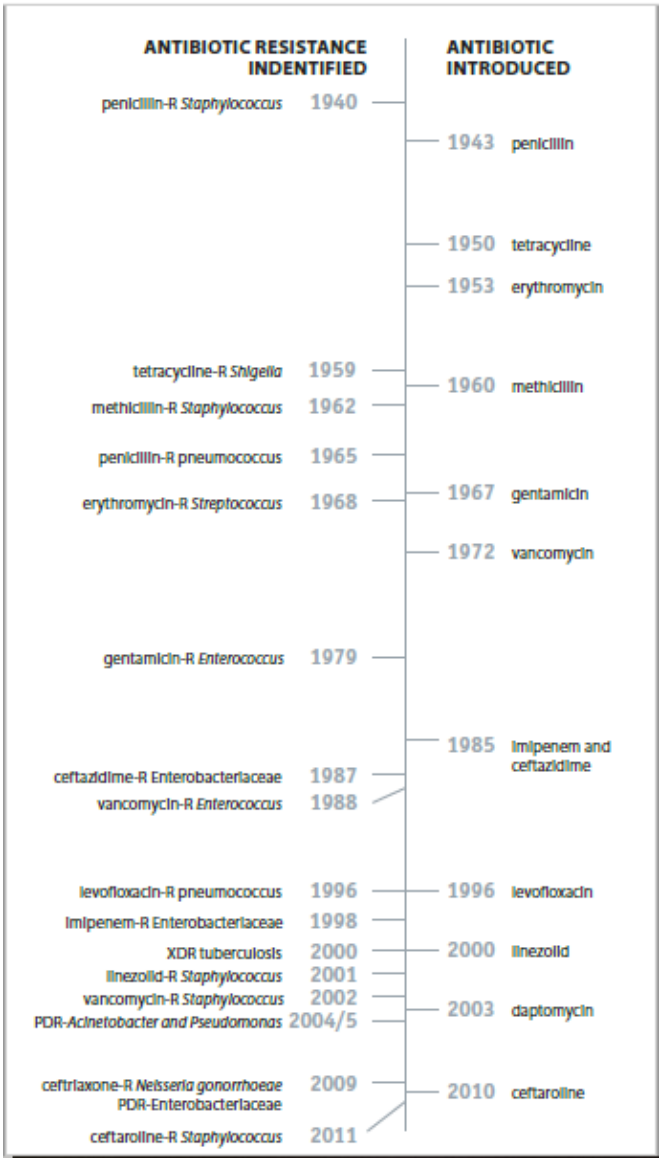


Figure 1.3 Timeline of antimicrobial resistance versus antimicrobial development. Cited from U.S Department of Health and Human Services, Center for Disease Control and Prevention (CDC), 2013 (29).

1.2.1 Intrinsic AMR

Some organisms are intrinsically or naturally resistant to specific antimicrobials. For example, a number of antimicrobials (e.g. vancomycin) are active against Gram-positive bacteria, but ineffective against Gram-negative bacteria, due to the distinct differences in their cell wall structures (Figure 1.2) (30). The coarse meshwork that makes up the thick peptidoglycan layer in Gram-positive bacteria favours the entry of small molecules. In contrast, the presence of the outer membrane in Gram-negative bacteria acts as a much finer molecular sieve, inhibiting the entry of these molecules (31). Therefore, treating an infection caused by Gram-negative bacteria with these antimicrobials would be ineffective.

1.2.2 Acquired AMR

Bacteria also have the ability to acquire resistance, meaning that antimicrobials which originally killed or inhibited them no longer have this effect (26). These new traits can be gained either by the occurrence of spontaneous mutations in existing genes or by acquiring new genetic material via horizontal gene transfer (HGT) (31) (32).

Spontaneous mutations

Spontaneous mutations can occur within existing genes, giving the bacterium newly acquired resistance properties. These mutations are subsequently passed down to daughter cells, via vertical transmission (31). An important example of this is chromosome-mediated quinolone resistance in members of the *Enterobacterales*. This resistance occurs through a series of mutations in the DNA gyrase encoding genes (*gryA* and *gryB)* and the topoisomerase IV encoding genes (*par*C and *parE*), with the level of resistance generally varying based on the number of mutations that have occurred and the type of quinolone (33). These mutations result in amino acid replacements and therefore an alteration in the target site structure, which reduces the binding affinity of the drug and the target site (34).

Acquisition of new genetic material via horizonal gene transfer

In addition to spontaneous mutations, bacteria can also acquire AMR by obtaining new genetic material through HGT. HGT is believed to play a significant role in bacterial evolution. It can occur via the following three routes: transformation, transduction, and conjugation (Figure 1.4) (25).

Transformation is the process whereby bacteria pick up naked DNA from the environment and incorporate it into their genome (25) (32). Several conditions need to be present for transformation to take place. The recipient bacterial cell must be competent (can accept the DNA), DNA must be available for uptake from the extracellular environment and the recipient must integrate the DNA that is picked up into its genome (25). A small number of clinically relevant species of bacteria (e.g *Neisseria*) are able to 'naturally' incorporate the acquired genetic material (27).

Transduction is the process by which a bacteriophage (a virus that infects bacteria) transfers DNA between bacterial cells, and it can be either generalised or specialised (32). In the case of generalised transduction, bacteriophages are capable of accidentally transferring any gene(s) from a donor to a recipient bacterial cell (35). Bacteriophages can have difficulty differentiating between bacterial DNA and their own viral DNA. Fragments of bacterial DNA can therefore get mistakenly packaged in the phage capsid, which in turn can be transferred into the genome of newly infected bacteria (e.g. *Salmonella enterica* with phage P22) (36). While generalised transduction occurs at low frequency, specialised/restricted transduction allows for very efficient transfer, but only a few closely linked genes can be transferred (e.g. *Escherichia coli* with lambda phage) (35). The transfer of antimicrobial resistance genes (ARGs) via transduction is well documented (e.g. the transfer of gentamycin resistance between *Enterococci*) (37) (32).

Conjugation or 'bacterial sex' is the most commonly studied of the three mechanisms of HGT and is regarded as a main facilitator in the transfer of

AMR encoding genes between bacteria (38). This is because bacteriophages have limited cargo regions and restricted host ranges (39). Conjugation is a multi-step process in which DNA is transferred from one bacterial cell to another. Unlike transformation and transduction, this method of HGT requires cell to cell contact. Specialised genes are needed for certain stages of this process, including the formation of the sex pilus (tube like structure that joins the two cells together) and for DNA export (36). These genes can be encoded either on conjugative plasmids or conjugative chromosomal elements (32). Transfer methods can differ based on the plasmid/element involved. The fertility factor (F factor) is a well-studied transferable plasmid often found in *E. coli* (35). Bacteria with this plasmid are said to be F+ and those without it to be F^-. In *E. coli,* the tip of the sex pilus from the F+ donor cell attaches to the F^- recipient cell via receptors. The pilus then contracts bringing the two cells together. A conjugation complex is formed as a result of the two cell envelopes fusing together. Single-stranded DNA is then passed through this complex from the donor to the recipient cell (36).

1.2.3 Mobile genetic elements

Mobile genetic elements (MGE) including plasmids, transposable elements and integrons are key vectors involved in HGT and play a central role in the mobilisation, acquisition and dissemination of AMR (40) (39).

Plasmids

Along with chromosomal DNA, many bacteria also carry plasmids, which are extra-chromosomal genetic elements capable of independent replication (41). While they are usually circular in shape, linear plasmids have also been identified, and they vary in size from 1 kb to greater than 1 Mb (35). Plasmids differ from chromosomes in that they carry non-essential (but often useful) genes, such as those associated with AMR (35). If two closely related plasmids are acquired by a bacterial cell, one will be lost following cell replication, as

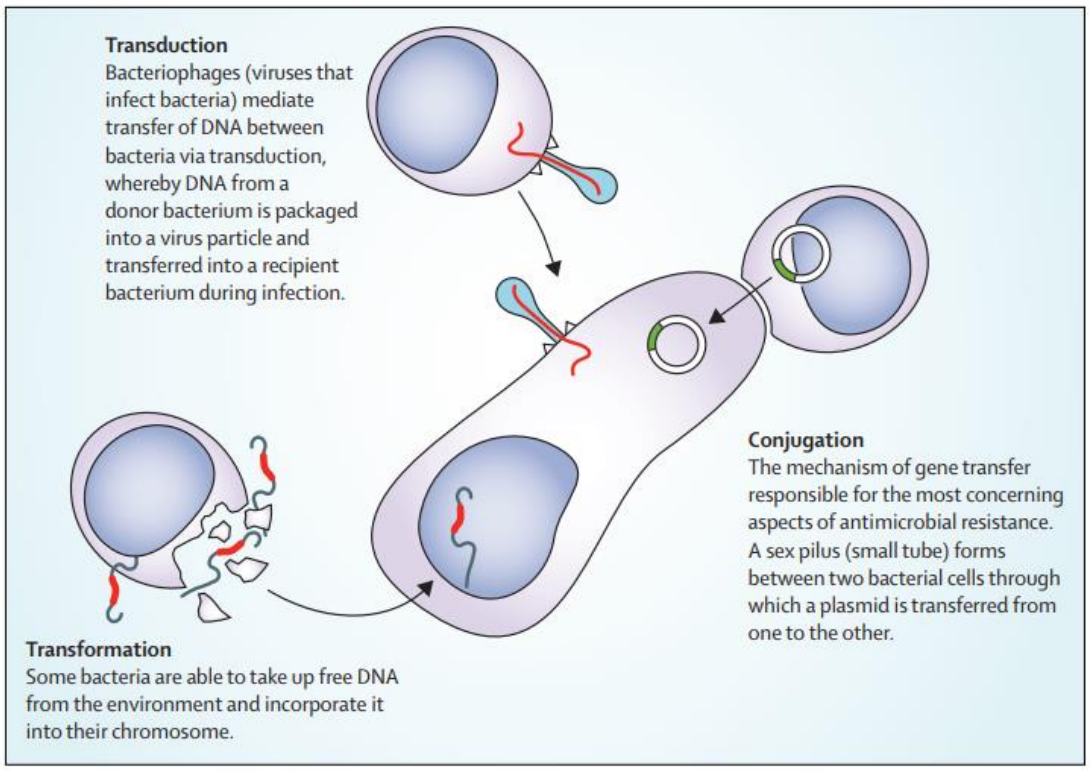


Figure 1.4 Transmission of genetic material between microorganisms via horizontal gene transfer **(25).** *Copyright obtained from Elsevier Ltd. via Copyright Clearance Centre, RightsLink® service.*

they are deemed incompatible. Similar plasmids are assigned to the same incompatibility (Inc) group. While plasmids cannot co-exist with plasmids from within the same Inc group, bacteria are frequently found to carry multiple plasmids from different Inc groups (36).

Mobility is a vital element of plasmid fitness. Plasmids can be classified into three groups based on mobility, which are conjugative, mobilizable and non-mobilizable plasmids (39). For a plasmid to be conjugative/self-transmissible it must contain genes which encode for a set of mobility genes, along with a membrane-associated mating pair formation complex (MPF) (to create the pilus). A plasmid is mobilizable if it uses the MPF of another genetic element within the cell, while a non-mobilizable plasmid spreads either by transduction or natural transformation (as it is not conjugative or mobilizable) (39).

In addition to the plasmid housekeeping encoding genes (including those necessary for plasmid replication and mobilisation), plasmids may also contain accessory regions. In plasmids associated with AMR (e.g. IncFII plasmids) these accessory regions commonly contain multiple AMR encoding genes, as well as MGEs such as insertion sequences, transposons and integrons (41) (40).

Transposable elements

Insertion sequences (IS) and transposons are described as transposable elements. Unlike plasmids, transposable elements (or jumping genes as they were originally known) cannot exist autonomously, and are essentially hitchhikers which have integrated into larger DNA molecules (either a plasmid, a chromosome or a bacteriophage genome) (42) (35). Both of these elements contain a gene which encodes for the enzyme transposase. This enzyme is responsible for catalysing the transfer/replication of the MGE from one molecule of DNA to another (36). IS and transposons also contain short inverted repeat sequences at either end, which are also necessary for transposition. IS are simple transposable elements, about 1000bp in size, while transposons are larger in size and commonly carry AMR encoding genes (e.g. Tn5 is associated with kanamycin resistance) (35).

Integrons

Integrons are versatile, genetic elements that capture and express exogenous genes on mobile pieces of DNA, known as gene cassettes. (43). Integrons are generally found on plasmids or transposons, but can also been integrated into the chromosome (35). All integrons have three crucial core features: *intI* (a gene which encodes for integron integrase (IntI)), *attI* (an integron-associated recombination site) and Pc (an-integron-associated promoter) (43). Integrons can be classed based on IntI sequence (e.g. IntI1, IntI2, IntI3), with IntI1 being the most commonly reported in AMR isolates of clinical origin (40). Integron gene cassettes are simple structures, usually containing a single open reading frame (ORF) flanked by *attC,* a cassette-associated recombination site. Gene

cassettes are integrated into integrons by site-specific recombination between *attC* and *attI* (43). Multiple cassettes may be inserted into the same integron, with several AMR encoding genes often present within this cassette array (40).

1.2.4 Mechanisms of AMR

Bacteria have developed several important mechanisms of AMR, as outlined in Figure 1.5.

Enzymatic degradation

One of the most successful of these mechanisms is the production of enzymes that inactivate antimicrobial molecules (26). Thousands of different enzymes capable of modifying or degrading antimicrobials from different classes such as the aminoglycosides, β-lactams, phenicols or macrolides have been identified (44). Among the most worrisome of these enzymes are the β-lactamases. These are a diverse group of enzymes (with a variable spectre of activity), which degrade β-lactam antimicrobials by cleaving the amide bond in their β-lactam ring, rendering the antimicrobial ineffective (45).

Prevention of access to the target site

As outlined in section 1.1.2, many antimicrobials have intracellular targets. Bacteria have developed two main mechanisms to prevent antimicrobials from reaching these intended sites.

The first mechanism is through the modification of their outer membrane permeability, which results in a reduction in the amount of antimicrobial molecules entering the cell (46). Reduced outer membrane permeability is achieved either by the replacement of porins with more-selective channels, or by the down-regulation of porins (44). This mechanism of resistance is particularly effective in Gram-negative bacteria, as many important hydrophilic antimicrobial molecules, including the β-lactams and the

tetracyclines, rely on these water-filled diffusion channels (porins) to penetrate the outer membrane (26).

The second mechanism by which bacteria prevent antimicrobial molecules from reaching their target site is via the activation of efflux pumps that work to expel the molecules out of the cell (46). Efflux pumps are an important source of intrinsic resistance in Gram-negative bacteria as they actively transport many types of antimicrobial molecules out of the cell. Many efflux pumps (referred to as multidrug resistance efflux pumps) can transport a variety of structurally different substrates out of the cell, while others, such as the Tet pumps, have narrow substrate specificity (44).

Modification of the target site

Another important mechanism of AMR is the modification of the target site (47). This is one of the most common mechanisms of resistance, affecting almost all classes of antimicrobials. It can occur as a result of point mutations in the target site encoding genes, via modifications in the target site due to enzymatic activity or through the bypass or replacement of the target site (26). A prime example of a mutation in the target site leading to resistance can be seen with the development of resistance to rifampicin. A single point mutation in the *rpoB* gene (which is the gene which encodes for RNA polymerase (the target of rifampicin)), results in a decreased affinity between rifampicin and its target site. This single mutation prevents the drug from inhibiting the transcription process (26).

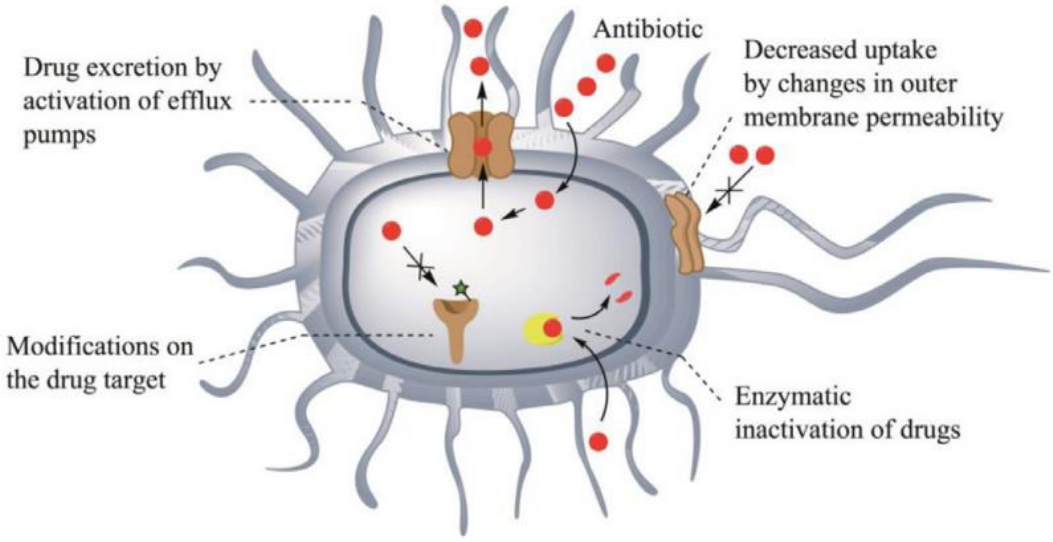


Figure 1.5 Main mechanisms of antimicrobial resistance in bacteria (46). *Copyright obtained from* (46) *via Copyright Clearance Centre, RightsLink® service.*

1.3 Drivers of AMR

When microbial communities are exposed to antimicrobials, this exerts a selective pressure on them (48). Bacteria capable of resisting the effects of the antimicrobials survive, while those that are susceptible are killed. The surviving bacteria multiply, passing on these resistance traits to their daughter cells, as well as to other bacteria, via HGT (49). All antimicrobial usage therefore results in increased selective pressure, which in turn promotes the development and dissemination of AMR (50).

AMR is by no means a recent phenomenon and selective pressures have long been exerted on microbial communities, due to the occurrence of antimicrobials in the natural environment (50). As far back as 1945, Alexander Fleming forewarned of the potential problems that might arise if antimicrobials were not used with care (51). However, his cautionary words were not heeded, which has led us to the AMR crisis that the world faces today. Not only have antimicrobials been overused and misused in human medicine, their great success also prompted their use outside of the clinical

setting, in veterinary medicine, agriculture and even aquaculture (52) (53) (38).

1.3.1 Antimicrobial use in human medicine

A key driver of AMR is the misuse of antimicrobials by humans, and this problem is only worsening (38). A study reported that worldwide antimicrobial consumption increased by 35% between 2000 and 2010, with five countries (Brazil, China, India, Russia and South Africa) responsible for 76% of this increase (54). A review published in 2011 reported that non-prescription use of antimicrobials was prevalent worldwide and consisted of between 19 and 100% of antimicrobial use in countries outside of North America and Northern Europe (55). In countries such as Chile, where new regulations banning the sale of non-prescription antimicrobials were introduced, levels of antimicrobial consumption has decreased, which in turn has resulted in a reduction in levels of AMR (56) (55). Other common misuses include doctors prescribing antimicrobials to treat non-bacterial infections and patients failing to complete their full prescriptions (38). In 2017, Ireland ranked 10th highest out of 28 European Union (EU)/European Economic Area (EEA) countries, based on the amount of antimicrobials consumed in the community and hospital sector (in the community Ireland used 22.9 defined daily dose (DDD) per 1,000 inhabitants per day vs. EU/EEA average of 21.8, and in the hospital sector Ireland used 1.77 DDD per 1,000 inhabitants per day vs. EU/EEA average of 2.03) (57).

1.3.2 Antimicrobial use in veterinary medicine, aquaculture and agriculture

Even if people rarely consume antimicrobials, they can still be exposed to antimicrobial resistant organisms (AROs) or ARGs via other routes, including through animals, the food chain, or the environment (58). Antimicrobials have been widely used in veterinary medicine since the 1950s, with many of the same antimicrobials/classes used in the treatment of both humans and animals (59). In Ireland, in 2017, 0.3 tonnes of third and fourth generation

cephalosporins and 0.85 tonnes of fluoroquinolones were sold for use in veterinary medicine (60). However, most of the antimicrobials used were older classes including tetracyclines, sulphonamides and trimethoprim (Figure 1.6) (60). Antimicrobials are also commonly used in aquaculture to control the spread of infection in this setting and can be administered either through feed or bath treatments (49).

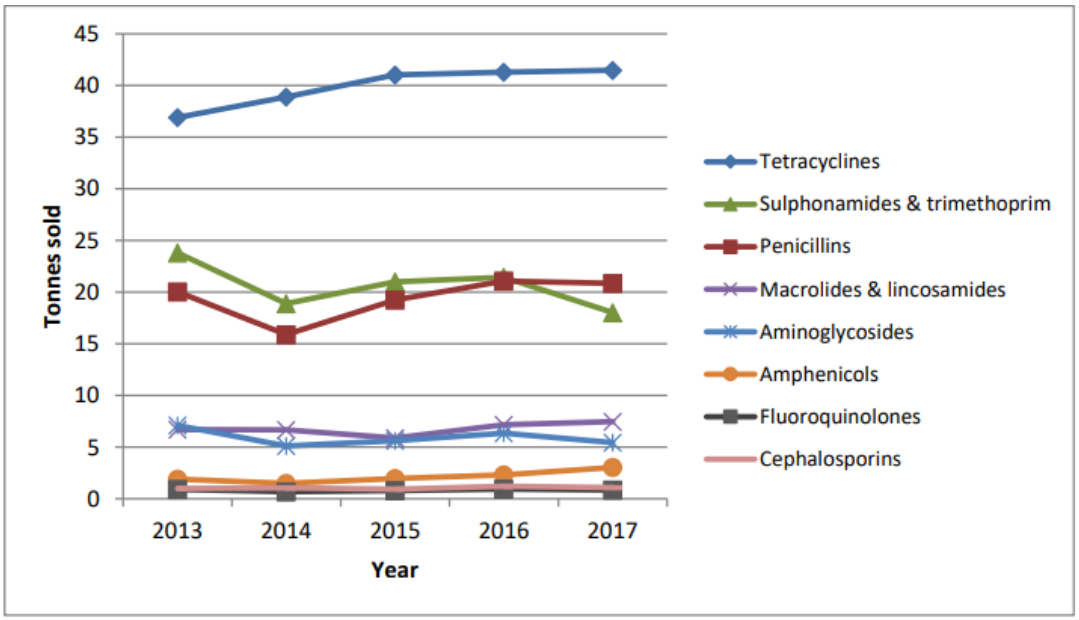


Figure 1.6 Sales (by tonnes sold) of veterinary antimicrobials in Ireland for the years 2013 to 2017. *Cited from Health Products Regulatory Authority (HPRA)* (60).

Similar to veterinary medicine, antimicrobials have been used as growth promoters in agriculture since the 1950s. Their use has been attributed to maximising livestock health and increasing production efficiencies (61). However, this subtherapeutic use also serves to promote the emergence of AMR, hereby compromising the effectiveness of these drugs in both human and animal medicine (61). In 2006, following successful implementation by both Sweden and Denmark, the EU introduced regulations banning the use of antimicrobials as growth promoters in food-producing animals (62). However, regulations governing antimicrobial use in food producing animals vary considerably on a global scale, with some countries estimated to use 70-80% of total antimicrobials within the animal sector (58).

1.3.3 Anthropogenic pollution of the environment

The environment can become contaminated with AROs, ARGs and antimicrobials, as a result of anthropogenic pollution, further driving the persistence and dissemination of AMR. These contaminants can enter the environment via a number of different routes (49). Unused antimicrobials are frequently disposed of incorrectly, either by being flushed down the toilet and ending up in wastewater systems, or being discarded into domestic bins intended for landfill, with the contaminants subsequently leaching into groundwater and soil (52). Pharmaceutical manufacturing waste released into the environment has also been found to contain significant quantities of active antimicrobial compounds (49) (52). Antimicrobial use is prevalent in hospital settings, and significant quantities of biologically active antimicrobial compounds and AROs are excreted into hospital sewage systems every day, which can then enter the environment via these wastewater systems (59) (52). A significant proportion of antimicrobials are also used in community settings, including long-term care facilities, meaning that municipal waste can also act as a source of these contaminants (57) (63). Similarly, in agriculture, large volumes of unmetabolized antimicrobials and AROs are shed in animal feces, which can enter groundwater and surface waters, via runoff from agricultural lands (52). This waste can be used as manure to fertilize food crops, further increasing the potential exposure to and dissemination of AMR (49). In addition to the selective pressure exerted by antimicrobials present in the environment, AMR genes may also be co-selected for by other substances, such as metals and biocides, as the resistance determinants for these compounds are often present on the same MGEs as the AMR genes (64).

1.3.4 Additional factors

Other factors contributing to the spread of AMR include poor infection control, inadequate sanitation and inappropriate food-handling, which can

result in increased levels of infection and therefore increased antimicrobial use (65). Foreign travel, migration, medical tourism and international trade all act to facilitate the global dissemination of AMR, meaning that different types of AROs and ARGs are not regionally confined (49) (66). Another important factor that may contribute to AMR dissemination is lack of knowledge on the subject. A European study which examined correlations between knowledge of AMR and levels of resistance in that country, found that the countries that had the highest prevalence of AMR had the lowest level of awareness about AMR (67).

1.4 Why AMR is a major public health concern

The rate at which bacteria have evolved to resist the effects of antimicrobials has exceeded the rate at which we are developing new antimicrobials to combat this resistance (5). The fast pace at which pathogens develop resistance to new antimicrobials, combined with the lack of novel bacterial cellular targets means there is little incentive for pharmaceutical companies to invest in the development of new antimicrobials (68). Although alternative options such as treatment of infections with phage therapy (69), as well as vaccination programs to prevent infection are promising, they also have limitations (70) (71). Increasing levels of AMR, coupled with limited new alternative drugs/treatments has resulted in AMR being considered one of the greatest threats to human health (72).

If left unchecked, AMR will have far reaching consequences, not only in relation to human and animal health, but also in terms of financial burden, socio-economic development, food security and environmental wellbeing (73). It is currently estimated that 700,000 people die every year globally as a result of infections caused by AROs, with this figure predicted to increase to 10 million by the year 2050 if effective action is not taken now (73) (74).

The cumulative costs of AMR are estimated to reach 100 trillion by this time (73).

1.5 *Enterobacterales*

A diverse range of ARGs and AROs have been identified to date, with some posing more of a threat to public health than others. Increasing levels of AMR among members of the *Enterobacterales* is of particular concern (75). The *Enterobacterales* are a Gram-negative order of bacteria that contain a diverse range of species, and up until recently its' members were referred to collectively as *Enterobacteriaceae* (76). This term was changed to reflect to phylogenetic diversity within this group. Members of this order can now be classified into the following seven families based on phylogeny: *Budviciaceae*, *Enterobacteriaceae, Erwiniaceae, Hafniaceae, Morganellaceae, Pectobacteriaceae* and *Yersiniaceae* (77). Several genera exist within this order and examples include *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Proteus* and *Salmonella* (77). *Enterobacterales* are so called because they are enteric organisms that reside in the gut of humans and animals. While generally commensal, many members are also considered important human pathogens, examples of which include *Klebsiella pneumoniae* and *E. coli* (78) (79).

1.5.1 *Klebsiella pneumoniae*

K. pneumoniae are rod shaped, non-motile, encapsulated bacteria, that are associated with a wide range of infections such as urinary tract infections (UTIs), pneumonia and bacteraemia (80) (81) (82). Infections caused by these opportunistic pathogens are particularly prevalent among vulnerable patients in hospital settings, including individuals who are immunocompromised, premature infants and the elderly (83) (84) (85). These organisms have the ability to disseminate rapidly among patients under the right conditions, with nosocomial outbreaks commonly reported

(86) (87) (88). Studies indicate that *K. pneumoniae* are especially successful at adapting to different niches through the acquisition of MGE via HGT, and thus act as an important reservoir of ARGs in healthcare settings (89).

1.5.2 *Escherichia coli*

E. coli are the most well studied of all microorganisms (90). As natural inhabitants of the gut of humans and animals, these rod-shaped bacteria are readily spread among both humans and animals via hand carriage and contaminated food and water, and are widely used as indicators of environmental faecal contamination (91) (92) (93). *E. coli* are one of the most important human pathogens and are a leading cause of both hospital and community-acquired infections, including UTIs, bacteraemia, diarrheal disease and meningitis (90) (91) (94).

1.6 Resistance to β-lactam antimicrobials

The β-lactams are an extensive collection of antimicrobials grouped together (into penicillin, cephalosporin, monobactam and carbapenem classes) by the commonality of a β-lactam ring in their structure (figure 1.1) (17). Each new β-lactam class was developed either to address the problem of emerging resistance against the current β-lactams, or to broaden the spectrum of activity to include further species of bacteria. Thousands of β-lactam derivatives have been developed since benzylpenicillin was first discovered in the 1920s (17). The β-lactams are among the most widely used antimicrobials worldwide, with many members listed as critical for use in human medicine (17). While the β-lactams are used to treat infections caused by a wide range of organisms, they are heavily relied upon for use in the treatment of infections caused by *Enterobacterales* (95).

A study published by the World Health Organization (WHO) in 2016, outlined a list of priority AROs for the guidance of research and development of new

antimicrobials (75). The AROs that made the list were ranked as either 'Priority 1: critical', 'Priority 2: high' or 'Priority 3: medium'. These priority organisms where selected based on the following criteria: mortality, AMR prevalence, 10-year AMR trend, community and healthcare burden, transmissibility, treatability, preventability in community and hospital settings and current pipeline (75). Both carbapenem-resistant *Enterobacterales* (CRE) and third generation cephalosporin-resistant *Enterobacterales* were ranked as 'Priority1: critical', alongside carbapenem-resistant *Acinetobacter baumannii* and carbapenem-resistant *Pseudomonas aeruginosa*.

1.6.1 β-lactamases

Bacteria utilise a wide range of resistance mechanisms to fight against antimicrobials (section 1.2.5). While *Enterobacterales* employ several mechanisms of resistance against the β-lactams, the mechanism that poses the greatest problem among these organisms is the production of β-lactamase enzymes (52) (95).

The β-lactamases are a well-studied family of enzymes that have a limited range of molecular structures. They act by hydrolysing the β-lactam ring (45). β-lactamases are classified using two major systems. Functional classification according to the Busy-Jacoby-Medeiros scheme is based on substrate and inhibitor profiles (Groups 1-4), while the Ambler classification is based on amino acid sequence homology (Classes A-D) (96).

These versatile enzymes have been reported in a diverse range of bacterial species and as of 2018, this group contained nearly 2,800 unique proteins (97). Many *Enterobacterales* contain chromosomally encoded AmpC β-lactamases, which have contributed to the problem of β-lactam resistance within these organisms (98). However, the bigger threat are β-lactamases encoded by genes carried on MGEs, as these can readily be transferred among and between bacterial species (97).

The first β-lactamase (penicillinase (now known as chromosomal AmpC)) was identified in 1940, in *E. coli* (28), while the earliest plasmid encoded β-lactamases (OXA, TEM-1 and SHV-1) emerged in the 1960s and 70s (99) (100) (99). Earlier β-lactamases such as these generally acted against penicillins and early generation cephalosporins (Figure 1.7) (97). To address this resistance problem a new generation of cephalosporins (extended-spectrum cephalosporins) were developed (in the early 80s), via semi-synthetic modifications to the original compounds. In addition to the development of new classes/modification of existing ones, β-lactams were also combined with β-lactamase inhibitors such as clavulanic acid, in an attempt to combat emerging resistance (101).

1.6.2 Extended-spectrum β-lactamases

Not long after the introduction of extended-spectrum cephalosporins into the clinical setting, several new variants of existing β-lactamases (SHV-1, TEM-1, TEM-2, oxacillinase-10 (OXA-10)) emerged. These variants had the ability to hydrolyse extended-spectrum cephalosporins and were therefore termed extended-spectrum β-lactamases (ESBLs) (102). An ESBL can be defined as a β-lactamase with the ability to hydrolyse penicillins, cephalosporins (first, second and third generation) and aztreonam, but not capable of hydrolysing carbapenems or β-lactamase inhibitors (e.g. clavulanic acid) (103).

A novel plasmid mediated ESBL was identified in 1989, which was coined CTX-M (due to increased activity against cefotaxime)(104). While previous ESBLs, such as SHV and TEM appear to have evolved vertically through point mutations in genes encoding for parent β-lactamases, CTX-M encoding genes are believed to have been mobilised from the chromosome of the environmental *species Kluyvera* (105). The likely occurrence of mutations and recombination events has led to a diverse family of CTX-M β-lactamases (over 172 variants, as of 2018) (106). These can be classified into five main clusters

based on sequence homology (CTX-M-Group-1, CTX-M-Group-2, CTX-M-Group-8, CTX-M-Group-9 and CTX-M-Group-25) (105).

1.6.3 Carbapenemases

Increased prevalence of ESBL-producing *Enterobacterales* (ESBL-PE) meant fewer treatment options were available for infections caused by these organisms, which led to an increase in carbapenem consumption (considered last-line antimicrobials) (107). This resulted in the emergence and dissemination of CRE. Resistance amongst *Enterobacterales* can arise via chromosomally encoded bla_{AmpC} genes, in combination with porin loss and efflux pumps (108). However, a more problematic resistance mechanism is the acquisition of carbapenemase encoding genes via MGE (these organisms are termed carbapenemase-producing *Enterobacterales* (CPE)) (109).

The first carbapenemase encoding gene (bla_{NmcA}) was identified in *Enterobacterales* (in *Enterobacter cloacae*) in 1993 (110). Since this first report, a number of carbapenemases have emerged, which can be grouped into either Ambler class A, B, or D (Figure 1.7) (97). Biochemically, carbapenemases can be classified into either serine or metallo-β-lactamases, based on their hydrolysis mechanism. Serine β-lactamases (Groups A and D) hydrolyse the β-lactam ring though formation of an acyl enzyme with an active serine site, while hydrolysis in metallo-β-lactamases (Group B) is facilitated by essential zinc ions in active sites (111).

Class A serine-β-lactamases include a variety of both chromosomally encoded carbapenemases such as Sme and NmcA, as well as a number plasmid encoded carbapenemases including *Klebsiella pneumoniae* carbapenemase (KPC), GES and IMI-2 (112). Class B metallo-β-lactamases (MBLs) include the plasmid encoded IMP-type metallo-beta-lactamase (IMP), Verona integron-encoded metallo-β-lactamase (VIM) and more recently New Delhi metallo- β-lactamase (NDM) (97). Class C serine-β-lactamases include OXA-48, which

was first identified in *K. pneumoniae* in 2003 (113) and OXA-181, which is derived from OXA-48 following a point mutation (112).

Some carbapenemases are very efficient at hydrolysing the carbapenems, while others, including OXA-48 and OXA-181 demonstrate poorer activity against these antimicrobials (114). As outlined in Figure 1.7, activity against β-lactamase inhibitors including clavulanic acid and avibactam, as well as broad-spectrum cephalosporins vary according to carbapenemase type (114).

1.6.4 Co-resistance

ESBL-PE and CPE commonly co-harbour resistance genes associated with other non-β-lactam antimicrobials including aminoglycosides, chloramphenicol, fluoroquinolones, sulphonamides, tetracycline, trimethoprim, and less commonly tigecycline and colistin (115) (116) (117) (118) These genes can be present on the same plasmid or on additional MGEs, or be chromosomally encoded (44). Not only does this further restrict treatment options, but it may also play a vital role in the dissemination and maintenance of these isolates, via co-selection (119). Reports of resistance to last line antimicrobials tigecycline and colistin is especially worrying as these are often relied upon for the treatment of infections caused by multi-drug resistant *Enterobacterales* (120).

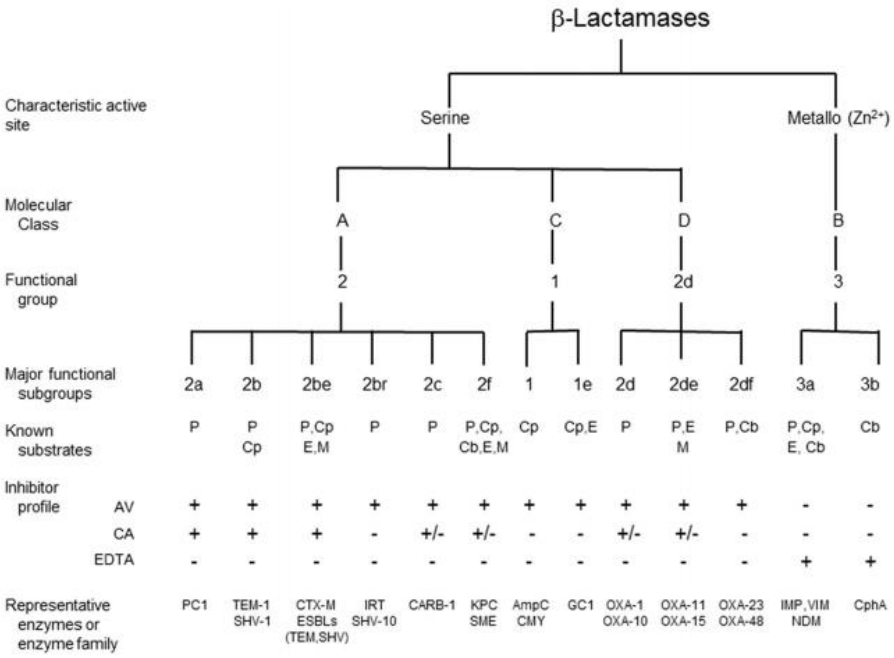


Figure 1.7 Functional and molecular relationships of β-lactamases (97). *AV: avibactam, CA: clavulanic acid, Cb: carbapenem, Cp: cephalosporin, E: extended-spectrum cephalosporin, M: monobactam, P: penicillin. Copyright obtained from* American Society for Microbiology *via Copyright Clearance Centre, RightsLink® service.*

1.7 Epidemiology of ESBL-PE and CPE

A number of factors have aided in the successful dissemination of ESBL-PE and CPE. One of the most important factors is the genetic environment of the ESBL or carbapenemase encoding genes, as some genetic structures, including plasmids, are more prone to mobility and gene plasticity than others (114). Another factor influencing their dissemination is the type of strain carrying the gene, with some so-called successful clones aiding in the rapid spread of the ESBLs and carbapenemases (121). Additionally, if the ARGs emerge in a geographical region with favourable conditions, such as poor sanitation and limited regulations regarding the use of antimicrobials, this further aids in the dissemination, with foreign travel to and from this region acting to disseminate the ARGs globally (114). The most prevalent

types of both ESBL-PE and CPE have disseminated rapidly as a result of a combination of these factors, with types of MGEs and clonal groups varying according to ESBL and carbapenemase (121) (114).

The European Antimicrobial Resistance Surveillance Network (EARS-Net) collects data on invasive isolates (from blood and cerebrospinal fluid), reported by 30 EU and EEA countries (118). This data indicates that although a much higher number of reported isolates are *E. coli* in comparison to *K. pneumoniae,* a higher proportion of *K. pneumoniae* are resistant to third generation cephalosporins and/or carbapenems. Total data for 2017 (118) shows that 31.2% (population-weighted mean) of *K. pneumoniae* invasive isolates (total n=32,453) were resistant to third generation cephalosporins (87.8% of which were ESBL producers), while 7.2% exhibited resistance to carbapenems. The populated-weighted mean for *E. coli* (total n=138,883) was 14.9% resistance to third generation cephalosporins (of which 87.4% were ESBL producers), while 0.1% were resistant to carbapenems. Collectively, the numbers of invasive isolates of both *E. coli and K. pneumoniae* from the 30 countries have consistently increased since 2014, although levels of third generation cephalosporin resistance and carbapenem resistance have remained at similar levels throughout this time.

1.7.1 Epidemiology of ESBL-PE

Initially, the two most prevalent ESBLs were TEM and SHV variants, which were most frequently associated with infections caused by *K. pneumoniae* in the hospital setting (102). However, the rapid dissemination of CTX-M in the early 2000s, meant it quickly replaced TEM and SHV as the most prevalent type of ESBL reported globally, with increasing levels of ESBLs detected among *E. coli* in the community (105). Today ESBL-producing *Enterobacterales* are widespread both in hospital (105) (122) (95) (123) and community settings (124) (125) (82) (126), with *E. coli* and *K. pneumoniae* as the two most commonly reported species. Studies indicate that the prevalence of ESBP-PE in both hospital and community settings in Ireland is

progressively increasing (127) (128), with an outbreak of CTX-M-15-producing *E. coli* reported in an Irish neonatal intensive care unit in 2017 (129).

CTX-M remains the most prevalent type of ESBL globally, with the CTX-M genotype varying according to geographical region. Overall CTX-M-15 (from CTX-M-Group-1) is the most commonly reported variant (frequently associated with *E. coli* sequence type ST131), with a high prevalence detected in ESBLs from Ireland (130), the United Kingdom (131), the United States (132) etc. Countries such as China and Korea have reported a high prevalence of CTX-M-14 among ESBLs (133) (134).

ESBL-PE from non-human sources

Not only are ESBL-PE widely disseminated among humans, they are also widely reported from other reservoirs. An extensive number of studies have detected ESBL-PE in food including, but not limited to, retail meat, fresh vegetables, ready to eat foods, seafood (135) (136) (137) (138). A particular focus has been on retail meats with a high prevalence of ESBL-PE commonly reported among retail chicken samples (139) (140) (141) (142).

Food-producing animals such as poultry, pigs and cattle have also been identified as reservoirs, with poultry most commonly associated with ESBL-PE (141) (143) (144) (145) (146) (147). While a high proportion of studies have focused on food-producing animals, other animal reservoirs include companion animals and wildlife (148) (149) (150). Another important reservoir is the aquatic environment, with ESBL-PE detected in fresh waters, seawater, irrigation and drinking waters (151) (152) (153) (92) (154) (155) (135) (156) (157). In addition, ESBL-PE are frequently reported in both hospital and municipal effluents, which are common sources of environment pollution with AROs and ARGs (154) (158) (92) (159) (160) (151).

While the CTX-M type generally varies according to geographical region it can also vary according to source. A study carried out examining ESBL-producing

E. coli (ESBL-PEc) from humans, animals and food in the United Kingdom, the Netherlands and Germany found that CTX-M-15 was the most common ESBL among human isolates in all three countries, while CTX-M-1 was the most common variant in isolates from poultry in the three countries (161).

1.7.2 Epidemiology of CPE

The rapid dissemination of CPE poses a serious public health threat. Infections caused by these organisms are associated with high morbidity and mortality (162). Carbapenemases are commonly associated with successful clonal groups including *K. pneumoniae* ST11, ST101 and ST258, and *E. coli* ST131, as well a variety of highly mobile genetic elements (162). The three most commonly detected carbapenemases are KPC, NDM and OXA-48.

KPC is the most prevalent carbapenemase globally (109). It was first identified in the United States in 1996, in *K. pneumoniae* (163), and since then has become widespread in countries such as the United States, Columbia, Greece and Italy (164) (165) (166). NDM was first isolated in *K. pneumoniae,* in 2007, from a Swedish patient who had recently returned from India (167). Subsequent studies identified widespread prevalence of NDM within the Indian subcontinent (168) (169). Other countries including Romania and Poland have reported inter-regional outbreaks of NDM-producing *Enterobacterales* (164).

The first report of OXA-48-producing *Enterobacterales* was in Turkey in 2001 (in *K. pneumoniae*) (113). Since then, countries including Belgium, France and Spain have reported a high prevalence of these organisms (164). In Ireland, OXA-48 is by far the most prevalent carbapenemase identified in the clinical setting, followed by KPC and NDM, to a much lesser extent (Figure 1.8) (170). The number of patients newly colonized with CPE has steadily increased since surveillance began in 2012, with *E. coli* and *K. pneumoniae* the most common species identified. Ireland's Minister for Health declared CPE a public health

emergency in late 2017 and a National Public Health Emergency Team was established to manage this growing threat (171).

Similar to ESBL-PE, which were initially predominantly associated with the clinical setting, studies indicate that CPE are gradually becoming established in the community globally, as highlighted by a recent review (172). While KPC-producers are most commonly associated with nosocomial *K. pneumoniae* infections, NDM and OXA-48 are frequently associated with community-acquired and nosocomial *E. coli* and *K. pneumoniae* infections (114).

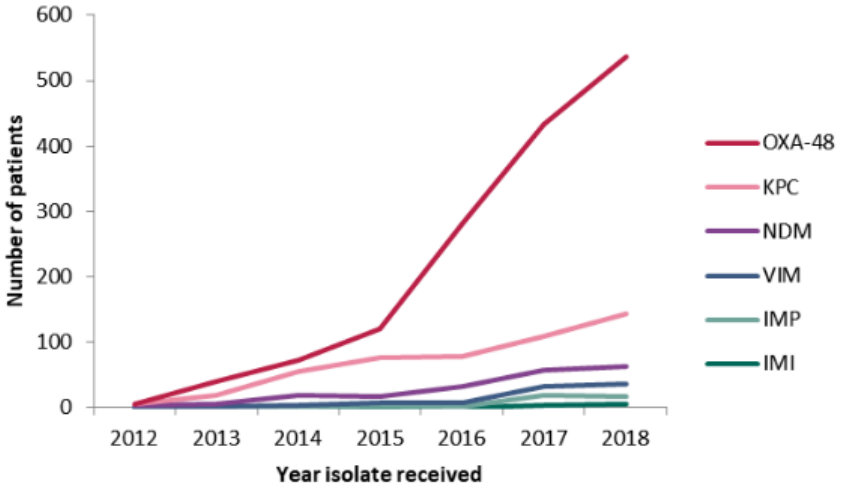


Figure 1.8 Annual numbers of patients with newly confirmed carbapenemase-producing Enterobacterales by carbapenemase type, Ireland, 2012 to 2018. *Cited from Health Protection Surveillance Centre (HSPC), 2019* (170).

CPE from non-human sources

Although CPE are not as widely disseminated as ESBL-PE, an increasing number of studies are reporting their detection from non-human sources (173). This includes the detection of CPE and/or carbapenemase encoding genes from food including retail chicken meat and seafood (174) (175) (135), as well as from companion animals, food-producing animals and wildlife (176) (177). CPE has also been identified in river sediment, freshwater, seawater, drinking water (178) (179) (151) (180) (181) (169) and from hospital and municipal wastewater (151) (182) (183).

1.8 Detection and characterisation of ESBL-PE and CPE

Several different methods have been developed to identify and characterise ESBL-PE and CPE, and these can be divided into either phenotypic or genotypic methods.

1.8.1 Phenotypic methods

Selective and differential chromogenic culture media have been developed to select for extended-spectrum cephalosporin resistance and/or CRE. This media contains agents which prevent the growth of undesired organisms, including Gram-positives, other Gram-negatives and yeast (108). The test samples can either be cultured directly onto the selective media or a sample enrichment step can be added, prior to culturing (184). Examples of chromogenic agar plates include Brilliance ESBL (Oxoid), chromID ESBL (Biomerieux), Brilliance CRE (Oxoid), chromID Carba and SUPERCARBA (Biomerieux) (185) (186).

Detection of ESBL and carbapenemase-production

Phenotypically, ESBL-production is most commonly identified using growth-based synergy tests, examples of which include combination disk method, double disc-synergy tests and E-tests. The tests work on the principal that a true ESBL-producer will be resistant to third-generation cephalosporins, while remaining susceptible to ß-lactamase inhibitors, including clavulanic acid (187).

Multiple phenotypic tests have been developed to identify carbapenemase production, which include growth-based assays, such as combination disk method, double disc-synergy tests, E-tests and the modified Hodge test (188), colorimetric-based assays, such as the Carba NP test (189), immunochromatogenic lateral flow assays (e.g. KPC K-SeT and OXA-48 K-SeT (Coris BioConcept)) for detection of carbapenemase specific antigens (190) (191) and matrix-assisted laser-desorption ionization time-of-flight (MALDI-

TOF) mass spectrometry for detection of carbapenem-hydrolysing activity (192).

While these methods are useful in identifying ESBL and carbapenemase-production, they do not allow for the rapid identification of these organisms (as they are culture based), and they do not identify the specific ESBL or carbapenemase type. A number of these tests have further limitations, with poor sensitivity for NDM-producers reported with the modified Hodge test, while the Carba NP test is reported as exhibiting poor sensitivity for OXA-48-producers (193).

Routine antimicrobial susceptibility testing against multiple antimicrobials is generally performed on isolates, and an antimicrobial susceptibility profile generated. This can be carried out either using an automated system (VITEK or VITEK 2), or manually via the disk diffusion method, with results interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/) or Clinical & Laboratory Standards Institute (CLSI) (https://clsi.org/) guidelines (193) (108).

1.8.2 Genotypic methods

The main methods used for the molecular detection of β-lactamase encoding genes are the polymerase chain reaction (PCR), loop mediated isothermal amplification (LAMP), microarrays, and whole-genome sequencing (WGS) (193). These methods have higher discriminatory power than phenotypic assays, and can also potentially be applied directly to samples, therefore eliminating the culture step and leading to more time-efficient results (193).

The use of real-time PCR (RT-PCR) allows for the rapid identification of both ESBL and carbapenemase encoding genes, with many multiplex assays designed for increased efficiency (194) (195) (196). While studies indicate that this methodology is very sensitive and allows for the accurate identification of targeted genes, a major limitation of RT-PCR is that only

known genes will be identified, while novel β-lactamase encoding genes may go undetected (196). LAMP is an isothermal nucleic acid amplification method that can be used to detect β-lactamase encoding genes (197). It has a number of advantages over PCR, including reaction simplicity and cost effectiveness. It also has a rapid turnaround time and is reported as having high sensitivity (197). However, it is less versatile than PCR and the primer-primer interactions are increased by the larger number of primers used (198). Similar to RT-PCR, microarray technologies use DNA probes that hybridize to DNA targets (199). However, unlike PCR which is limited to four or five targets, the use of microarrays allow for the simultaneous detection of between dozens and hundreds of targets (depending on the type of platform used), enabling the differentiation of closely related variants of ESBL and carbapenemase encoding genes (200) (201). While the use of microarray technologies provide excellent sensitivity and specificity, like RT-PCR and LAMP it only detects known genes (193).

The use of WGS provides the highest level of discrimination. The whole bacterial genome can be examined for all known ARGs, plasmids and other MGEs, as well as genes associated with virulence etc. Relatedness to other isolates, as well as evolutionary lineages can also be assessed (193). Additionally, this data can be used both to investigate the potential origin of the isolate and to inform suspected outbreak investigations. As outbreaks of CPE and ESBL-PE are commonly associated with the dissemination of MGEs, rather than clonal spread, this level of discriminatory power is often necessary to identify if an outbreak has occurred or is occurring (202). This genomic data can then be stored and examined retrospectively, as new genes of interest are discovered. Since next generation sequencing was first developed the cost of sequencing an entire genome has decreased considerably and this technology is now being used widely in both research and in surveillance/clinical settings (203) (204).

Pulsed-field gel electrophoresis (PFGE) was up until recently considered the 'gold standard' of molecular typing methods. It is well standardised and is highly reproducible and it has been widely used to characterise bacterial strains such as *E. coli* (205). However, it is very laborious, and while it holds higher discriminatory power over other traditional typing techniques such as multi-locus sequence typing (MLST), it is slowly becoming obsolete with the growing affordability and advances of next generation sequencing technologies (205).

1.9 How the problem of AMR is currently being addressed

The rapid dissemination of AMR is considered to be one of the biggest threats to human and animal welfare (52). The seriousness of the situation is reflected in the number of high-level policy documents and initiatives introduced during the last decade, at national, European and global levels. In 2015, the WHO published a Global Action plan (GAP) on AMR, which included five main objectives to act as a framework in the development of action plans at National levels (World Health Organisation (WHO), 2015). This GAP was then adopted by the Food and Agriculture Organization (FAO), the World Animal Health Organisation (OIE),and recognised as the world's blueprint for addressing the problem of AMR at the United Nations' General Assembly (206). The five main objectives of the GAP include: improving understanding and awareness of AMR, increased surveillance and research, improved infection control and prevention measures, optimisation of antimicrobial use, and investment in diagnostic tools, alternative medicines, vaccines and other interventions (World Health Organisation (WHO), 2015).

All national and international action plans aim to use a 'One Health' approach when addressing the problem of AMR, as it is well recognised that the health of humans, animals and the environment is interconnected (World Health Organisation (WHO), 2015). As of 2014, EU legislation (Decision

2013/652/EU) requires Ireland and other EU countries to monitor AMR in zoonotic and indicator organisms originating from both animals and food of animal origin, with monitoring for commensal *E. coli,* ESBL, AmpC and carbapenemase-producing *E. coli* also required (207). An important step for coordinated surveillance was made in January 2015, when ECDC, together with European Medicines Agency (EMA) and European Food Safety Authority (EFSA) published the first joint report on integrated analysis of antimicrobial use and AMR in bacteria from humans and food-producing animals (208).

In 2014, a national Interdepartmental AMR Consultative Committee was established by the Irish Department of Health and the Department of Agriculture, Food and the Marine, followed by the release of Ireland's National Action plan in 2017 (52). Ireland have also since established a National Public Health Emergency Team to address to rise in CPE in the Irish clinical setting (171).

Ireland's first One Health Report on antimicrobial use & AMR was published in January 2019, and represented Ireland's first cross-sectional report on antimicrobial use and AMR in humans and animals (59). This report recognised that the environment is also an important reservoir of AMR. However, it stated that 'currently there is no systematic surveillance for AMR in, or of antimicrobial discharges to, the environment', meaning that no data on AMR in the environmental was included in this One Health report. Similarly, a One Health report published by the UK (209) also stated 'there is no structural, statutory surveillance dedicated to assessing the level of AMR in the environment in the UK'. Ireland and the UK are not unique in this, and while the potential role of the environment in the emergence, persistence and dissemination of AMR is recognised at both national and international levels, it is acknowledged that this role remains largely unknown. However, few measures have been put in place to rectify this lack of knowledge and understanding. Unlike the food chain, it is not mandatory to assess the aquatic environment for the presence of AMR. There therefore exists a major

gap in our knowledge in relation to the environment's role in the transmission and persistence of AMR.

1.10 Potential Role of the Aquatic Environment in the Dissemination of AMR

Water is present in all forms of life and acts as the perfect medium for the transmission of microorganisms between humans, animals and the environment (210). Clean and healthy water systems are essential for health and wellbeing (211). Contaminated water is a potential public health hazard (212). According to the WHO, waterborne and water-related infectious diseases result in approximately 3.4 million deaths every year globally (213).

Overall, the risk associated with acquiring AROs or ARGs following exposure to contaminated water is largely unknown. However, evidence suggests that people who frequently use bathing waters for recreational purposes are more commonly associated with colonization with AROs than people who do not (214) (215). A study in France reported that an IMP-2-producing *Enterobacter asburiae*, causing a case of bacteraemia was potentially transmitted to the patient following river exposure, as a closely related strain was isolated from a river sample taken at the exposure site one month later (216).

Copious amounts of AROs and antimicrobials are shed in human and animal feces every day, as highlighted in section 1.3.3. Ireland, along with many other countries, has seen vast improvements in sanitation practices in the last century (210). However, these practices vary greatly on an international level and it is estimated that up to 80% of the worlds wastewater flows untreated into the environment (217). Wastewater treatment plants (WWTPs) have proved to be a vital component in improving sanitation and have resulted in a significant reduction in levels of contaminants, such as AROs, entering the environment (49). However, studies have shown that WWTPs are ineffective

at removing all AROs, ARGs and antimicrobial residues, which can subsequently enter the environment via wastewater effluent (218) (158) (173). In Sweden, comparison of VIM-1 producing *K. oxytoca* isolated from a river, sewage and patients at a local hospital found that the isolates were genotypically related, indicating dispersion from the hospital to the aquatic environment, via treated wastewater effluent (219).

Levels of treatment vary according to treatment system type, which are classed as preliminary, primary, secondary and tertiary. Factors affecting the efficiency of WWTPs include increased water volume (as a result of heavy rainfall), poorly maintained systems and the use of older, outdated systems (49) (220). A report published by the Environmental Protection agency (EPA) Ireland stated that in 2017, 28 out of 179 large urban areas in Ireland did not meet EU wastewater treatment standards, and sewage from 38 towns and villages in Ireland (from approximately 88,000 people) was being released untreated into the environment (220).

In Brazil there have been multiple reports of the detection of CPE in coastal waters and other aquatic environments, with poor sewage treatment practices reported in these areas (180) (181) (221) (179). Findings also indicate that NDM is widespread in the aquatic environment in India, with reports from drinking water and seepage water, with similar associations with poor sanitation practices in this region (169).

Multiple studies have investigated the potential role that animals play in the dissemination of AROs to humans, and while shared reservoirs of ESBL or carbapenemase encoding genes, plasmids or strains have been reported, evidence for the direct transfer of these organisms/resistance determinants between animals and humans is limited (222) (223). A study carried out in the United Kingdom compared the genomes of 431 *E. coli* from livestock and retail meat in England to the genomes of 1,517 *E. coli* associated with

bloodstream infection, and found distinct clonal lineages and MGEs between those originating from human and those from animal sources (224).

An extensive study carried out in the Netherlands pooled data from 35 studies (from 27,000 samples) relating to ESBL and AmpC-producing *E. coli* from humans, animals, food and the environment (all studies were carried out in the Netherlands) (225). This One Health approach compared the frequency distribution of ESBL/AmpC encoding genes (of 5805 isolates) and plasmid replicons (of 812 isolates) across 22 reservoirs. The study identified predominant ESBL and AmpC encoding genes in each reservoir. Similarities between ESBL and AmpC encoding genes were identified between isolates originating from humans within farming communities and their animals (pigs and broilers). Higher levels of similarities were identified between isolates originating from humans in the general population and those from clinical settings, wild birds, surface and sewage water, then those originating from food reservoirs and livestock.

A very important discovery made in 1973 was that AMR was not solely restricted to pathogenic bacteria (226). While these non-pathogenic organisms might not pose a direct risk to human or animal health, they can act as reservoirs of AMR, which can potentially be mobilised and transferred to human or animal pathogens via HGT. Approximately 70% of the earth is covered in water, with seawater constituting 97% of this (227). It is estimated that our planet may be home to one trillion microbial species, many of which may reside in our oceans, with 99.99% of these organisms yet to be discovered (228). The environment has already proven to be a crucial vector in the mobilisation and transfer of ARGs, with many clinically important genes including $bla_{\text{CTX-M}}$ (originating from *Kluyvera species*) and $bla_{\text{OXA-48}}$ (originating from *Shewanella species*) believed to have been mobilised from the chromosome of environmental species into the genomes of clinically relevant species (105) (229). Yet the environment remains by far the most under investigated element of the One Health initiative.

1.11 Research Aims and Objectives

The aim of this thesis was to improve our understanding of the role of the aquatic environment in the dissemination of AMR of public health significance.

The hypothesis of this thesis is that antimicrobial resistant bacteria of public health significance and their MGEs are shared between humans and the aquatic environment.

The main objectives were as follows:

Objective 1: Assessment of recreational waters and sewage for the presence of ESBL-PE and CPE.

Objective 2. Preliminary characterisation of bacterial isolates and selection for whole genome sequencing.

Objective 3. Comparison of environmental isolates to clinical isolates via high-resolution genomic analysis and examination for linkages.

CHAPTER 2: METHODS

2.1 Methods

To avoid repetition for the reader the methodology is presented in this thesis in the following format: an individual methods section has been included in the following chapters: Chapter 3 (Section 3.3), Chapter 4 (Section 4.3) and Chapter 5 (Section 5.3). Additional information relating to the methodologies has been included in the Appendices and is referred to in Sections 3.3, 4.3 and 5.3, where relevant.

CHAPTER 3: INDISTINGUISHABLE NDM-PRODUCING *ESCHERICHIA COLI* ISOLATED FROM RECREATIONAL WATERS, SEWAGE, AND A CLINICAL SPECIMEN IN IRELAND, 2016 TO 2017

Preamble to Chapter 3: Statement of contribution

This chapter consists of work carried out as part of a collaborative project between the Antimicrobial Resistance and Microbial Ecology Group, National University of Ireland, Galway; the National Carbapenemase-Producing *Enterobacterales* Reference Laboratory, Department of Medical Microbiology, University Hospital Galway; the Environmental Health Service, HSE West and Galway County Council. I carried out sample collection along with Dr. Carina Brehony, Dr. Dearbháile Morris, Mr. Paul Hickey and Mr. Shane Keane. I performed filtration of water samples alongside Dr. Carina Brehony and Mr. James Killeen. I screened all samples for CPE and carried out MALDI-TOF, antimicrobial susceptibility testing and PFGE. Ms. Elaine McGrath carried out real-time PCR. Ms. Ann Dolan facilitated gaining access to the sewage supply. I prepared the published manuscript with Dr. Dearbháile Morris and Prof. Martin Cormican. All co-authors reviewed the manuscript prior to submission. This study was published in Eurosurveillance, in April 2017 (230).

3.1 Abstract

The role that the aquatic environment plays in the dissemination of carbapenemase-producing *Enterobacterales* (CPE) is largely unknown. This study aimed to investigate this by examining recreational water for the presence of CPE. New Delhi metallo-β-lactamase (NDM)-producing *Enterobacterales* were identified in fresh water and seawater samples collected at two beaches located near an untreated human sewage ocean discharge. NDM-producing *Escherichia coli* isolated from the sewage collection system, the sewage storage tank and the outflow were 100% identical by pulsed-field gel electrophoresis to those isolated from a fresh water stream on one of the beaches, and to a clinical isolate. Similarly, NDM-producing *Klebsiella pneumoniae* isolated from the sewage collection system and the sewage storage tank were closely related to isolates obtained from seawater samples collected at the two beaches, and to a human isolate. These findings raise concerns regarding the potential for sewage discharges to contribute to the spread of CPE.

3.2 Background

The rapid dissemination of carbapenemase-producing *Enterobacterales* (CPE) in Europe and worldwide is making the delivery of effective healthcare an increasing challenge (164). The number of CPE confirmed by the national reference laboratory in humans in Ireland has increased every year, rising from 48 in 2013, to 369 in 2016 (231). In 2016, the three most commonly reported carbapenemases in Ireland were *Klebsiella pneumoniae* carbapenemase (KPC), carbapenem-hydrolysing oxacillinase-48 (OXA-48), and New Delhi metallo β-lactamase (NDM) (231). The NDM gene is primarily plasmid-encoded, enabling its easy transfer between bacterial species. The plasmids are diverse and usually harbour a large number of other resistance genes (112). The NDM gene has been detected extensively in the Indian

subcontinent where it has been reported from both environmental and clinical sources (232). Rapid global spread has been aided greatly by intercontinental travel (233). However, a recent study in 2016 reported an outbreak of NDM-1-producing *Enterobacterales* in a number of hospitals in Ireland, where links to foreign travel were not identified (234).

In Europe, a number of studies have reported the presence of CPE in recreational water, including Verona integron-encoded metallo-beta-lactamase (VIM) producing *K. pneumoniae* in a river in Switzerland in 2013 (235), KPC-producing *E. coli* in a river in Portugal in 2012 (236), VIM-1, VIM-34, and IMP-type metallo-beta-lactamase (IMP)-8 producing *E. coli* in the same Portuguese river in 2016 (237), and NDM-1 producing *K. pneumoniae* in the River Danube in Serbia, in 2016 (238). This study aimed to examine recreational water as a reservoir for CPE.

3.3 Methods

3.3.1 Recreational water and sewage sample sites

In 2016, we identified a beach (Beach A) in Ireland, used for bathing and recreation, which is crossed by two fresh water streams (Stream A and Stream B), flowing from the surrounding countryside (Figure 3.1). Sampling was performed in the period May to September 2016 (Table 3.1). The detection of NDM-producing *E. coli* in these waters prompted subsequent additional sampling of the streams. As untreated human sewage was being discharged into the sea in the vicinity of the beach, and the fresh water streams can become immersed in seawater at high tide, sewage was evaluated as a potential source. The sewage system is not linked to any hospital or long-term care facility that we are aware of. Sewage samples included samples from the collection system, the storage tank and the outflow (Figure 3.1). Further sampling of the fresh water streams and sewage

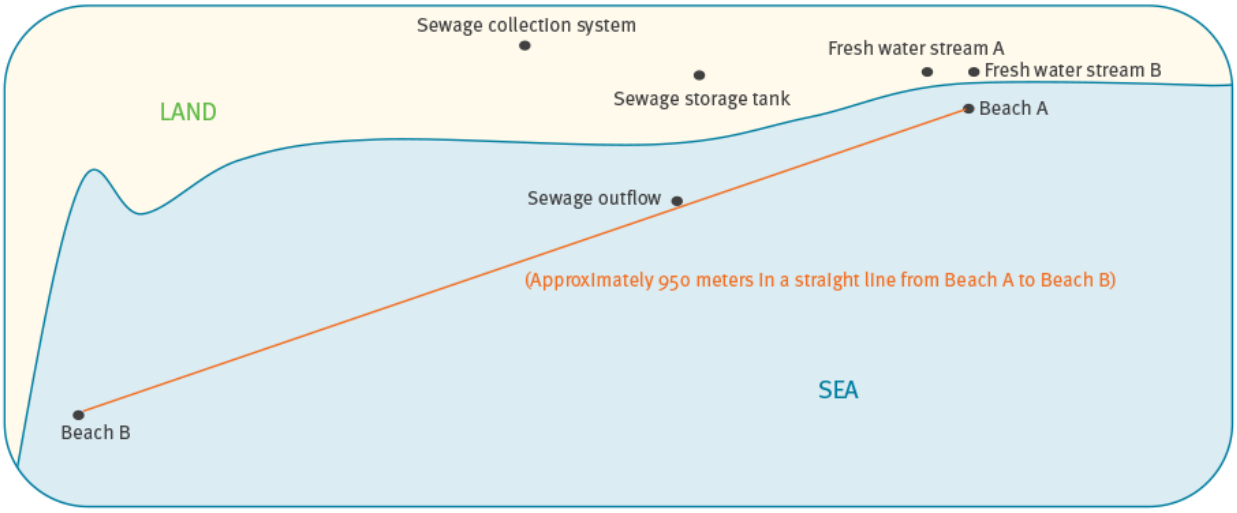


Figure 3.1 Schematic diagram of water and sewage sampling points and their location relative to each other, Ireland, 2016–2017.

sites was carried out in January 2017. In addition to this, seawater from Beach A and from a second beach (Beach B), ca 950m in a direct line from Beach A were examined. Water samples were collected (30L/sample) in sterile 5 L containers (x6), while the sewage samples were collected in sterile 1 L glass bottles. The majority of the seawater samples were collected by local Environmental Health Officers, in line with their sampling schedule for quality testing of the recreational water. All samples were transported to the laboratory and processed within 24 h of collection. Samples which were not processed immediately were stored in the dark at 2-8 °C until processed.

3.3.2 Processing of samples

We applied a previously described method (CapE), to examine the fresh water and seawater samples for the presence of CPE (239). This large volume filtration system was developed via modifications of the Filta-Max system, and contains a high-pressure stainless steel 142 mm filter holder. This holder was attached to the outlet hose of the sampling rig and the bacteria captured on 0.45 µm membrane filters (Merck Millipore Ltd. Cork, Ireland) (239). Following filtration and overnight enrichment of the membrane filters, the

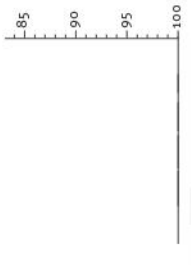
samples were sub-cultured onto Brilliance CRE agar (Oxoid). Sewage samples were examined by direct plating onto Brilliance CRE agar. Following purification, presumptive isolates were identified to species level by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry, and antimicrobial susceptibility testing was performed and interpreted in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (Appendix 1, Appendix 3) (240). Carbapenemase-encoding genes were detected by real-time PCR, as outlined in Appendix 2, and as previously described (241) (242) (194) (243). Typing of NDM-producing *Enterobacterales* was not performed at the variant level, but pulsed-field gel electrophoresis (PFGE) was performed on all isolates, as outlined previously (244). PFGE profiles of NDM-producing *Enterobacterales* isolated from recreational water and sewage samples were compared with PFGE profiles of NDM-producing *Enterobacterales* isolated from clinical specimens.

3.4 Results

3.4.1 Findings of New Delhi metallo β-lactamase (NDM)-producing *E. coli*

Of eight fresh water samples from Stream B, NDM-producing *E. coli* were isolated from two samples, which were collected on 13 July and 24 August 2016. NDM-producing *E. coli* were also isolated in samples collected on 15 September 2016 from the sewage collection system (one of two samples), the storage tank (one of two samples) and the sewage outflow (one of one sample) (Table 3.1). All isolates were resistant to ampicillin, cefotaxime, cefoxitin, cefpodoxime, ceftazidime, ciprofloxacin, ertapenem, meropenem and nalidixic acid. Based on comparison of the environmental and clinical NDM-producing *E. coli* isolates via PFGE profiles, one clinical *E. coli* was included for further comparison. The isolates obtained from the fresh water and sewage samples were indistinguishable by PFGE analysis from this clinical

isolate, which was submitted to the National Carbapenemase-Producing *Enterobacterales* Reference Laboratory Service (NCPERLS) in early 2016 (Figure 3.2).



Isolate number	Bacterium	Source of isolate	Date sample was taken
418	*E. coli*	Fresh water stream B	13 Jul 2016
523	*E. coli*	Fresh water stream B	24 Aug 2016
567	*E. coli*	Sewage storage tank	15 Sep 2016
574	*E. coli*	Sewage outflow	15 Sep 2016
581	*E. coli*	Sewage collection system	15 Sep 2016
C2	*E. coli*	Clinical specimen	Feb 2016

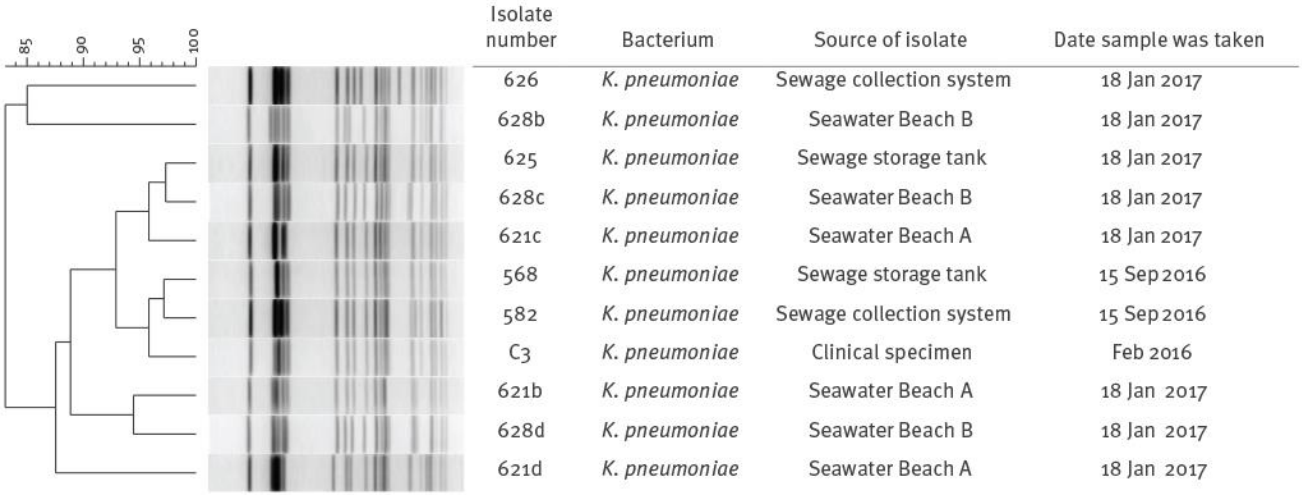
Figure 3.2 PFGE analysis of New Delhi metallo-beta-lactamase-producing Escherichia coli isolated from fresh water, sewage and a clinical source in Ireland, 2016–2017.
PFGE: pulsed-field gel electrophoresis. PFGE typing was performed with Xba1. All five isolates were 100% similar by PFGE.

Table 3.1 Overview of sampling sites, dates and detection of carbapenemase-producing Enterobacterales in a coastal region in Ireland, 2016–2017.

Sample site	Date of sampling	Carbapenemase-producing *Enterobacteriaceae*
Fresh water Stream A	25 May 2016	Not detected
	22 Jun 2016	Not detected
	13 Jul 2016	Not detected
	10 Aug 2016	Not detected
	24 Aug 2016	Not detected
	7 Sep 2016	Not detected
	15 Sep 2016	Not detected
	18 Jan 2017	Not detected
Fresh water Stream B	25 May 2016	Not detected
	22 Jun 2016	Not detected
	13 Jul 2016	NDM-producing *E. coli*
	10 Aug 2016	Not detected
	24 Aug 2016	NDM-producing *E. coli*
	7 Sep 2016	Not detected
	15 Sep 2016	Not detected
	18 Jan 2017	Not detected
Sewage storage tank	15 Sep 2016	NDM-producing *E. coli*
		NDM-producing *K. pneumoniae*
	18 Jan 2017	NDM-producing *K. pneumoniae*
Sewage collection system	15 Sep 2016	NDM-producing *E. coli*
		NDM-producing *K. pneumoniae*
	18 Jan 2017	NDM-producing *K. pneumoniae*
Sewage outflow	15 Sep 2016	NDM-producing *E. coli*
Seawater Beach A	18 Jan 2017	NDM-producing *K. pneumoniae*
Seawater Beach B	18 Jan 2017	NDM-producing *K. pneumoniae*

3.4.2 Findings of New Delhi metallo beta-lactamase (NDM)-producing *K. pneumoniae*

NDM-producing *K. pneumoniae* were isolated from two of three sewage sampling sites on 15 September 2016 and two of two sewage sampling sites on 18 January 2017. NDM-producing *K. pneumoniae* were also detected in seawater samples collected at Beach A and Beach B on 18 January 2017 (Table 3.1). These isolates were resistant to ampicillin, cefotaxime, cefoxitin, cefpodoxime, ceftazidime, ciprofloxacin, ertapenem, gentamicin, kanamycin, meropenem, nalidixic acid and tetracycline. Again, based on comparison of the environmental and clinical NDM-producing isolates via PFGE profiles, one clinical *K. pneumoniae* was included for further comparison. PFGE analyses of isolates from sewage, seawater and this clinical isolate (from NCPERLS) showed isolates to be between 83% and 97% similar (Figure 3.3).



Isolate number	Bacterium	Source of isolate	Date sample was taken
626	*K. pneumoniae*	Sewage collection system	18 Jan 2017
628b	*K. pneumoniae*	Seawater Beach B	18 Jan 2017
625	*K. pneumoniae*	Sewage storage tank	18 Jan 2017
628c	*K. pneumoniae*	Seawater Beach B	18 Jan 2017
621c	*K. pneumoniae*	Seawater Beach A	18 Jan 2017
568	*K. pneumoniae*	Sewage storage tank	15 Sep 2016
582	*K. pneumoniae*	Sewage collection system	15 Sep 2016
C3	*K. pneumoniae*	Clinical specimen	Feb 2016
621b	*K. pneumoniae*	Seawater Beach A	18 Jan 2017
628d	*K. pneumoniae*	Seawater Beach B	18 Jan 2017
621d	*K. pneumoniae*	Seawater Beach A	18 Jan 2017

Figure 3.3 PFGE analysis of New Delhi metallo-beta-lactamase-producing Klebsiella pneumoniae isolated from seawater, sewage and a clinical source in Ireland, 2016–2017. *PFGE: pulsed-field gel electrophoresis. PFGE typing was performed with Xba1. The 11 isolates were between 83% and 97% similar by PFGE (n = 11 isolates).*

3.5 Discussion

In this study, we identified NDM-producing *Enterobacterales* in environmental water samples collected at two adjacent beach sites in Ireland. As far as we are aware, this is the first such finding in bathing seawater in Europe. We consider that contamination of the environment with NDM-producing *Enterobacterales* from a human sewage outflow is likely to be the source, and that the fresh water streams were contaminated by backwash of sewage onto the beach by tidal currents. The presence of NDM-producing *Enterobacterales* in the bathing water (seawater) and at a separate bathing site ca 950m in a direct line indicates the extent of this contamination. It is important to note that by the established regulatory standards, the bathing water quality in the area concerned has been consistently of sufficient quality (245). Notwithstanding compliance with regulatory standards however, it is reasonable to conclude that those using a beach such as this for recreational purposes might be at least intermittently exposed to NDM-producing *Enterobacterales*. Although, to date, there is no evidence that NDM-producing *Enterobacterales* have been acquired as a result of exposure to this beach environment, Leonard et al. have recently reported on the level of risk of exposure to antibiotic resistant bacteria in coastal waters and its relationship to different types of water sports (214).

It appears therefore that there is potential for environmental contamination to contribute to a transition of CPE from largely healthcare-associated organisms, to organisms affecting the general population and the veterinary sector. From a public health perspective, these findings focus attention on the need to accelerate programmes to cease discharge of untreated sewage into the environment. This practice should be unacceptable in the context of discharges in the vicinity of popular bathing and recreation areas where human exposure is highly likely.

We consider that our findings point to potential limitations of the use of *E. coli* as an indicator organism for bathing water quality based on the number of colony forming units (CFU) per 100 mL (246). In our view, this approach does not reflect the pathogenicity of some variants of *E. coli*, such as Shiga-toxigenic *E. coli* for which the infectious dose is very low, (>10 CFU/mL) (247).

CHAPTER 4: DETECTION OF OXA-48-LIKE-PRODUCING ENTEROBACTERALES IN IRISH RECREATIONAL WATER

Preamble to Chapter 4: Statement of contribution

This chapter consists of work completed as part of a collaborative project between the Antimicrobial Resistance and Microbial Ecology Group, National University of Ireland, Galway; the National Carbapenemase-Producing *Enterobacterales* Reference Laboratory, the Department of Medical Microbiology, University Hospital Galway; the Environmental Health Service, HSE West; J. Murphy & Sons Limited, Galway; University of Oxford, Oxford, United Kingdom and Institut Pasteur, Paris, France. Influent and effluent sewage samples were processed by Ms. Niamh Cahill, Ms. Aine Varley and Dr. Louise O'Connor, with collection of sewage samples carried out by Ms. Martina Mulligan and Mr. Bryan Ruane. Seawater samples were collected by Mr. Paul Hickey and Mr. Shane Keane. I carried out filtration of seawater and processing of samples for CPE and performed MALDI-TOF and antimicrobial susceptibility testing alongside Ms. Sinéad Ryan. I carried out real-time PCR and prepared DNA for whole genome sequencing. Short-reads were assembled and quality checks were performed by Dr. Keith Jolley, and the genomes uploaded to BIGSdb by Dr. Carina Brehony. Ms. Elaine McGrath provided access to the genomes of the clinical isolates. I performed whole genome sequence analysis, with occasional input from Dr. Carina Brehony. I prepared the manuscript with contributions from Dr. Dearbháile Morris and Prof. Martin Cormican. All co-authors reviewed the manuscript prior to submission. This study was published in Science of the Total Environment, in June 2019 (248).

4.1 Abstract

The rapid dissemination of carbapenemase-producing *Enterobacterales* (CPE) is a major public health concern. The role that the aquatic environment plays in this dissemination is underexplored. This study aimed to examine seawater as a reservoir for CPE. Seawater sampling took place at a bathing site throughout the 2017 bathing season. Each 30 L sample (*n* = 6) was filtered using the CapE filtration system. Wastewater samples (200 mL) (pre-treatment (*n* = 3) and post-treatment (n = 3)) were obtained from a nearby secondary wastewater treatment plant, during the same time period. All samples were examined for CPE. Whole genome sequencing of confirmed CPE was carried out using Illumina sequencing. Isolate genomes were hosted in corresponding BIGSdb databases and analyses were performed using multiple web-based tools. CPE was detected in 2/6 seawater samples. It was not detected in any wastewater samples. OXA-48-like-producing ST131 *Escherichia coli* (Ec_BM707) was isolated from a seawater sample collected in May 2017 and OXA-48-like-producing ST101 *Klebsiella pneumoniae* (Kp_BM758) was isolated from a seawater sample collected in August 2017. The genomes of the environmental isolates were compared to a collection of previously described Irish clinical OXA-48-like-producing *Enterobacterales* (*n* = 105). Ec_BM707 and Kp_BM758 harboured $bla_{\text{OXA-48}}$ on similar mobile genetic elements to those identified in the clinical collection (pOXA-48 fragment in Ec_BM707 and IncL(pOXA-48) plasmid in Kp_BM758). Genetic similarities were observed between Ec_BM707 and several of the clinical *E. coli* ST131, with allele matches at up to 98.2% of 2513 core genome multilocus sequence type (cgMLST) loci. In contrast, Kp_BM758 and the 34 clinical *K. pneumoniae* were genetically distant. The source of the CPE at this site was not identified. The detection of OXA-48-like-producing *E. coli* ST131 and OXA-48-like-producing *K. pneumoniae* ST101 in Irish recreational water is a concern. The

potential for contamination of the aquatic environment to contribute to dissemination of CPE in Europe warrants further study.

4.2 Background

Antimicrobial resistance (AMR) is recognised as one of the biggest threats to public health and global efforts are being made to control this escalating problem (72). Carbapenemase-producing *Enterobacterales* (CPE) are one of the antimicrobial resistant organisms of greatest concern (52). Infections caused by CPE are associated with increased mortality rates and higher healthcare costs (249) (250). In addition to being resistant to the carbapenems, these organisms are frequently co-resistant to several other antimicrobial classes due to the presence of multiple resistance genes (91).

There are many different types of carbapenemases, the most common of which are Oxacillinase-48-like (OXA-48-like), *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-beta-lactamase (NDM) and Verona integron-encoded metallo-beta-lactamase (VIM) (251). The prevalence of each varies between geographical regions (251). OXA-48-like is the most common carbapenemase identified in Ireland, with 394 (73.5%) of 536 clinical cases of CPE in 2018 confirmed as OXA-48-like producers (252). The $bla_{\text{OXA-48}}$ encoding gene is most commonly associated with an IncL(pOXA-48) plasmid (within transposon Tn1999), and this plasmid has been reported in multiple species of the *Enterobacterales* (253) (229). There have also been several recent reports of the detection of a fragment of pOXA-48 (containing $bla_{\text{OXA-48}}$) chromosomally integrated into strains of *Escherichia coli* and *Klebsiella pneumoniae* (253) (254) (255).

The 'One Health' approach recognises that human, animal and environmental health are inextricably linked (72). The environment remains the most under investigated aspect of the One Health triad and the role that it plays in the dissemination of AMR is not well understood (256). A number of studies have

reported the detection of CPE in freshwater bodies in Europe, with several types of carbapenemases including OXA-48-like, KPC, NDM, VIM and IMP identified (219) (235) (237) (238) (257). We recently reported the first detection of NDM-producing *Enterobacterales* in European seawaters, with untreated human sewage discharges identified as the source of CPE (230). The aim of our current study was to investigate if seawaters not receiving any apparent raw sewage discharges may also act as a reservoir of CPE. We also aimed to establish if CPE present in the aquatic environment was similar to CPE present in humans in Ireland during the same time period.

4.3 Methods

4.3.1 Sampling sites and sample collection

Seawater from a bathing site located in the West of Ireland was assessed for the presence of CPE, throughout the 2017 bathing season (Figure 4.1, Appendix 5). A total of six 30 L samples were collected between May and September 2017. Samples were collected in sterile 5 L containers (x6) and were obtained approximately 30 cm below the water's surface, in water at least one metre in depth. The bathing site is located within close proximity to a secondary waste water treatment plant (SWWTP) (Figure 4.1). To the authors knowledge this is the only point source release in this area. Samples (200 mL) of influent (pre-treatment, n = 3) and effluent (post-treatment, n = 3) were collected from this treatment plant during the same time period. The sewage samples were collected in sterile 1 L glass bottles. All seawater and sewage samples were subsequently transported to the laboratory and processed within 24 h of collection. Samples which were not processed immediately were stored in the dark at 2-8 °C until processing took place. The rainfall measurements for the 24 h preceding the seawater sampling dates were obtained from the Informatics Research Unit for Sustainable Engineering (IRUSE) (http://www.iruse.ie/). This data was examined to

determine if stormwater overflows could potentially be associated with the presence of any CPE detected in the samples taken on the corresponding dates.

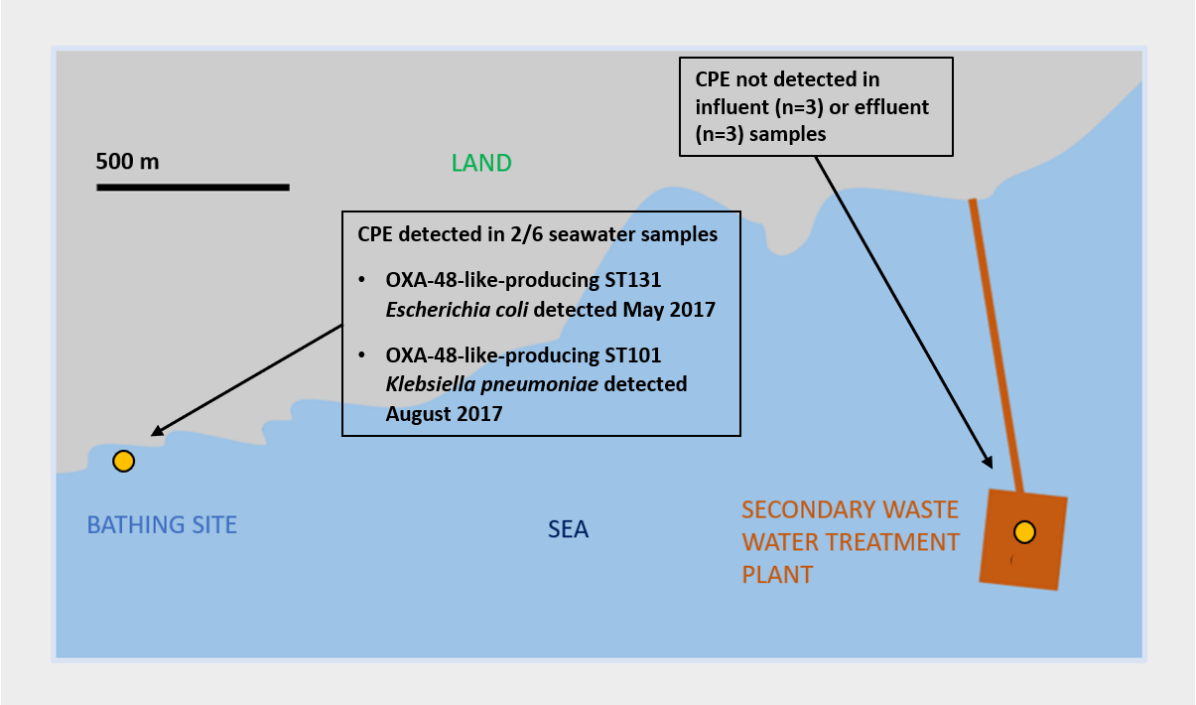


Figure 4.1 Schematic diagram of bathing site and secondary waste water treatment plant, Ireland, 2017. *The sampling points are indicated in yellow.*

4.3.2 Processing of samples and preliminary characterisation of CPE

Following collection, each 30 L seawater sample was filtered through a 0.45 µm membrane filter (Millipore) using the CapE large volume filtration system (239). This filter was then placed into 100 mL of buffered peptone water and incubated overnight at 42 °C. The enrichments were subsequently sub-cultured onto Brilliance CRE agar plates (Oxoid) using a sterile cotton swab. The pre-treatment and post-treatment sewage samples were cultured directly onto this selective agar. The plates were then incubated overnight at 37 °C. Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry (Bruker; Biotyper version 4.1) was used to identify suspect *Enterobacterales* to species level (in line with the manufacturer's

guidelines). Isolates identified as members of the *Enterobacterales* family were stored for further characterisation. Antimicrobial susceptibility profiles were generated for each isolate by performing susceptibility testing against 14 antimicrobials (Appendix 1), via the disk diffusion method (European Committee on Antimicrobial Susceptibility Testing guidelines) (240). Suspect CPE were screened for carbapenemase encoding genes by real-time PCR (bla_{OXA-48}, bla_{KPC} and bla_{NDM} as previously described (241) (242) and bla_{VIM} and bla_{IMP} using an unpublished duplex assay developed by the National Carbapenemase-Producing *Enterobacterales* Reference Laboratory Services (NCPERLS)) (Appendix 2). Appropriate ATCC/NCTC strains were used for quality control purposes (Appendix 3).

4.3.3 Whole genome sequencing analysis

Total genomic DNA was extracted from confirmed CPE using the Qiagen EZ1 Advanced XL automated extraction system. Paired-end short-read sequencing of prepared DNA was performed using an Illumina HiSeq platform. Following quality checks, *de novo* assembly of short reads was carried out using the VelvetOptimiser algorithm (258). The Center for Genomic Epidemiology web tools (http://www.genomicepidemiology.org/) were used to identify AMR determinants and plasmid replicon content (Appendix 4).

4.3.4 Comparison to clinical CPE

Further analysis was carried out to determine if the strains/mobile genetic elements identified in this study were similar to those present in the human population in Ireland during the same time period. The Irish NCPERLS provided access to the genomes of 105 OXA-48-like-producing *Enterobacterales* for this purpose. This clinical collection originated from healthcare facilities throughout Ireland (all isolated during 2016/2017) and have previously been described (259). Genome analysis tools within the *Klebsiella* (http://bigsdb.pasteur.fr/klebsiella/)

and *Escherichia* (https://pubmlst.org/escherichia/) BIGSdb databases were used to examine the genetic relatedness of the two collections. Core genome multi-locus sequence type (cgMLST) comparisons at 2513 loci for *E. coli* and 629 for *K. pneumoniae* were visualized using minimum-spanning trees. This was performed with genomes within the same ST and was generated using the GrapeTree tool in BIGSdb (260). pOXA-48 plasmid comparisons were performed by comparing isolates at 71 loci (present in the pOXA-48 plasmid (JN626286)), as previously described (259).

4.4 Results

4.4.1 Detection of CPE in seawater samples and WWTP samples

CPE was detected in two of six seawater samples examined (Appendix 5). OXA-48-like-producing *E. coli* (isolate ID: Ec_BM707) was detected in a sample collected on May 29th, 2017, while OXA-48-like-producing *K. pneumoniae* (isolate ID: Kp_BM758) was detected in a sample collected on August 14th, 2017. According to data from IRUSE, the daily rainfall measurements in this region during the sampling time period (27th May to 11th September) ranged from 0 mm to 49 mm (average daily rainfall of 4.7 mm). There was a total of 0 mm rainfall recorded in the 24 h preceding May 29th, and 8 mm in the 24 h preceding August 14th. Samples of influent and effluent were collected from a nearby SWWTP during the same time period and examined for CPE. CPE was not detected in any of these influent (n = 3) or effluent (n = 3) samples.

4.4.2 Characterisation of OXA-48-like-producing *E. coli* (Ec_BM707)

Ec_BM707 was non-susceptible to 8/14 of the antimicrobials tested (Table 4.1). It remained susceptible to cefoxitin, chloramphenicol, gentamicin, kanamycin, tetracycline, trimethoprim. *In silico* analysis identified Ec_BM707 as a member of ST131. In addition to $bla_{\text{OXA-48}}$, it co-harboured $bla_{\text{CTX-M-}}$

$_{174}$ and *mdf(A)*. A total of four plasmid replicon types were detected: Col156, IncFIA, IncFIB and IncFII. Ec_BM707 did not harbour an IncL(pOXA-48) plasmid, but analysis indicated that *bla*$_{oxa-48}$ was harboured on a pOXA-48 fragment, as 29/71 pOXA-48-like genes were detected in this isolate. The location of this fragment within the genome is unknown. Ec_BM707 was compared to a collection of previously described clinical OXA-48-like-producing *Enterobacterales* (*n* = 105) (259). Nine of these clinical isolates (eight *E. coli* and one *K. pneumoniae*) carried *bla*$_{OXA-48}$ on a similar pOXA-48-like fragment (the same 29 genes were identified in these isolates). However, the alleles present in Ec_BM707 differed to those found within the nine clinical isolates, with genetic diversity varying at between 14% and 21% of the 29 loci. Of the 55 *E. coli* within the clinical collection, nine belonged to ST131. A cgMLST comparison of Ec_BM707 to the nine ST131 isolates at 2513 loci showed that the closest related clinical isolate (ERR2124250) had only 1.8% allele differences (46/2513) (Figure 4.2). Of the nine clinical ST131, three (including ERR2124250) harboured *bla*$_{OXA-48}$ on a pOXA-48 fragment, with the remaining six harbouring the encoding gene on an IncL(pOXA-48) plasmid.

Table 4.1 Overview of results of antimicrobial susceptibility testing and in silico analyses of genomic data of OXA-48-like-producing Enterobacterales isolated from recreational water, Ireland, 2017.

Isolate ID (sampling date)	Antimicrobial susceptibility testing	Sequence type	Plasmid replicons	Resistance genes
Ec_BM707 (29/05/2017)	**Non- susceptible to:** ampicillin, cefpodoxime, cefotaxime, ceftazidime, ciprofloxacin, ertapenem, meropenem, nalidixic acid	ST131	Col156, IncFIA, IncFIB, IncFII	*blaOXA-48, blaCTX-M-174, mdf(A)*
Kp_BM758 (14/08/2017)	**Non- susceptible to:** ampicillin, cefpodoxime, cefotaxime, cefoxitin, ceftazidime, chloramphenicol, ciprofloxacin, ertapenem, gentamicin, kanamycin, meropenem, nalidixic acid, tetracycline, trimethoprim	ST101	Col440II, IncFIA, IncFIB, IncFII, IncL(pOXA-48), IncR	*aac(3)-Iia, aac(6')-Ib-cr, aadA1, aac(6')-Ib, blaSHV-28, blaOXA-1, blaOXA-9, blaOXA-48, blaCTX-M-15, blaTEM-1A, oqxA, oqxB, fosA, catB3, tet(D), dfrA14*

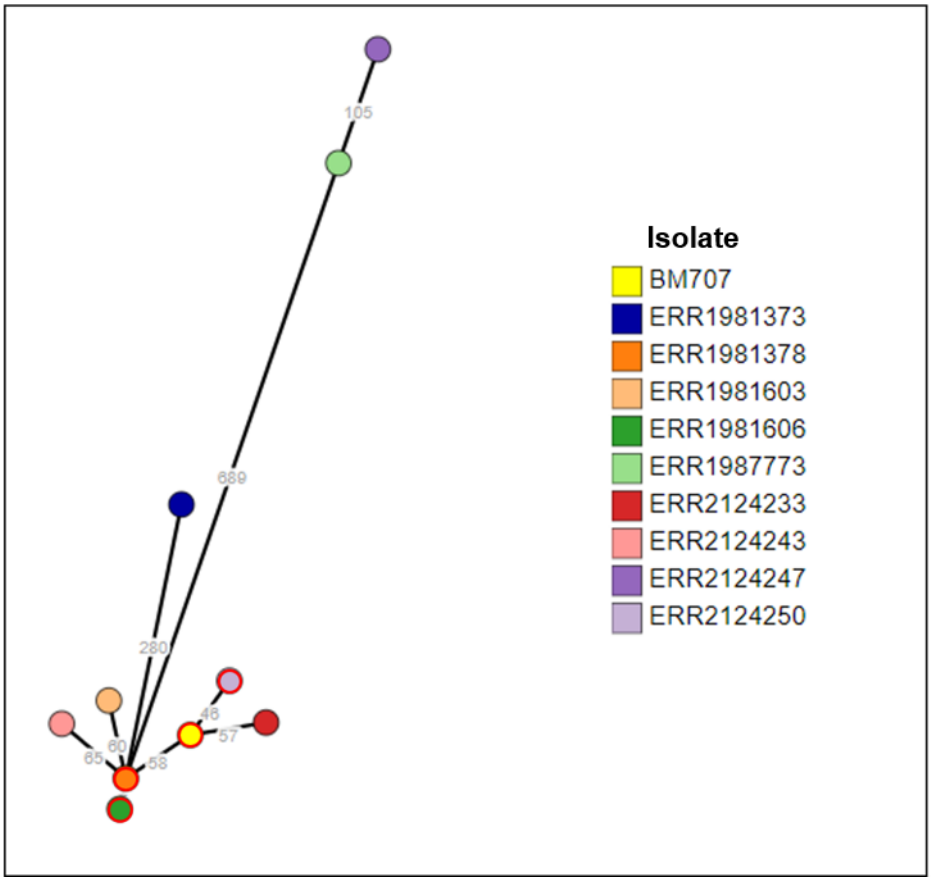


Figure 4.2 Minimum-spanning tree based on cgMLST locus (n = 2513) allele differences among OXA-48-like-producing ST131 E. coli (n = 10). *There are nine clinical isolates and one environmental isolate (BM707). Each node/circle represents one isolate. The number of locus allele differences between nodes are represented by the number on the connecting line. Four of the isolates harboured* bla_{OXA-48} *on a pOXA-48 fragment. These isolates are circled in red. The remaining six isolates all harboured* bla_{OXA-48} *on an IncL(pOXA-48) plasmid.*

4.4.3 Characterisation of OXA-48-like-producing *K. pneumoniae* (Kp_BM758)

Kp_BM758 was non-susceptible to all 14 antimicrobials tested (Table 4.1). It was identified as ST101, and along with bla_{OXA-48}, it co-harboured 15 additional resistance genes, including $bla_{CTX-M-15}$ (Table 4.1). Six plasmid replicon types were detected: Col440II, IncFIA, IncFIB, IncFII, IncL(pOXA-48) and IncR, with bla_{OXA-48} carried on the IncL(pOXA-48) plasmid. Kp_BM758 was compared to the same collection of 105 clinical OXA-48-like-

producing *Enterobacterales.* Like Kp_BM758, 89% (*n* = 93) of the clinical isolates harboured bla_{OXA-48} on an IncL(pOXA-48) plasmid. Kp_BM758 differed at between 9% (*n* = 6) and 14% (*n* = 10) of the 71 pOXA-like loci, when compared to the IncL(pOXA-48) plasmids from those 93 isolates. Of the 34 *K. pneumoniae* within the clinical collection, none were ST101, and BM_758 and the 34 isolates varied at between 72% (*n* = 454) and 85% (*n* = 533) of 629 cgMLST loci.

4.5 Discussion

The dissemination of CPE poses a significant threat to public health. The potential reservoirs and dissemination routes of AMR are complex and are only partially understood. Studies indicate that many clinically relevant resistance genes, such as bla_{OXA-48} and bla_{CTX-M} originated in environmental bacteria, demonstrating that the environment can act as an ideal setting for the horizontal gene transfer of mobile genetic elements (261) (262). To date, many different types of carbapenemases have been isolated from freshwater sources (219) (238) (237) (235) (257). This study investigated the presence of CPE in seawater samples collected at a bathing site during the 2017 bathing season. Over the course of the bathing season we detected two different species of *Enterobacterales* harbouring bla_{OXA-48} (*E. coli* (Ec_BM707) and *K. pneumoniae* (Ec_BM758)).

In a previous study carried out in 2016–2017, we reported the first identification of CPE (NDM-producing *Enterobacterales*) in seawater in Europe (230). The source of CPE at this site was identified as untreated human sewage, which was being discharged nearby. In the present study there were no apparent sources of untreated human sewage discharges in this area. Rainfall levels in the 24 h prior to sampling were low, which makes storm water overflows an unlikely source. CPE was not detected in any of the samples of treated wastewater effluent collected from a nearby SWWTP.

However, this does not rule out treated effluent as a potential source of CPE at this site, as the effluent sample size was small (n = 3), the sample volume was much lower (200 mL) when compared to the bathing water sample volume (30 L) and the method applied (direct plating of sample onto selective agar) had limitations. In a recent publication we have shown that CPE are regularly present in untreated sewage that is processed in this SWWTP (263). The last century has seen vast improvements in the infrastructure of WWTPs. However, WWTPs have not been specifically designed to combat AMR (264), and multiple studies have detected AMR *Enterobacterales* including extended-spectrum β-lactamase-producing *Enterobacterales* and CPE, as well as their mobile genetic elements in effluent from WWTPs (158) (265) (173).

A recent study (259) characterised a collection of 105 OXA-48-like-producing *Enterobacterales* isolated from clinical specimens in Ireland during a similar time-period (2016/2017). High-resolution genomic analysis was used to investigate the genetic relatedness of environmental isolates Ec_BM758 and Ec_BM707 to this collection of clinical CPE. Along with the OXA-48-like gene, Ec_BM707 harboured two additional resistance genes, including CTX-M-174. This CTX-M variant is relatively uncommon and was not identified among the collection of clinical CPE (259). Ec_BM707 belonged to ST131. This sequence type has contributed to the rapid global dissemination of $bla_{\text{CTX-M-15}}$ (266) and is increasingly associated with OXA-48-like-producing *E. coli* (267) (268). ST131 was the most common sequence type identified among the *E. coli* within the Irish clinical collection (9/55). The nine ST131 *E. coli* originated from five different hospitals throughout Ireland showing that ST131 is widely disseminated among OXA-48-like producing *E. coli* within the Irish health care setting (259). A high level of genetic similarity among cgMLST genes was observed between Ec_BM707 and several of the clinical ST131 isolates, indicating that they were closely related (Figure 4.2). Like Ec_BM707, 33% of the clinical ST131 *E. coli* harboured $bla_{\text{OXA-48}}$ on a

pOXA-48 fragment. While chromosomal integration of this fragment type into ST131 *E. coli* has previously been reported (254), without the addition of long read sequencing, the exact location of this fragment within the genome of Ec_BM707 is unknown. Another limitation associated with the use of short read sequencing is the potential for genes of interest to go undetected if they are located at the end of a contig following short read assembly.

Kp_BM758 harboured 15 additional resistance genes, including five further beta-lactamase encoding genes, and these genes were similar to those reported in the clinical collection (259). Kp_BM758 was a member of ST101 which is considered a pandemic clone (269), and has previously been associated with outbreaks of OXA-48-like-producing *K. pneumoniae* in several countries (270) (271). Overall, a high level of genetic diversity was observed between the environmental Kp_BM758 and the 34 Irish clinical *K. pneumoniae* and ST101 was not identified among this clinical *K. pneumoniae* collection. Correspondence with the NCPERLS has confirmed that to date two OXA-48-like-producing ST101 *K. pneumoniae* (both in 2019) have been identified in the Irish healthcare setting (unpublished data, NCPERLS). This indicates that this sequence type is present among clinical isolates of OXA-48-like-producing *K. pneumoniae* in Ireland. Kp_BM758 harboured bla_{OXA-48} on an IncL(pOXA-48) plasmid, the plasmid most commonly associated with carrying this gene, globally (252) (253) (229). Analysis indicated that the IncL(pOXA-48) plasmid carried by Kp_BM758 was not closely related to plasmids found within the clinical collection.

In conclusion, we report the detection of both OXA-48-like-producing ST131 *E. coli* (Ec_BM707) and OXA-48-like-producing ST101 *K. pneumoniae* (Kp_BM758) in Irish recreational water. Similarities were identified between the two environmental isolates and a collection of clinical CPE, with both collections harbouring bla_{OXA-48} on similar mobile genetic elements. While Kp_BM758 was genetically distant when compared to the

34 clinical *K. pneumoniae*, a high level of genetic similarity was found between Ec_BM707 and a number of clinical *E. coli*, with the nearest isolate matching at 98.2% of cgMLST loci. The findings of this study are a cause for concern, especially given the potential for the aquatic environment to act as a favourable setting for the horizontal gene transfer of these important mobile genetic elements between different bacterial strains and species. These findings highlight the need for the environment to be examined more closely for its role in the dissemination of AMR of public health significance.

CHAPTER 5: HIGH-RESOLUTION GENOMIC ANALYSIS OF CARBAPENEMASE-PRODUCING *ENTEROBACTERALES* AND EXTENDED SPECTRUM-BETA LACTAMASE-PRODUCING *ENTEROBACTERALES* ISOLATED FROM THE AQUATIC ENVIRONMENT, SEWAGE AND CLINICAL SPECIMENS

Preamble to Chapter 5: Statement of contribution

This chapter contains work carried out as part of a collaborative study between the Antimicrobial Resistance and Microbial Ecology Group, National University of Ireland, Galway; the National Carbapenemase-Producing *Enterobacterales* Reference Laboratory, the Department of Medical Microbiology, University Hospital Galway; University of Oxford, Oxford, United Kingdom and Institut Pasteur, Paris, France. I carried out preliminary characterisation of isolates and prepared DNA for whole genome sequencing. Short-reads were assembled and quality checks were performed by Dr. Keith Jolley, and the genomes uploaded to BIGSdb by Dr. Carina Brehony and Dr. Sylvain Brisse. Ms. Elaine McGrath provided access to the genomes of the clinical isolates. I performed whole genome sequence analysis, with occasional input from Dr. Carina Brehony and Dr. Carla Rodrigues. I prepared the manuscript for publication with contributions from Dr. Dearbháile Morris and Prof. Martin Cormican. This manuscript is currently under review by co-authors and will be submitted to Water Research by 14th October.

5.1 Abstract

There is a growing understanding that the environment plays an important role in the dissemination of antimicrobial resistance (AMR). However, this role remains largely under investigated. This study aimed to determine if antimicrobial resistant bacterial strains and their mobile genetic elements are shared between humans and the aquatic environment. This was carried out by comparing the genomes of environmental isolates to clinical isolates, using whole genome sequence (WGS) analysis. The environmental isolates consisted of 24 NDM-producing *Enterobacterales* and 31 extended-spectrum β-lactamase-producing *E. coli* (ESBL-PEc). The isolates originated from seawater, fresh water and sewage samples taken at a site in the West of Ireland. The clinical collection contained three NDM-producing *Enterobacterales* and 16 ESBL-PEc. WGS was carried out using an Illumina HiSeq platform and assembly was performed using VelvetOptimiser. AMR gene profiling and plasmid replicon content were assessed using web-based tools. Multilocus-sequence typing (MLST) and core gene MLST (cgMLST) was performed using the online BIGSdb *Klebsiella* and *Escherichia* websites. Plasmid analysis was carried out using reference mapping and comparative analysis was performed using BRIG.

WGS analysis confirmed that the same two strains of NDM-19-producing *Enterobacterales* were repeatedly detected in recreational waters. This was linked to the release of untreated human sewage in this area. The eight NDM-producing *E. coli* belonged to ST167, while the 16 NDM-producing *K. pneumoniae* were ST11. cgMLST results indicate that both the environmental NDM-producing *E. coli* and the environmental NDM-producing *K. pneumoniae* were closely related to two clinical NDM-19-producing *Enterobacterales* (Ec_C2 matched at 2508/2513 loci, Kp_C3 matched at 625/629 loci). All 27 NDM-19-producing Enterobacterales harboured the $bla_{\text{NDM-19}}$ gene on similar/identical IncX3 plasmids. Overall, 12 of the same AMR-associated genes and five plasmid replicon types were identified among

ESBL-PEc originating from seawater, fresh water, sewage and clinical samples, with the closest environmental ESBL-PEc matching with a clinical isolate at 2506/2513 loci (*E. coli* S131). The most prevalent $bla_{CTX\text{-}M}$ gene identified was $bla_{CTX\text{-}M\text{-}15}$, while the most common ST was ST131, both of which were identified among isolates originating from all sample types.

The findings of this study show that indistinguishable or very closely related bacterial strains and their mobile genetic elements are found among humans and the aquatic environment. These findings highlight the importance of including environmental contamination with antimicrobial resistant bacteria within a One-Health approach in the control of AMR.

5.2 Background

A study carried out by the European Centre for Disease Prevention and Control (ECDC) estimates that approximately 33,000 people die in Europe every year due to infections caused by antimicrobial resistant bacteria (within the European Union (EU) and European Economic Area (EEA)) (272). This figure is expected to increase dramatically in the future, unless effective action is taken (74). The 'One Health' approach recognises that antimicrobial resistance (AMR) is a multifaceted problem and that coordinated efforts across human, animal and environmental sectors are necessary to effectively address this major public health concern (World Health Organisation (WHO), 2015). The majority of AMR research to date has focused on the clinical setting and to a lesser extent on animals and food of animal origin (273) (3). While studies relating to AMR in the environment are not as frequent, a growing number of reports are pointing towards the environment acting as an important reservoir, that is potentially aiding in the persistence, transmission and dissemination of AMR (274) (275) (49).

The aquatic environment can become contaminated with antimicrobial resistant bacteria of human and animal origin by several routes, including from sewage outflows and run off from agricultural lands (276). The *Enterobacterales* are a family of gram-negative bacteria that are normal flora of the intestinal tract of humans and animals, but also are frequently associated with human infection (112). Examples include *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*), which are leading causes of both community-associated and hospital-acquired infection (78) (277). Increasing levels of resistance to the cephalosporins and carbapenems has limited treatment options for infections caused by the *Enterobacterales* (112), most notably through the production of enzymes known as extended-spectrum β-lactamases (ESBLs) and carbapenemases. Such is the concern, that carbapenem-resistant *Enterobacterales* (CRE) and third generation cephalosporin-resistant *Enterobacterales* are both on the WHO global priority pathogens list of antimicrobial resistant bacteria for the guidance of research and development of new antimicrobials (75).

Most recent European Antimicrobial Resistance Surveillance Network (EARS-Net) data (278), reveal that the number of invasive infections caused by both *E. coli* (n=3239) and *K. pneumoniae* (n=483) were at their highest levels to-date in Ireland in 2018, with 6.2% of *E. coli* and 8.3% of *K. pneumoniae* reported as being multi-drug resistant (non-susceptible to three or more antimicrobial classes). Overall, 11.6% of *E. coli* and 16.9% of *K. pneumoniae* were confirmed as ESBL producers, while 0.2% (n=5) of *E. coli* and 0.8% (n=4) of *K. pneumoniae* were carbapenemase-producers (all OXA-48) (278). The number of patients with newly confirmed CPE in the Irish health care setting is increasing year on year, with a ten-fold increase observed between 2013 (n=50) and 2018 (n=537) (279). CPE was declared a national public health emergency in Ireland in October 2017 (52).

There are growing reports of the detection of both CPE and ESBL-PE in the aquatic environment (219) (236) (265) (248). In April 2017, we reported the

first detection of CPE in European seawaters. This contamination was as a result of the release of untreated human sewage in this area (230). In our current study we carried out high-resolution genomic analysis on this collection of isolates, as well as on additional CPE and ESBL-producing *E. coli* (ESBL-PEc) obtained from human sewage, fresh water and seawater sources at this site. These genomes were then compared to CPE and ESBL-PEc from clinical specimens to investigate if antimicrobial resistant bacterial strains and their mobile genetic elements are shared between humans and the aquatic environment.

5.3 Methods

5.3.1 Strain collection and origin of isolates

A collection of 74 *Enterobacterales* were examined in this study. This collection consisted of 27 CPE and 47 ESBL-PEc, which were isolated from clinical specimens, seawater, fresh water and human sewage, as outlined in Figure 5.1.

Environmental isolates (n=55)

The environmental isolates examined in this study were obtained from seawater, fresh water and sewage samples collected at a site in the West of Ireland (Figure 5.1). A subset of these isolates were obtained during a previously published study carried out between May 2016 and January 2017 (230), with additional isolates added to this collection following further sampling. In total, 53 samples were collected from 17 sample points (Figure 5.1), between May 2016 and September 2017. Seawater samples (n=11) were collected at two adjacent beaches at this site (point 6 and point 17, Figure 5.1). Fresh water samples (n=29) were collected from two streams which flow onto Beach A, and a river which flows onto Beach B. Sewage samples (n=13) were collected at five different points within the same

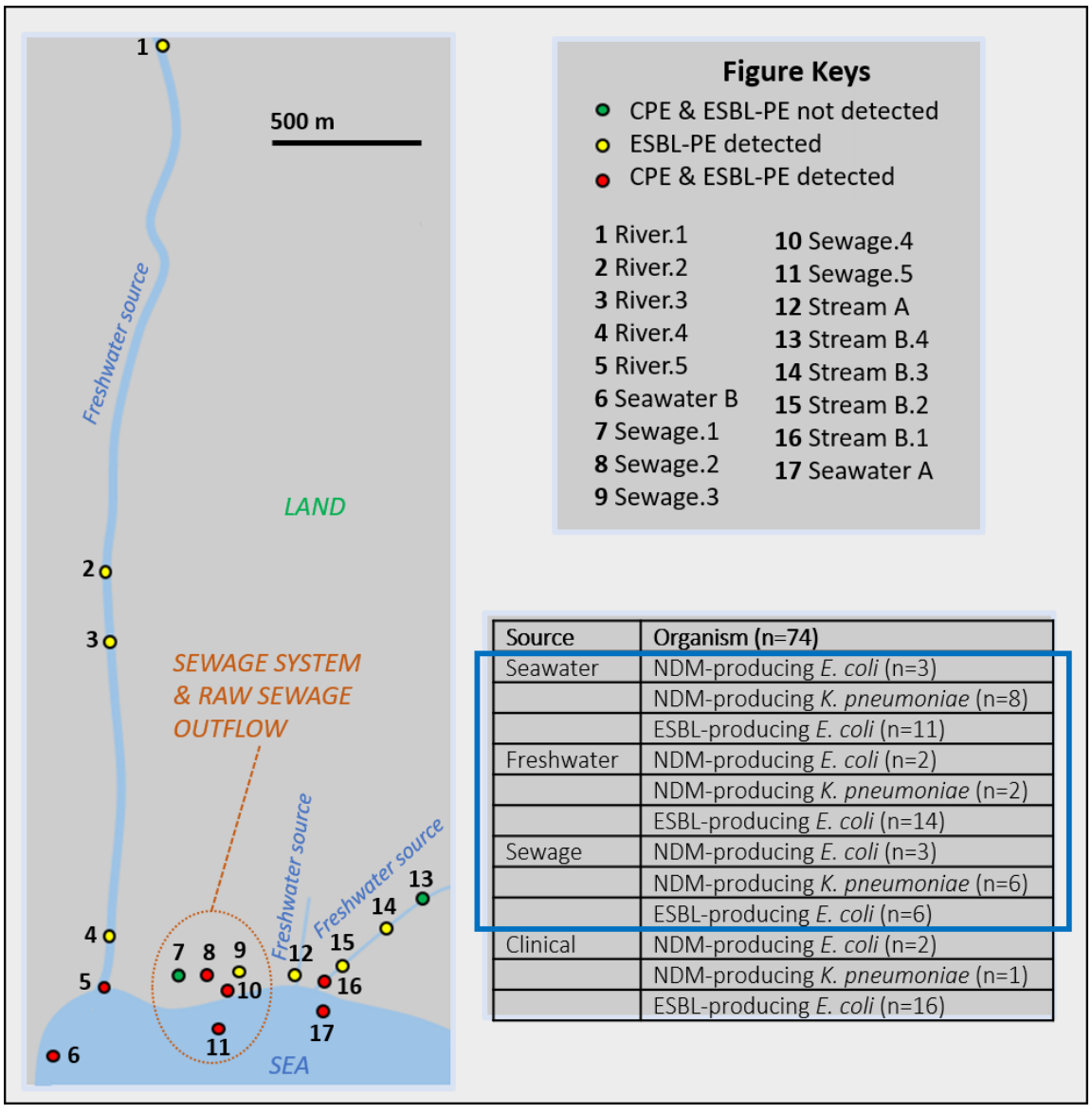
municipal sewage collection system. This sewage is released untreated into the sea at a point between the two beaches (point 11, Figure 5.1). Details relating to the 53 individual samples, including sample dates, are outlined in Appendix 6.

Samples were collected, processed and examined for CPE as described in Sections 4.3.1 and 4.3.2 (248). In addition to CPE, the samples were also examined for ESBL-PE. This was carried out using ChromID ESBL agar plates (Biomerieux). Antimicrobial susceptibility testing against 14 antimicrobials (248) was carried out on all suspect ESBL-PE isolates and ESBL-production was confirmed phenotypically (cefpodoxime (10µg) and cefpodoxime (10µg) plus clavulanic acid (1µg) (Mast Diagnostics)) by the combination disk diffusion method, in accordance with EUCAST criteria (Appendix 1) (240). All isolates phenotypically positive for ESBL-production were examined for CTX-M encoding genes (*bla*$_{\text{CTX-M Group-1}}$, *bla*$_{\text{CTX-M Group-2}}$ and *bla*$_{\text{CTX-M Group-9}}$) by real-time PCR, as previously described (194). Only one isolate of CPE and/or ESBL-PE per species type was selected per sample for whole genome sequencing (WGS) if the antimicrobial susceptibility profiles were the same. A total of 55 isolates were selected following this preliminary characterisation.

Clinical isolates (n=19)

The National Carbapenemase-Producing *Enterobacterales* Reference Laboratory Services (NCPERLS), based at University Hospital Galway routinely sequences the genomes of confirmed CPE. Following correspondence with the NCPERLS the genomes of two clinical NDM-producing *E. coli* and one clinical NDM-producing *K. pneumoniae* were selected for inclusion in this study. These genomes were selected based on the NDM variant that they were harbouring, with two of these isolates previously confirmed (by pulsed-field gel electrophoresis) as being closely related to the initial collection of environmental NDM-producing *Enterobacterales* (230).

The Antimicrobial Resistance and Microbial Ecology group has a large archived collection of clinical ESBL-PE, which were obtained from the clinical microbiology lab, University Hospital Galway. A total of 16 ESBL-PE were selected from this collection for WGS, based on the corresponding time frame that the environmental sampling took place (one isolate per month was selected at random between June 2016 and September 2017).



Source	Organism (n=74)
Seawater	NDM-producing *E. coli* (n=3)
	NDM-producing *K. pneumoniae* (n=8)
	ESBL-producing *E. coli* (n=11)
Freshwater	NDM-producing *E. coli* (n=2)
	NDM-producing *K. pneumoniae* (n=2)
	ESBL-producing *E. coli* (n=14)
Sewage	NDM-producing *E. coli* (n=3)
	NDM-producing *K. pneumoniae* (n=6)
	ESBL-producing *E. coli* (n=6)
Clinical	NDM-producing *E. coli* (n=2)
	NDM-producing *K. pneumoniae* (n=1)
	ESBL-producing *E. coli* (n=16)

Figure 5.1 Schematic diagram of seawater, fresh water and sewage sampling points, Ireland, 2016-2017. *The organisms obtained at this site are outlined in the table, within the blue box. CPE: carbapenemase-producing Enterobacterales, ESBL-PE: extended-spectrum ß-lactamase-producing Enterobacterales.*

5.3.2 Whole genome sequencing and analysis

DNA extraction was performed on selected isolates using the Qiagen EZ1 Advanced XL automated extraction system. Prepared DNA was sequenced (paired-end short-read sequencing) via an Illumina Hiseq platform, housed at Wellcome Trust Centre for Human Genetics, University of Oxford. Following *de novo* assembly of short reads using the VelvetOptimiser algorithm (258), the genomes were uploaded onto online *Escherichia* BIGSdb (https://pubmlst.org/escherichia/) and *Klebsiella* BIGSdb (http://bigsdb.pasteur.fr/klebsiella/) databases.

Multilocus-sequence typing (MLST) of all genomes and core gene MLST (cgMLST) of *K. pneumoniae* (629 genes) and *E. coli* (2513 genes) genomes was performed using corresponding BIGSdb databases. Minimum spanning trees based on cgMLST were generated for collections of NDM-producing *E. coli*, NDM-producing *K. pneumoniae* and ESBL-PEc using the Grapetree tool within BIGSdb (260). *E. coli* genomes were assigned to ST clonal complexes using the EnteroBase website (http://enterobase.warwick.ac.uk/species/index/ecoli). AMR gene profiling, plasmid replicon content and Fim type (for *E. coli* ST131) were assessed using the Center for Genomic Epidemiology web tools (http://www.genomicepidemiology.org/) (Appendix 4).

Plasmid reference mapping was carried out for all NDM-producing *Enterobacterales* using SMALT (version 0.7.4) (280) and Samtools (version 01.1.11) (281). Appropriate reference genomes were selected using the National Center for Biotechnology Information website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Short reads were mapped against IncX3 reference plasmids: pSCM96-2 (NZ_CP028718.1 (46161 bp)) and pKW53T (KX214669.1 (46161 bp)) and viewed using Artemis (version 18.0.2) (282). A comparative analysis of five NDM-producing *Enterobacterales* genomes was performed against the two reference plasmids and a graphic representation obtained using BRIG (http://sourceforge.net/projects/brig/).

The five isolates selected for the comparative analysis included the first *E. coli* and *K. pneumoniae* isolated from an environmental sample and the three clinical isolates.

5.4 Results

5.4.1 Characterisation of CPE

CPE was detected in 39.5% (21/53) of samples taken at a site in the West of Ireland (Figure 5.1), in 91% (10/11) of seawater samples,13.8% (4/29) of fresh water samples, and 53.8% (7/13) of sewage samples taken over a 14-month time period. NDM-producing *K. pneumoniae* were detected in 16 samples and NDM-producing *E. coli* were detected in eight samples, with both species detected in three samples. The genomes of these 24 environmental NDM-producing *Enterobacterales* were examined in this study. *In silico* analysis identified NDM-19 as the NDM variant harboured by all 24 isolates. This collection of genomes was compared to the genomes of three clinical NDM-19 producing *Enterobacterales* (two *E. coli* and one *K. pneumoniae*). These isolates are the only NDM-19-producing *Enterobacterales* that have been identified in the Irish clinical setting to date (correspondence with NCPERLS).

All 17 *K. pneumoniae* (16 environmental, one clinical) belonged to ST11 and in addition to NDM-19, they harboured 13 additional genes associated with AMR, along with four plasmid replicon types (Table 5.1). A cgMLST comparison of the 17 NDM-producing *K. pneumoniae* (at 629 loci) showed only a 0.6% (4/629) genetic divergence within this collection, with 11/17 isolates matching at all 629 loci (Figure 5.2).

The eight environmental NDM-producing *E. coli*, along with one of the two clinical NDM-producing *E. coli* (Ec_C2), belonged to ST167. A total of three plasmid replicon types were detected in all nine ST167 isolates (Table 5.1). No other genes associated with AMR were identified among these isolates.

Less than a 0.2% (5/2513) genetic divergence within the 2513 cgMLST alleles was identified among the collection of ST167 *E. coli* (Figure 5.3). The second clinical NDM-producing *E. coli* (Ec_C1) belonged to ST205. Overall, 14 additional AMR-associated genes, along with nine plasmid replicon types were detected within this genome.

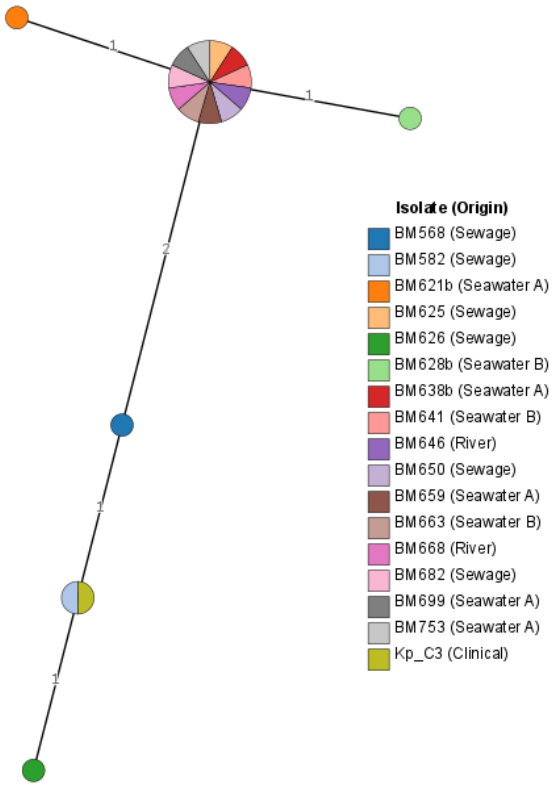


Figure 5.2 Minimum-spanning tree based on cgMLST locus (n=629) allele differences amongst NDM-19-producing ST11 K. pneumoniae (n=17). *Each node/circle represents one isolate. The number of locus allele differences between nodes are represented by the number on the connecting line.*

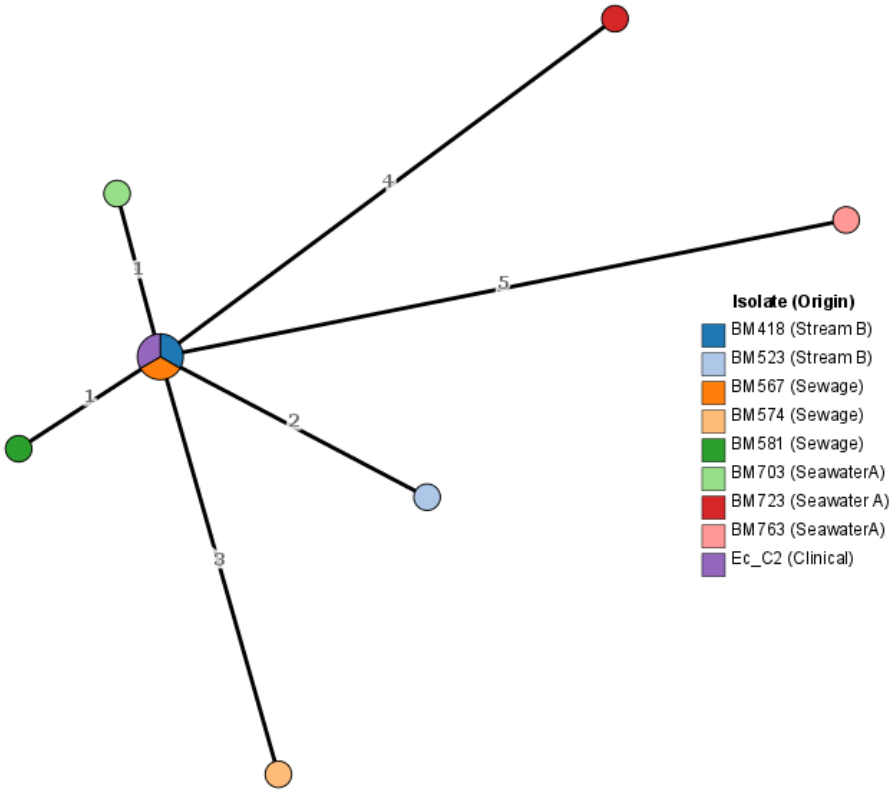


Figure 5.3 Minimum-spanning tree based on cgMLST locus (n=2513) allele differences amongst NDM-19-producing ST167 E. coli (n=9). *Each node/circle represents one isolate. The number of locus allele differences between nodes are represented by the number on the connecting line.*

Table 5.1 Overview of sequence types, antimicrobial resistance genes and plasmid replicon types identified in a collection of environmental and clinical NDM-19-producing Enterobacterales. **cgMLST results indicates that isolates are closely related. Amino: aminoglycoside, β-lac: β-lactam, Fluor: fluoroquinolone, Fos: fosfomycin, MLS: macrolide-lincosamide-streptograminB, Sul: sulphonamide, Tet: tetracycline, Trim: trimethoprim.*

Sequence type, count (source)	Antimicrobial Resistance genes								Plasmid Replicons
	Amino.	β-lac.	Fluor.	Fos.	MLS.	Sul.	Tet.	Trim.	
ST11 *K. pneumoniae,* n=17* (sewage (n=6), seawater (n=8), fresh water (n=2), clinical (n=1))	*aadA2* (n=17), *armA* (n=17)	*bla*$_{NDM-19}$ (n=17), *bla*$_{SHV-182}$ (n=17), *bla*$_{TEM-1B}$ (n=17)	*oqxA* (n=17), *oqxB* (n=17)	*fosA* (n=17)	*mph(A)* (n=17), *mph(E)* (n=17), *msr(E)* (n=17)	*sul1* (n=17)	*tet(B)* (n=17)	*dfrA12* (n=17)	IncFIB (n=17), IncFII (n=17), IncHI1B (n=17), IncX3 (n=17)
ST167 *E. coli,* n=9* (sewage (n=3), seawater (n=3), fresh water (n=2), clinical (n=1))		*bla*$_{NDM-19}$ (n=9)							Col (n=9), Col440II (n=9), IncX3 (n=9), IncI1 (n=1)
ST205 *E. coli,* n=1 (clinical)	*aac(3)-IId aac(6')-Ib-cr aadA1 aadA5 aph(3'')-Ib aph(6)-Id*	*bla*$_{CMY-42}$ *bla*$_{NDM-19}$ *bla*$_{OXA-1}$ *bla*$_{TEM-1B}$			*mph(A)*	*sul1*	*tet(B)*	*dfrA1 dfrA17*	Col440II, Col8282, ColpVC, IncFIA, IncFIB, IncFII, IncI, IncX3, IncY

Analysis indicated that the 27 CPE all harboured *bla*$_{NDM-19}$ on an IncX3 plasmid. Isolate short reads were mapped to two IncX3 reference plasmids (pSCM96-2 and pKW53T). All 27 NDM-producing *Enterobacterales* mapped to the whole length of both reference plasmid sequences (46161 bp). Comparative analysis which was carried out on five NDM-producing *Enterobacterales* from different isolation sources indicates that the isolates all harboured *bla*$_{NDM}$ on the same/very similar IncX3 plasmid, as illustrated in Figure 5.4. This analysis also identified the presence of *ble*$_{MBL}$, the gene associated with resistance to the cancer drug Bleomycin, which was found to be located beside *bla*$_{NDM-19}$. This gene was present in all 27 CPE isolates.

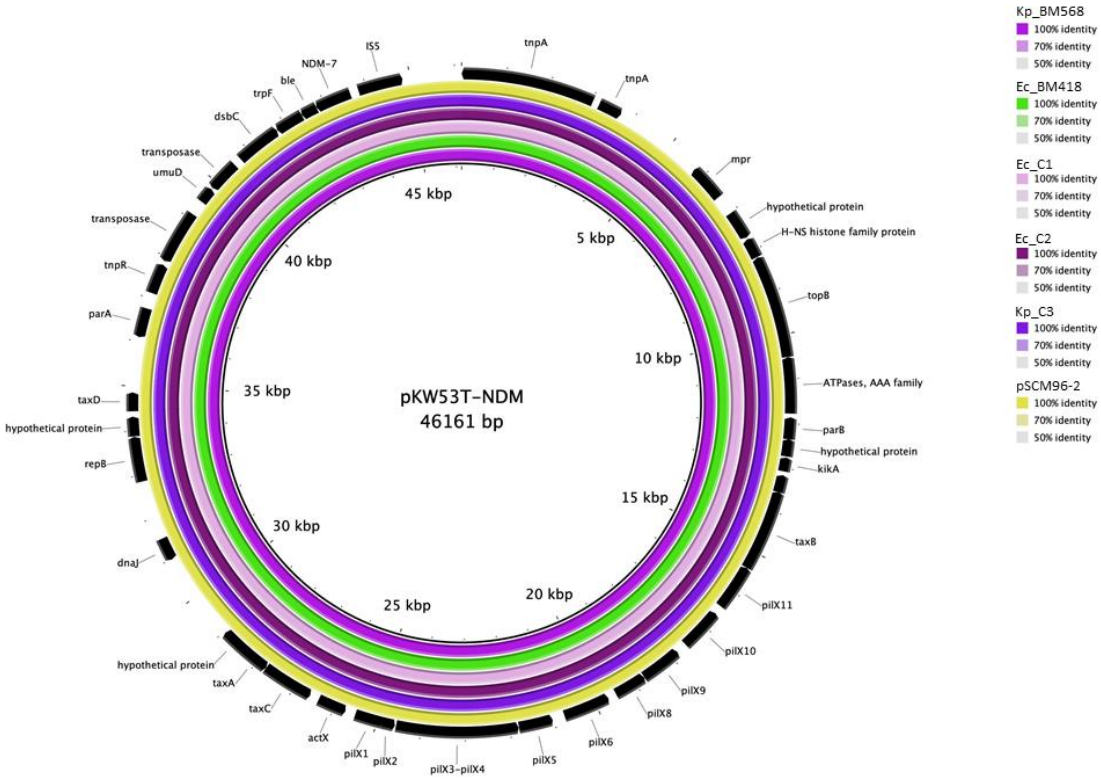


Figure 5.4 Comparative analysis of bla$_{NDM}$ harboring IncX3 plasmids from different isolation sources. *The blaNDM-7–harboring plasmid pKW53T-NDM was used as a reference plasmid. The outermost circle is an annotation of this reference plasmid and shows the direction of transcriptional open-reading frames. Kp_BM558 and Ec_BM418 originated from environmental samples, Ec_C1, Ec_C2 and Kp_C3 from clinical specimens, while pSCM96-2 is an additional reference plasmid.*

5.4.2 Characterisation of ESBL-PEc

ESBL-PE (all *E. coli*) were detected in 31/53 environmental samples collected. Overall, ESBL-PEc were isolated from 55% (16/29) of fresh water samples, 38.5% (5/13) of sewage samples and 91% (10/11) of seawater samples. A total of 31 isolates were selected from this collection for high resolution genomic analyses, along with 16 ESBL-PEc previously isolated from clinical specimens.

In total, 16 different ST were identified within this collection of 47 *E. coli*, with ST131 being the most commonly detected (Figure 5.5, Table 5.2). Of the 16 clinical isolates, 13 were ST131, with the remaining three clinical isolates belonging to ST316, ST2159 and ST8029. ST131 was the only ST that was identified among ESBL-PEc isolated from all four isolation sources (seawater, fresh water, sewage and clinical). ST131 isolates were further classified according to the presence of marker alleles for *fimH* (type 1 fimbriae). FimTyper identified four different *fimH* types among the 20 *E. coli* ST131: H41, which is associated with Clade A, H30, which is associated with Clade C, H89 and H99 (unknown Clades).

Overall, a high level of diversity was found among the collection of 47 *E. coli* genomes, with differences at up to 2420 of 2513 cgMLST loci (Figure 5.5). However, high levels of homology between ESBL-PEc originating from different sources was also identified. A number of strains that originated from seawater samples were also detected in sewage and/or fresh water samples. Isolates identified as being closely related/identical by cgMLST are indicated by the * symbol in Table 5.2 and can be viewed in the minimum spanning tree (Figure 5.5). Several clinical ST131 isolates appear to be closely related to the environmental isolates, with the closest isolate matching at 2506/2513 loci. The collection of 20 ST131 grouped into two distinct clusters, as illustrated in Figure 5.5. The majority of isolates (n=17) made up one cluster, with between 0% and 3.4% (86/2513) allele differences based on 2513 cgMLST detected

among these 17 isolates. A total of 14 isolates in this cluster belonged to ST131-H30, while the remaining three were ST131-H99. The three isolates in the second cluster were ST131-H41 (n=2) and ST131-H89 (n=1).

The most common CTX-M gene identified was *bla*$_{\text{CTX-M-15}}$, with this variant along with *bla*$_{\text{CTX-M-27}}$ detected among isolates originating from all four isolation sources (Table 5.2). Overall, 14 of the ST131 isolates harboured *bla*$_{\text{CTX-M-15,}}$ while the remaining six harboured *bla*$_{\text{CTX-M-27}}$. The ST131 isolates harbouring *bla*$_{\text{CTX-M-27}}$ were two ST131-H30 clinical isolates, one ST131-H41 environmental isolate and three closely related/identical ST131-H89 environmental isolates. Of the three non-ST131 clinical isolates, two carried *bla*$_{\text{CTX-M-15}}$ (*E. coli* ST316 and ST8029), while the remaining isolate (*E. coli* ST2179) harboured *bla*$_{\text{CTX-M-65}}$. *bla*$_{\text{CTX-M-1}}$ (in *E. coli* ST10 and ST23) was only detected from fresh water isolates.

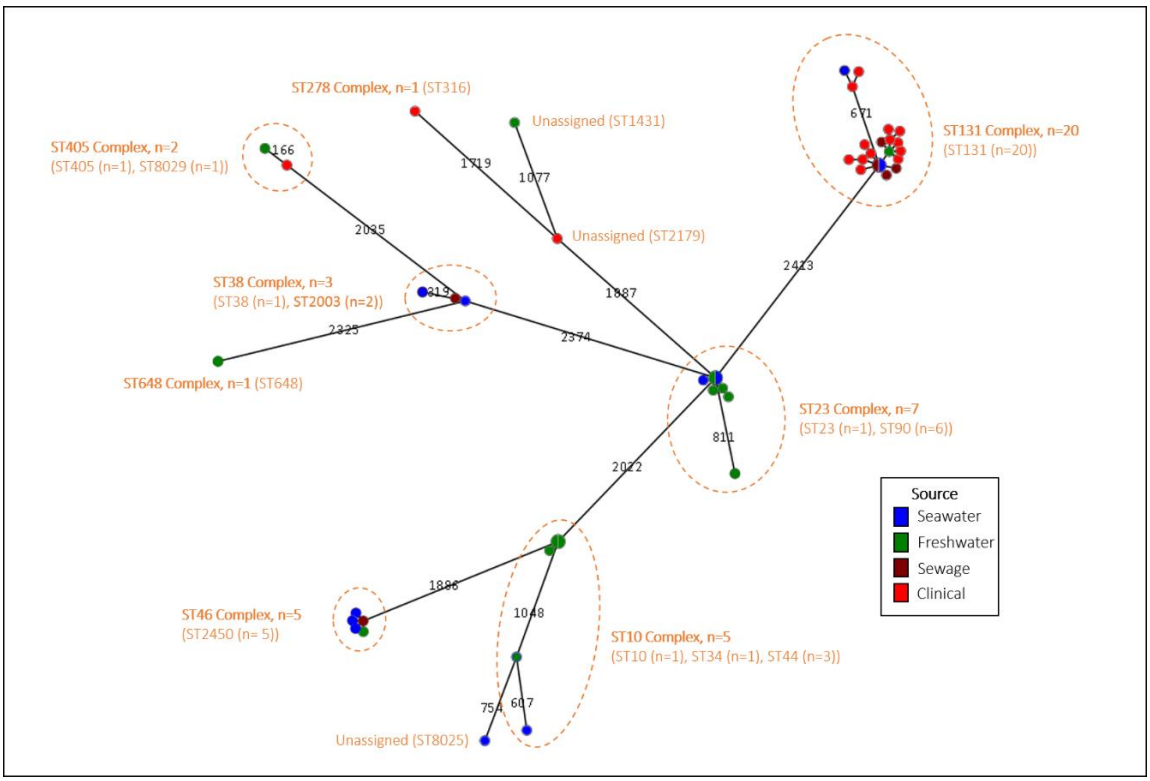


Figure 5.5 Minimum-spanning tree based on 2513 cgMLST allele differences among 47 extended-spectrum β-lactamase-producing E. coli. *Sequence types (ST) and ST complexes are indicated in orange with members of same the complex grouped together by orange dotted circles.*

All isolates were examined for chromosomal mutations associated with AMR and of the 55 ESBL-PEc, only four had no mutations in the *gryA*/*parC*/*parE* genes, which are associated with quinolone resistance. All other isolates had between one and five mutations in these genes. Of the ST131 isolates 18/20 had a total of five mutations in the *gryA* (x2), *parC* (x2) and *parE* (x1) genes. ST131 was the only ST that had up to five mutations in these genes. Of the four ESBL-PEc with no mutations, three of those originated from environmental sources (*E. coli* ST10, ST23 and ST38), while the remaining one was isolated from a clinical specimen (*E. coli* ST316). The ST316 clinical isolate was, however, one of four isolates harbouring an acquired gene associated with quinolone-resistance (*qnrS1*) (Table 5.2).

Table 5.2: Overview of sequence types, antimicrobial resistance genes and plasmid replicon types identified among a collection of environmental and clinical extended-spectrum ß-lactamase-producing-Enterobacterales. **cgMLST results indicates that isolates are closely related. Amino: aminoglycoside, ß-lac: ß-lactam, Fluor: fluoroquinolone, MLS: macrolide-lincosamide-streptograminB, Phen: phenicol, Sul: sulphonamide, Tet: tetracycline, Trim: trimethoprim.*

Sequence type, count (source)	Antimicrobial Resistance genes								Plasmid Replicon
	Amino.	β-lac.	Fluor.	MLS.	Phen.	Sul.	Tet.	Trim.	
ST10, n= 1 (fresh water)	*aadA5*	*bla*$_{CTX-M-1}$				*sul2*		*dfrA17*	IncI1
ST23, n=1 (fresh water)	*aadA5*	*bla*$_{CTX-M-1}$				*sul2*		*dfrA17*	IncFIB, IncFIC, IncI1, IncX1
ST34, n=1 (seawater)	*aadA1*	*bla*$_{CTX-M-15}$		*mph(A)*				*dfrA1*	IncFIB, IncFII
ST38, n=1 (seawater)	*aadA1*, *aph(3')-Ia*, *aph(3'')-Ib*, *aph(6)-Id*	*bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$			*catA1*	*sul2*	*tet(D)*	*dfrA1*	IncFIB, IncFII, IncQ1

ST44, n=3* (fresh water (n=3))	*aac(6')-Ib-cr* (n=3), *aadA5* (n=3)	*bla*$_{CTX-M-15}$ (n=3), *bla*$_{OXA-1}$ (n=3)		*mph(A)* (n=3)	*catB3* (n=3)	*sul1* (n=3)	*tet(B)* (n=3)	*dfrA17* (n=3)	Col (n=3), IncFIA (n=3), IncFIB (n=3), IncFII (n=3), IncI1 (n=2)
ST90, n=6* (fresh water (n=4), seawater (n=2))	*aadA5* (6), *aph(3'')-Ib* (6), *aph(6)-Id* (6)	*bla*$_{CTX-M-27}$ (n=6)		*mph(A)* (n=6)	*floR* (n=6)	*sul1* (n=6), *sul2* (n=6)	*tet(A)* (n=6)	*dfrA17* (n=6)	IncFIA (n=6), IncFIB (n=6), IncFII (n=6), IncN (n=6), Col156 (n=5)
ST131 Clade A (fimH41) (n=2) (clinical (n=1), seawater (n=1))	*aadA5* (n=2), *aph(3'')-Ib* (n=1), *aph(6)-Id* (n=1)	*bla*$_{CTX-M-15}$, (n=1) *bla*$_{CTX-M-27}$ (n=1)		*mph(A)* (n=2), *erm(B)* (n=1)		*sul1* (n=2), *sul2* (n=1)	*tet(A)* (n=1)	*dfrA17* (n=2)	Col156 (n=2), IncB/O/K/Z (n=2), IncFIA (n=2), IncFIB (n=2), IncFII (n=2), IncFI2 (n=1)
ST131 H30 (Clade C), n=14 (clinical (n=11), sewage (n=2), fresh water (n=1))	*aadA5* (n=12), *aac(6')-Ib-cr* (n=11), *aph(3'')-Ib* (n=2), *aph(6)-Id* (n=2)	*bla*$_{CTX-M-15}$ (n=12), *bla*$_{OXA-1}$ (n=11), *bla*$_{CTX-M-27}$ (n=2), *bla*$_{TEM-1B}$ (n=2)		*mph(A)* (n=12)	*catB3* (n=11), *catA1* (n=1)	*sul1* (n=12), *sul2* (n=2)	*tet(A)* (n=9), *tet(B)* (n=1)	*dfrA17* (n=12)	IncFIA (n=14), IncFII (n=14), IncFIB (n=5), Col (n=2), IncX4 (n=2), IncN (n=1), IncI1 (n=1)
ST131 H89 (unknown Clade), n=1 (clinical)		*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$							Col156, IncFIB, IncFII, IncI1
ST131 H99 (unknown Clade), n=3* (SW (n=1), sewage (n=2))	*aadA5* (n=3), *aph(3'')-Ib* (n=3), *aph(6)-Id* (n=3)	*bla*$_{CTX-M-27}$ (n=3)		*mph(A)* (n=3)		*sul1* (n=3), *sul2* (n=3)	*tet(A)* (n=3)	*dfrA17* (n=3)	Col156 (n=3), IncFIA (n=3), IncFIB (n=3), IncFII (n=3)

ST316, n=1 (clinical)		*bla*$_{CTX-M-15}$	*qnrS1*						IncFII
ST405, n=1 (fresh water)	*aac(3)-Iia*, *aac(6')-Ib-cr*, *aadA5*	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$		*mph(A)*	*catB3*	*sul1*	*tet(B)*	*dfrA17*	IncFIA, IncFIB, IncFII
ST648, n=1 (fresh water)	*aac(3)-Iia*, *aac(6')-Ib-cr*	*bla*$_{CTX-M-15}$, *bla*$_{DHA-1}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$	*qnrB4*	*mph(A)*	*catA1*, *catB3*	*sul1*	*tet(B)*	*dfrA17*	Col, IncFIA, IncFIB
ST1431, n=1 (fresh water)	*aph(3'')-Ib*, *aph(6)-Id*	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$	*qnrS1*				*tet(A)*	*dfrA14*	IncY
ST2003, n=2* (seawater (n=1), sewage (n=1)),	*aac(6')-Ib3* (n=2), *aac(3)-Iid* (n=2)	*bla*$_{CTX-M-14}$ (n=2), *bla*$_{TEM-1B}$ (n=2)		*erm(B)* (n=1), *mph(A)* (n=2)	*cmlA1* (n=2)		*tet(B)* (n=2)	*dfrA1* (n=2)	IncFIA (n=2), IncFIB (n=2), IncFII (n=2), IncI2 (n=1)
ST2179, n=1 (clinical)	*aac(3)-Iid*, *aac(6')-Ib-cr*, *aadA5*, *aph(3'')-Ib*, *aph(6)-Id*	*bla*$_{CTX-M-65}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$	*qnrS2*	*mph(A)*	*catB3*, *floR*	*sul1*, *sul2*	*tet(A)*	*dfrA17*	IncFIB, IncFIC
ST2450, n=5* (seawater (n=3), fresh water (n=1), sewage (n=1))	*aadA2* (n=5)	*bla*$_{CTX-M-15}$ (n=5)		*mph(A)* (n=5)		*sul1* (n=5)	*tet(A)* (n=5)	*dfrA12* (n=5)	IncFIA (n=5), IncFIB (n=5), IncFII (n=5)
ST8025, n=1 (seawater)		*bla*$_{CTX-M-15}$, *bla*$_{TEM-35}$		*mph(A)*			*tet(B)*		Col440I, IncFIA, IncFIB, IncFII, IncI1
ST8029, n=1 (clinical)	*aac(3)-Iid*	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$		*mph(A)*		*sul1*		*dfrA5*	Col, IncB/O/K/Z, IncFIB, IncFII

A comparison of the type of AMR-associated genes and plasmid replicon types detected in isolates according to isolation source was carried out. Overall, 12 different AMR-genes and five plasmid replicon types were common among isolates from all four sample types (Figure 5.6). In addition to β-lactamase encoding genes, genes associated with resistance to aminoglycosides, macrolide-lincosamide-streptograminB, sulphonamides, tetracycline and trimethoprim were detected among isolates from all four sample types, along with chromosomal mutations associated with quinolone resistance. The two ESBL-PEc with the fewest AMR genes both originated from clinical samples (*E. coli* ST131-H89 harbouring *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B}}$ and *E. coli* ST316 harbouring *bla*$_{\text{CTX-M-15}}$ and *qnrs1*).

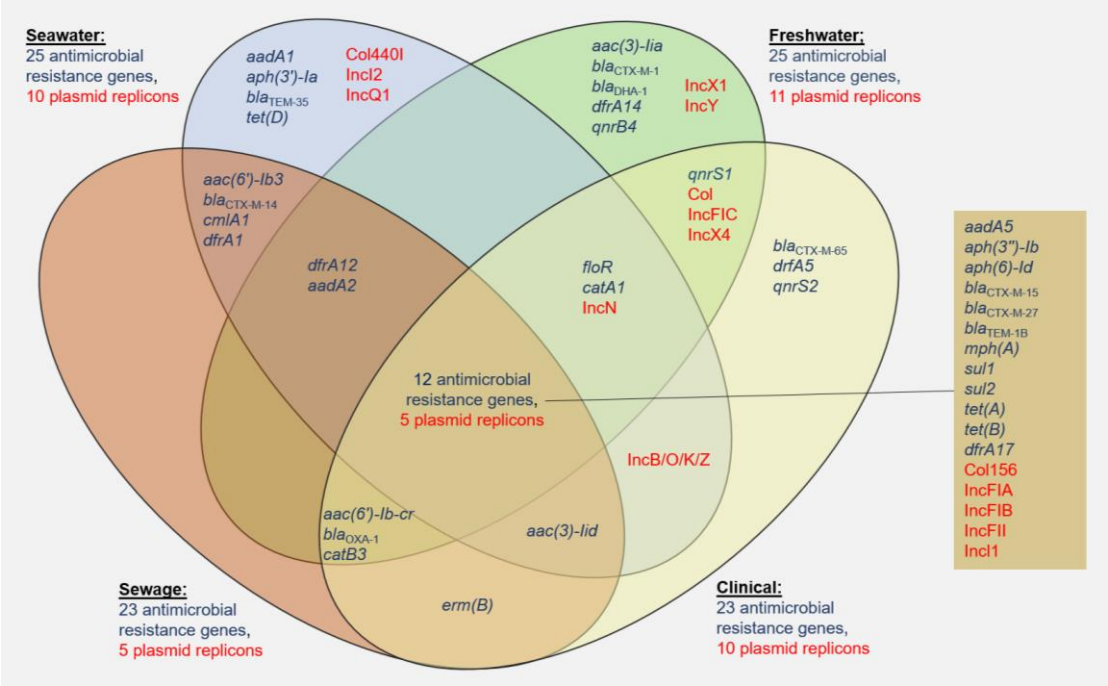


Figure 5.6 Venn diagram illustrating the antimicrobial resistance genes and plasmid replicon types from a collection of 47 extended-spectrum β-lactamase-producing E. coli, according to isolation source. *The antimicrobial resistance genes are in blue and the plasmid replicon types are in red. The genes/replicons that were common to all four sources are listed in the brown box.*

5.4 Discussion

The role of the aquatic environment in the dissemination of AMR is increasingly recognised. However, more data are required to fill the substantial knowledge gaps before the environmental aspect of AMR can be fully understood. This study investigated if antimicrobial resistant bacterial strains and their mobile genetic elements were shared between humans and the aquatic environment. This was determined by carrying out high-resolution genomic analysis on a collection of CPE and ESBL-PEc isolated from human sewage, fresh water and seawater sources at a site in the West of Ireland and comparing them to a collection of clinical CPE and ESBL-PEc isolated during a similar time period.

NDM-producing *Enterobacterales* are very prevalent in certain regions of the world, including the Indian subcontinent. Some European countries including Romania and Poland have reported inter-regional outbreaks of these organisms (Nordmann et al., 2011a) (164). The number of patients colonised with NDM-producing *Enterobacterales* is relatively low in Ireland, but data from NCPERLS have shown an increase in numbers in recent months (170). NDM-19 was identified as the variant harboured by all 24 environmental NDM-producing *Enterobacterales*. This variant remains uncommon both in Ireland and around the world, with only three additional reports to date, from China (clinical *K. pneumoniae* isolate) (283), Switzerland (clinical *E. coli* isolate) (284) and Canada (environmental *E. coli* isolate) (data from the National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/nuccore/MF370080.1).

A total of three NDM-19-producing *Enterobacterales* have been identified from clinical specimens in Ireland to date, which consist of one NDM-19-producing *K. pneumoniae* (ST11) and two NDM-19-producing *E. coli* (ST167 and ST205). cgMLST confirmed that the collection of 16 environmental NDM-19-producing *K. pneumoniae* ST11 were closely related to the clinical *K.*

pneumoniae isolate (only 0.6% genetic divergence). Similarly, one of the clinical *E. coli* had less than a 0.2% genetic divergence, when compared to the collection of eight environmental NDM-19-producing *E. coli* ST167. Comparative analysis identified the presence of the *bla*$_{\text{NDM-19}}$ gene on the same/closely related IncX3 plasmid carried by all 27 isolates. This plasmid type is frequently associated with *bla*$_{\text{NDM}}$ (285) (286). While there were no other AMR-associated genes identified on the IncX3 plasmid, a gene (*ble*$_{\text{MBL}}$) associated with conferring resistance to the cancer drug Bleomycin was identified. This is commonly associated with the *bla*$_{\text{NDM}}$ gene and may potentially aid in the dissemination of NDM through co-selection (287). Evidence suggests that *E. coli* ST167 may act as an important reservoir of *bla*$_{\text{NDM}}$, with studies from several countries reporting the detection of this gene on IncX3 plasmids carried by this ST. This includes reports from Switzerland (*bla*$_{\text{NDM-19,}}$ clinical isolate) (284), China (*bla*$_{\text{NDM-7}}$, *bla*$_{\text{NDM-5}}$, *bla*$_{\text{NDM-1}}$, clinical isolates) (288) (289) and Finland (*bla*$_{\text{NDM-7}}$, companion animal isolates). There has also been a report of *bla*$_{\text{NDM-1}}$ being chromosomally inserted into an *E. coli* ST167 isolate in China (290). Similarly, *K. pneumoniae* ST11, which is considered a pandemic clone known to be associated with human infection, is widely reported to harbour *bla*$_{\text{NDM}}$, with reports from Bulgaria (*bla*$_{\text{NDM-1}}$, clinical isolates) (291), Poland (*bla*$_{\text{NDM-1}}$, clinical isolates), (292) and Greece (*bla*$_{\text{NDM-1}}$, clinical isolates) (293).

In addition to CPE, a collection of 31 environmental ESBL-PEc obtained at the same site, were also examined in this study and compared to a collection of 16 ESBL-PEc from clinical specimens (Figure 5.1). Similar to the NDM-producing *Enterobacterales*, a number of ESBL-PEc strains identified in the seawater samples were also identified in the sewage and/or fresh water samples, indicating that these outflows act as dissemination routes for these strains to receiving bathing waters. The most prevalent *bla*$_{\textit{CTX-M}}$ variant identified among both environmental and clinical isolates was *bla*$_{\text{CTX-M-15}}$, which is reported as being the most common *bla*$_{\text{CTX-M}}$ genotype worldwide

(215). This genotype, along with *bla*$_{\text{CTX-M -27,}}$ the second most prevalent genotype identified in this study, has frequently been reported among environmental ESBL-PE in previous studies (257) (215). As *bla*$_{\text{CTX-M-1}}$ was only identified among isolates from fresh water sources and not those from clinical or sewage specimens, this indicates that this genotype remains uncommon among humans in Ireland. However, the clinical sample size was relatively small (n=16), so a larger sample size would be necessary to confirm this.

ST131 was the most common ST identified among the collection of ESBL-PEc and was the only ST identified in isolates originating from all four sample types, with the ST131-*H3*0 subclone making up 70% (14/20) of ST131 isolates. In the past, ST131-*H3*0 was most frequently associated with carrying *bla*$_{\text{CTX-M-15}}$ (294). However, in recent years, several countries including Japan, Germany and France have reported a shift in the CTX-M variant being harboured by this subclone, with the presence of *bla*$_{\text{CTX-M-27}}$ frequently reported (295) (296) (297). Similarly, in this study, *bla*$_{\text{CTX-M-27}}$ was identified among two *E. coli* ST131-H30, which originated from clinical isolates. A number of STs were only identified among isolates originating from water samples and not from sewage or clinical samples. These included STs from clonal complexes ST10 and ST23, which have previously been reported among ESBL-PEc isolated from animals in Ireland (ST10 and ST34 from pigs) (298), Ghana (ST10 from poultry) (299), Spain (ST10 from pigs) (300) and Denmark (ST34 from pigs) (301). This suggests that the isolates could be of animal origin. However, a limitation of this study was lack of access to ESLB-PE originating from animals, so this element of the One Health approach was not addressed.

A high level of similarity was identified between the collection of environmental and clinical ESBL-PEc, with the closest environmental isolate matching with a clinical isolate at 2506/2513 loci (*E. coli* S131). Additionally, 12 of the same genes associated with AMR and five plasmid replicon types

were identified among isolates from all four sample types (seawater, fresh water, sewage and clinical samples). Of the 23 AMR-associated genes identified among clinical isolates, only three were not identified in isolates from environmental sources and variants of these three genes ($bla_{\text{CTX-M-65}}$, *drfA5*, *qnrS2*) were detected in ESBL-PEc originating from environmental samples (Table 4.2). This indicates that these genetic elements are widely disseminated among ESBL-PEc, regardless of the source.

A report published by the Environmental Protection Agency (EPA (Ireland)) identified the site in this study as one of 38 sites around Ireland that discharges untreated waste water into the environment (220). In sites such as this, where there are multiple outflows, including from fresh water sources and untreated sewage, an ideal setting is created for the exchange of genetic material such as AMR genes, via horizontal gene transfer. While certain potential reservoirs, including food and food-producing animals are monitored for antimicrobial resistant bacteria (3), the same requirements do not apply to recreational waters. Ireland, like other EU member states, currently assess the water quality of its beaches by identifying the number of colony forming units of both *E. coli* and intestinal *Enterococci* per 100 mL sample (which are the established regulatory standards) (276). According to Ireland's 2017 annual bathing water quality report (276) the water quality of the two beaches in this study met the minimum requirement of 'sufficient' quality. There is no reason to consider that this site differs from many other comparable bathing sites with waters graded as being of sufficient quality, where quality is measured based solely on the number of *E. coli* present and not on the characteristics of the organisms' present (such as carbapenemase or ESBL production), or the configuration of the surrounding area.

Currently there is insufficient data available to quantify the human health risk associated with exposure to antimicrobial resistant bacteria and genes in recreational water. However, several studies suggest that people who routinely use bathing water for recreational purposes have a higher chance

of colonisation with antimicrobial resistant bacteria (214) (215). It is well established that the collection and appropriate treatment of human sewage has played a vital part in lowering the global incidences of infectious disease, including infections caused by antimicrobial resistant bacteria (264). The continued discharge of untreated human sewage, particularly in the vicinity of recreational bathing waters, may increases the human health risk in relation to exposure to AMR.

In conclusion, this study identified closely related bacterial strains, plasmids and AMR-associated genes among CPE and ESBL-PEc isolated from both environmental sources and clinical specimens. These findings support the hypothesis that bacterial strains and their mobile genetic elements are shared between humans and the aquatic environment. These findings also highlight that recreational seawaters are vulnerable to contamination with AMR bacteria of public health significance via multiple routes. Our findings emphasise the risk associated with discharging untreated human sewage into the environment in an era of rapidly spreading AMR and highlight a critical limitation of current EU bathing water quality monitoring regulations. These regulations place great emphasis on relatively infrequent microbiological testing of small volumes of water for indicator organisms, with no requirement for an environmental health assessment of the site to ensure that there is no uncontrolled source of contamination. Any revision of the EU bathing water regulations must require site assessment and certification as prerequisite for the application of microbiological methods.

CHAPTER 6: DISCUSSION

6 Discussion

The aim of this thesis was to improve our understanding of the role of the aquatic environment in the dissemination of antimicrobial resistance (AMR) of public health significance. This was achieved by assessing recreational waters and sewage for the presence of extended-spectrum β-lactamase-producing *Enterobacterales* (ESBL-PE) and carbapenemase-producing *Enterobacterales* (CPE). Following preliminary characterisation, a subset of these isolates were selected for whole genome sequencing (WGS) and the genomes compared to those of clinical isolates.

A main finding of this thesis was the continuous detection of CPE in recreational seawaters at a site in the West of Ireland, over a 14 month-time period, as outlined in Chapters 3 and 5. This represents the first finding of CPE in seawater in Europe. The type of CPE identified was New Delhi metallo-β-lactamase-(NDM)-19 producing *E. coli* (n=8) and NDM-19-producing *K. pneumoniae* (n=16). Given the complex nature of the interactions between different reservoirs of AMR it is often difficult to determine the directionality of transmission of AROs between reservoirs. In the case of the NDM-producing *E. coli* and *K. pneumoniae*, findings indicate that it was the same two strains that were repeatedly detected at this site, as a result of an untreated human sewage discharge point in this area. Similarly, in Brazil there have been multiple reports of the presence of CPE in coastal waters, with poor sewage treatment practices reported in these areas. The types of CPE isolated from these sources included KPC-producing *Citrobacter spp*., *Enterobacter spp*. and *Klebsiella spp*. (180) and NDM-producing *K. pneumoniae* (181) (221).

In total, three strains of NDM-19-producing *Enterobacterales* have been identified in the Irish clinical setting to date (National Carbapenemase-Producing Reference Laboratory Service (NCPERLS)). Analysis indicates that these three clinical isolates harboured the NDM-19 encoding gene on a

similar IncX3 plasmid as the 24 environmental isolates, with two of the three isolates identified as being closely related to the environmental strains (*E. coli* and *K. pneumoniae*) (via core genome multi-locus sequence typing (cgMLST)). To date, there have only been three other reports of NDM-19-producing *Enterobacterales* worldwide, which were isolated from clinical specimens (n=2, Switzerland and China) and an environmental sample (Canada), indicating that this variant of NDM is not widely disseminated globally (283) (284) (data from the National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/nuccore/MF370080.1).

OXA-48 is the most common carbapenemase reported in the Irish clinical setting (278). This thesis reports the identification of a strain of both OXA-48-like-producing *E. coli* and OXA-48-like-producing *K. pneumoniae* in recreational seawater samples collected at a second site in the West of Ireland (Chapter 4). These isolates harboured $bla_{\text{OXA-48}}$ on similar mobile genetic elements (MGEs) to those identified in a clinical collection of OXA-48-like producing *Enterobacterales* (259), with *E. coli* harbouring $bla_{\text{OXA-48}}$ on a pOXA-48 fragment, while *K. pneumoniae* harboured it on an IncL(pOXA-48) plasmid. The source of CPE at this site is unknown. CPE was not detected in samples of treated and untreated sewage collected at a nearby secondary waste water treatment plant (SWWTP). However effluent from this SWWTP cannot be ruled out as a potential source, as the sewage effluent sample size was small and the method for detecting CPE had limitations, as highlighted in Chapter 4. Several studies have demonstrated that WWTPs may not be effective at removing all antimicrobial resistant organisms (AROs) and genes, and ESBL-PE and CPE have been reported in treated effluent samples in multiple studies (158) (265) (173).

ESBL-producing *E. coli* (ESBL-PEc) were commonly detected in seawater, fresh water and sewage samples examined in this study (Chapter 5). This supports the findings of previous studies, which indicate that ESBL-PEc are widespread in the aquatic environment (153) (92) (154) (155). A Dutch study (302) which

compared a collection of ESBL-PEc isolated from recreational waters to ESBL-PEc originating from WWTPs in the same area, identified isolates from the two collections with the same ESBL encoding genes, AMR profiles, sequence type and phylogenetic background. This study also identified ESBL-PEc in connecting water bodies which were not impacted by WWTP effluents, indicating additional sources of contamination with ESBL-PEc. This is in keeping with the findings of this thesis (Chapter 5), with similar ESBL-PEc identified in fresh water samples as those isolated from receiving recreational seawater samples, indicating that these outflows act as additional routes of dissemination of ESBL-PEc to receiving waters.

A Dutch study which compared ESBL and AmpC-producing *E. coli* obtained from 35 studies found that isolates originating from human sources were more similar to those originating from wild birds and environmental samples than from livestock and food sources (225). This thesis reports the identification of 12 AMR-associated genes and five plasmid replicon types that were common among ESBL-PEc originating from seawater, fresh water, sewage and clinical samples (Chapter 5). ST131 was the only sequence type (ST) identified among ESBL-PEc originating from the four sample types, and it was the most commonly detected ST overall. This ST has previously been attributed to the rapid global dissemination of CTX-M-15 and studies indicate that it has successfully disseminated globally (266). CTX-M-15 is the most prevalent type of ESBL reported both in Ireland and globally (130) (132) (131), and it was most the most common CTX-M variant identified in this study, both among environmental and clinical isolates.

Studies show that ESBL-PE have rapidly disseminated outside of the hospital setting, into the community, as well as among animals, food and as highlighted above, within the aquatic environment (141) (142) (82) (126) (154). It therefore stands to reason that CPE could potentially follow a similar pattern of widespread dissemination, as carbapenemase and ESBL encoding genes are often harboured on similar highly transmissible MGEs, and reports

of CPE in the community and from non-human sources are beginning to emerge (172) (173). The identification of CPE belonging to several successful clonal groups, including *K. pneumoniae* ST11 (NDM-producer), *K. pneumoniae* ST101 (OXA-48-like-producer) and *E. coli* ST131 (OXA-48-like-producer) among the environmental collection is of particular concern, given the ability of these clonal groups to rapidly disseminate (114). These findings highlight the need for further research to be carried out to identify potential routes of dissemination of CPE outside of the hospital setting, in order to attempt to curb this escalating problem.

It is well established that anthropogenic pollution can result in contamination of the environment with AROs and antimicrobial resistant genes (ARGs). According to the most recently published Environmental Protection Agency report (220), there are currently 38 locations around Ireland where raw sewage is being released untreated into the environment, with the findings of Chapters 3 and 5 illustrating the potential human health hazards associated with this practice. Although, by the established regulatory standards, the bathing water quality in the areas concerned have consistently been of sufficient quality (245), it is reasonable to conclude that those using a recreational site, where AROs (such as CPE) are continuously detected, might be at least intermittently exposed to these organisms. From a public health perspective, these findings focus attention on the need to accelerate programmes to cease discharge of untreated sewage into the environment. Intervention strategies that target sources of contamination with AROs and ARGs could act to limit the emission of these contaminants into the environment, thereby reducing potential human and animal exposure.

Although the number of studies reporting AMR in the environment are steadily increasing, it is difficult to make comparisons on findings between different sources/regions, as there is currently no standardised methodology for assessing the aquatic environment for AMR. Additionally, there is currently no routine surveillance of the environment for AMR. If standardised

methodologies were developed and routine monitoring implemented this would generate comparable data to help gain insight into the environmental aspect of AMR and the local dimensions that impact on this. It would also potentially provide the necessary data for policy makers to identify and implement appropriate regulations in order to minimise the role of the environment in the dissemination of AMR.

In conclusion, the findings of this thesis support the hypothesis that antimicrobial resistant bacteria of public health significance and MGEs are shared between humans and the aquatic environment. These findings call for further research into the role of the aquatic environment in the emergence, persistence and dissemination of AMR.

6.2 Limitations

The initial findings of CPE in fresh water samples prompted an investigation into the potential source(s) of CPE at this site, as outlined in Chapter 3. The sampling plan at this site was therefore guided based on these findings. We did not infer any statistical conclusions in this study, as the application of statistical analysis to these data would not have added any additional beneficial information.

The study was limited to two sampling sites, as outlined in Chapters 3, 4 and 5, so the findings of this study are not generalizable in terms of other sites and is therefore an area for future research.

The use of a culture-based method facilitates isolation of specific target organisms and was therefore an appropriate choice for the purposes of this study. However, this methodology has certain limitations. Studies have shown that bacteria can survive for long periods of time in the aquatic environment (303) (304) (305). This time frame can vary according to environmental conditions, which include exposure to UV radiation, salinity,

microbial predation, temperature, nutrient availability and physical factors (such as dilution, sedimentation and resuspension) (306) (307). In response to unfavorable environmental conditions, many bacteria enter a so called viable but non-culturable (VBNC) state (303) (308). VBNC cells are living cells that are metabolically active, but which have lost the ability to grow on suitable media (309). The use of culture-based testing to assess environmental samples for the presence of AROs therefore has inherent culture bias, as VBNC organisms go undetected.

6.3 Future Work

Metagenomics is the study of microbial communities using genetic material extracted directly from environmental samples, and it is culture independent (310). Unlike culture-based methods, instead of examining single organisms, metagenomics can be used to study the entire complement of genes and species within a microbial community (311). Both culture-based and metagenomic approaches have limitations when used independently to assess environmental samples for AMR. Therefore, future work which combines both approaches would be beneficial.

This study examined the role of the aquatic environment in the dissemination of AMR using a One Health approach. However, we did not have access to ESBL-PE of animal origin, so this component of the One Health triad was not addressed in this thesis. It would be of interest to examine food-producing animals, companion animals and wildlife in the same region for the presence of ESBL-PE and CPE and compare the genomes of any isolates obtained those identified in this study.

As outlined in the limitations, this study was limited to two sampling sites, so the findings are not generalizable in terms of other sites. The Environmental Protection agency, in collaboration with the Health Service Executive have

funded a four-year project titled: Antimicrobial Resistance and the Environment –Sources, persistence, Transmission and risk management (AREST), to address these limitations, as well as to answer additional questions. The AREST project aims to: generate national level data on the key sources, hot spots and drivers of AMR in the environment. This data will be generated by: mapping hotspots of drivers of AMR in different regions around Ireland, assessing the contributions of healthcare and agriculture to AMR in the environment, assessing the efficiency of water treatment processes, and developing a risk ranking protocol to determine the contribution of various sectors on the sources and levels of AMR in the environment. This study will be used to inform relevant policies and to support Irelands National Action Plan on AMR.

CHAPTER 7: BIBLIOGRAPHY

1. Taylor NGH, Verner-Jeffreys DW, Baker-Austin C. Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? Trends Ecol Evol. 2011;26(6):278–84.
2. Wright GD. Q&A: Antibiotic resistance: where does it come from and what can we do about it? BMC Biol. 2010;8(123):1–6.
3. FSAI (Food Safety Authority of Ireland). Potential for Transmission of Antimicrobial Resistance in the Food Chain [Internet]. 2015. Available from: https://www.fsai.ie/publications_AMR/
4. Gould K. Antibiotics: from prehistory to the present day. J Antimicrob Chemother. 2016;71:572–5.
5. Aminov RI. A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. Front Microbiol. 2010;1(134):1–7.
6. Lobanovska M, Pilla G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? YALE J Biol Med. 2017;90:135–45.
7. The Nobel Prize. The Nobel Prize in Physiology or Medicine 1945. [Internet]. 1945. Available from: http://www.nobelprize.org/nobel_prizes/medicine/laureates/1945/
8. Epand RM, Walker C, Epand RF, Magarvey NA. Molecular mechanisms of membrane targeting antibiotics. Biochim Biophys Acta. 2016;1858:980–7.
9. Krause KM, Serio AW, Kane TR, Connolly LE. Aminoglycosides: An Overview. Cold Spring Harb Perspect Med. 2016;6:1–18.
10. Meletis G. Carbapenem resistance: overview of the problem and future perspectives. Ther Adv Infect Dis. 2016;3(1):15–21.
11. Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Cámara M. Quinolones: from antibiotics to autoinducers. FEMS Microbiol Rev. 2011;35:247–74.
12. Nguyen F, Starosta AL, Arenz S, Sohmen D, Dönhöfer A, Wilson DN. Tetracycline antibiotics and resistance mechanisms. Biol Chem. 2014;395(5):559–75.

13. Lee D, Das S, Dawson NL, Dobrijevic D, Ward J, Orengo C. Novel Computational Protocols for Functionally Classifying and Characterising Serine Beta-Lactamases. PLoS Comput Biol. 2016;12(6):1–33.
14. Yoneyama H, Katsumata R. Antibiotic Resistance in Bacteria and Its Future for Novel Antibiotic Development. Biosci Biotechnol Biochem. 2006;70(5):1060–75.
15. Sarkar P, Yarlagadda V, Ghosh C, Haldar J. A review on cell wall synthesis inhibitors with an emphasis on glycopeptide antibiotics. Medchemcomm. 2017;8:516–33.
16. Kohanski MA, Daniel J Dwyer, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol. 2010;8(6):423–35.
17. Bush K, Bradford PA. Beta-Lactams and beta-Lactamase Inhibitors: An Overview. Cold Spring Harb Perspect Med. 2016;6:1–22.
18. Allen NE, Nicas TI. Mechanism of action of oritavancin and related glycopeptide antibiotics. FEMS Microbiol Rev. 2003;26:511–32.
19. Silhavy TJ, Kahne D, Walker S. The Bacterial Cell Envelope. Cold Spring Harb Perspect Biol. 2010;2:1–16.
20. Pogliano J, Pogliano N, Silverman JA. Daptomycin-Mediated Reorganization of Membrane Architecture Causes Mislocalization of Essential Cell Division Proteins. J Bacteriol. 2012;194(17):4494–504.
21. Velkov T, Roberts KD, Nation RL, Thompson PE, Li J. Pharmacology of polymyxins: new insights into an “old” class of antibiotics. Future Microbiol. 2013;8(6):1–20.
22. Hawkey PM. Mechanisms of quinolone action and microbial response. J Antimicrob Chemother. 2003;51(Suppl. S1):29–35.
23. Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, et al. Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase. Cell. 2001;104:901–12.
24. Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. Aminoglycosides: Activity and resistance. Antimicrob Agents Chemother.

1999;43(4):727–37.

25. Holmes AH, Moore LSP, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, et al. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2016;387:176–87.
26. Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. Microbiol Spectr. 2016;4(2):1–37.
27. Lupo A, Coyne S, Berendonk TU. Origin and evolution of antibiotic resistance: The common mechanisms of emergence and spread in water bodies. Front Microbiol. 2012;3:1–13.
28. Abraham EP, Chain E. An Enzyme from Bacteria able to Destroy Penicillin. Nature. 1940;146:837.
29. CDC (Center for Disease Control and Prevention). About Antimicrobial Resistance [Internet]. 2013. Available from: https://www.cdc.gov/drugresistance/about.html
30. Domalaon R, Idowu T, Zhanel G., Schweizer F. Antibiotic Hybrids: the Next Generation of Agents and Adjuvants against Gram-Negative Pathogens? Clin Microbiol Rev. 2018;31(2):1–45.
31. Cox G, Wright GD. Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. Int J Med Microbiol. 2013;303:287–92.
32. von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, et al. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Front Microbiol. 2016;7(173):1–10.
33. Jacoby GA. Mechanisms of Resistance to Quinolones. Clin Infect Dis. 2005;41:S120–6.
34. Correia S, Poeta P, Hébraud M, Capelo JL, Igrejas G. Mechanisms of quinolone action and resistance: where do we stand? J Med Microbiol. 2017;66:551–9.
35. Madigan M, Martinko J, Stahl D, Clark D. Brock Biology of Microorganisms. 13th ed. Pearson; 2011. 1–1152 p.
36. Slonczewski JL, Foster JW. Microbiology an Evolving Science. 3rd ed.

W. W. Norton & Company, Inc; 2013. 1–1408 p.

37. Mazaheri Nezhad Fard R, Barton MD, Heuzenroeder MW. Bacteriophage-mediated transduction of antibiotic resistance in Enterococci. Lett Appl Microbiol. 2011;52:559–64.
38. Berglund B. Environmental dissemination of antibiotic resistance genes and correlation to anthropogenic contamination with antibiotics. Infect Ecol Epidemiol. 2015;5(28564).
39. Smillie C, Garcillan-Barcia MP, Francia M V., Rocha EPC, de la Cruz F. Mobility of Plasmids. Microbiol Mol Biol Rev. 2010;74(3):434–52.
40. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin Microbiol Rev. 2018;31(4):1–61.
41. San Millan A. Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context. Trends Microbiol. 2018;26(12):978–85.
42. Klemm EJ, Wong VK, Dougan G. Emergence of dominant multidrug-resistant bacterial clades: Lessons from history and whole-genome sequencing. Proc Natl Acad Sci USA. 2018;115(51):12872–7.
43. Gillings MR. Integrons: Past, Present, and Future. Microbiol Mol Biol Rev. 2014;78(2):257–77.
44. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ V. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2015;13:42–51.
45. Bonnet R. Growing Group of Extended-Spectrum Beta-Lactamases: the CTX-M Enzymes. Antimicrob Agent Chemother. 2004;48(1):1–14.
46. González-Bello C. Antibiotic adjuvants – A strategy to unlock bacterial resistance to antibiotics. Bioorganic Med Chem Lett. 2017;27:4221–8.
47. Lambert PA. Bacterial resistance to antibiotics: Modified target sites. Adv Drug Deliv Rev. 2005;57:1471–85.
48. Kolar M, Urbanek K, Latal T. Antibiotic selective pressure and development of bacterial resistance. Int J Antimicrob Agents. 2001;17:357–63.

49. CDC, Wellcome Trust, UK Science & Innovation Network. Initiatives for Addressing Antimicrobial Resistance in the Environment [Internet]. 2018. Available from: https://wellcome.ac.uk/sites/default/files/antimicrobial-resistance-environment-report.pdf

50. WHO (World Health Organisation). Antimicrobial Resistance: An Emerging Water, Sanitation and Hygiene Issue [Internet]. 2014. Available from: https://apps.who.int/iris/bitstream/handle/10665/204948/WHO_FWC_WSH_14.7_eng.pdf;jsessionid=8D7B292324CF99840E37C0A9052D0022?sequence=1

51. The Nobel Prize. Sir Alexander Fleming Nobel Lecture [Internet]. 1945. Available from: https://www.nobelprize.org/prizes/medicine/1945/fleming/lecture/

52. Department of Health. Ireland's National Action Plan on Antimicrobial Resistance 2017-2020 [Internet]. 2017. Available from: http://health.gov.ie/national-patient-safety-office/patient-safety-surveillance/antimicrobial- resistance-amr/%0AIn

53. Santos L, Ramos F. Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. Int J Antimicrob Agents. 2018;52:135–43.

54. Van Boeckel TP, Gandra S, Ashok A, Caudron Q, Grenfell BT, Levin SA, et al. Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data. Lancet Infect Dis. 2014;14:742–50.

55. Morgan DJ, Okeke IN, Laxminarayan R, Perencevich EN, Weisenberg S. Non-prescription antimicrobial use worldwide: a systematic review. Lancet Infect Dis. 2011;11:692–701.

56. Stelling JM, Travers K, Jones RN, Turner PJ, Brien TFO, Levy SB. Integrating Escherichia coli Antimicrobial Susceptibility Data from Multiple Surveillance Programs. Emerg Infect Dis. 2005;11(6):873–82.

57. ECDC (European Centre for Disease Prevention and Control).

Antimicrobial consumption Annual Epidemiological Report for 2017 [Internet]. 2018. Available from: https://ecdc.europa.eu/sites/portal/files/documents/AER_for_2017-antimicrobial-consumption.pdf

58. Burki TK. Tackling antimicrobial resistance in food-producing animals. Lancet Respir Med. 2018;6:93–4.
59. The Department of Health. One Health Report on Antimicrobial Use & Antimicrobial Resistance [Internet]. 2019. Available from: https://health.gov.ie/wp-content/uploads/2019/01/One-Health-Report-on-Antimicrobial-Use-Antimicrobial-Resistance.pdf
60. Health Products Regulatory Authority. Report on consumption of veterinary antibiotics in Ireland during 2017. 2019. p. 1–4.
61. Brown K, Uwiera RRE, Kalmokoff ML, Brooks SPJ, Inglis GD. Antimicrobial growth promoter use in livestock: a requirement to understand their modes of action to develop effective alternatives. Int J Antimicrob Agents. 2017;49:12–24.
62. Castanon JIR. History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. Poult Sci. 2007;86:2466–71.
63. Health Protection Surveillance Centre. Point Prevalence Survey of Healthcare-Associated Infections & Antimicrobial Use in Long-Term Care Facilities (HALT)- May 2016 [Internet]. 2017. Available from: https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/infectioncontrolandhai/surveillance/hcaiinlongtermcarefacilities/haltreports/2016report/File,16218,en.pdf
64. Bengtsson-Palme J, Kristiansson E, Larsson DGJ. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol Rev. 2018;42(1):68–80.
65. WHO (World Health Organization). Antimicrobial resistance [Internet]. 2018. Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

66. Memish ZA, Venkatesh S, Shibl AM. Impact of travel on international spread of antimicrobial resistance. Int J Antimicrob Agents. 2003;21:135–42.

67. Grigoryan L, Burgerhof JGM, Degener JE, Deschepper R, Lundborg C, Monnet DL, et al. Attitudes, beliefs and knowledge concerning antibiotic use and self-medication: a comparative European study. Pharmacoepidemiol Drug Saf. 2007;16(11):1234–43.

68. Savic M, Årdal C. A Grant Framework as a Push Incentive to Stimulate Research and Development of New Antibiotics. J Law, Med Ethics. 2018;46:9–24.

69. Lin DM, Koskella B, Lin HC. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. World J Gastrointest Pharmacol Ther. 2017;8(3):162–73.

70. Dagan R. Impact of pneumococcal conjugate vaccine on infections caused by antibiotic-resistant Streptococcus pneumoniae. Clin Microbiol Infect. 2009;15(SUPPL. 3):16–20.

71. Klugman KP, Black S. Impact of existing vaccines in reducing antibiotic resistance: Primary and secondary effects. Proc Natl Acad Sci USA. 2018;115(51):12896–901.

72. WHO (World Health Organisation). Global Action Plan on Antimicrobial Resistance. [Internet]. 2015 [cited 2018 Dec 10]. Available from: http://www.who.int/drugresistance/global_action_plan/en/

73. HM Government. Contained and controlled: The UK's 20-year vision on antimicrobial resistance [Internet]. 2019. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773065/uk-20-year-vision-for-antimicrobial-resistance.pdf

74. O'Neill J. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations [Internet]. 2016. Available from: https://amr-review.org/sites/default/files/160525_Final paper_with cover.pdf

75. WHO (World Health Organization). Global Priority List Of Antibiotic-

Resistant Bacteria To Guide Research, Discovery, And Development Of New Antibiotics [Internet]. 2016. Available from: http://www.cdc.gov/drugresistance/threat-report-2013/

76. Health Protection Surveillance Centre. mcr-positive Enterobacteriaceae infection or colonisation [Internet]. 2019. Available from: https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/mcrpositiveenterobacteriaceae/casedefinition/

77. Adeolu M, Alnajar S, Naushad S, Gupta RS. Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morgane. Int J Syst Evol Microbiol. 2016;66:5575–99.

78. Zhou K, Lokate M, Deurenberg RH, Tepper M, Arends JP, Raangs EGC, et al. Use of whole-genome sequencing to trace, control and characterize the regional expansion of extended-spectrum β-lactamase producing ST15 Klebsiella pneumoniae. Sci Rep. 2016;6(20840):1–10.

79. Day MJ, Doumith M, Abernethy J, Hope R, Reynolds R, Wain J, et al. Population structure of Escherichia coli causing bacteraemia in the UK and Ireland between 2001 and 2010. J Antimicrob Chemother. 2016;71(8):2139–2142.

80. Paczosa MK, Mecsas J. Klebsiella pneumoniae: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev. 2016;80(3):629–61.

81. Chen P, Seth AK, Abercrombie JJ, Mustoe TA, Leung KP. Activity of Imipenem against Klebsiella pneumoniae Biofilms In Vitro and In Vivo. Antimicrob Agents Chemother. 2014;58(2):1208–13.

82. Caneiras C, Lito L, Melo-Cristino J, Duarte A. Community- and Hospital-Acquired Klebsiella pneumoniae Urinary Tract Infections in Portugal: Virulence and Antibiotic Resistance. Microorganisms. 2019;7(138):1–

14.

83. Viale P, Giannella M, Lewis R, Trecarichi EM, Petrosillo N, Tumbarello M. Predictors of mortality in multidrug-resistant Klebsiella pneumoniae bloodstream infections. Expert Rev Anti Infect Ther. 2013;11(10):1053–63.

84. Zakariya BP, Vishnu Bhat B, Harish BN, Arun Babu B, Joseph NM. Risk factors and outcome of Klebsiella pneumoniae sepsis among newborns. Curr Pediatr Res. 2012;16(2):115–8.

85. Martin RM, Bachman MA. Colonization, Infection, and the Accessory Genome of Klebsiella pneumoniae. Front Cell Infect Microbiol. 2018;8(4):1–15.

86. Hoenigl M, Valentin T, Zarfel G, Wuerstl B, Leitner E, Salzer HJF, et al. Nosocomial Outbreak of Klebsiella pneumoniae Carbapenemase-Producing Klebsiella oxytoca in Austria. Antimicrob Agents Chemother. 2012;56(4):2158–61.

87. Onori R, Gaiarsa S, Comandatore F, Pongolini S, Brisse S, Colombo A, et al. Tracking Nosocomial Klebsiella pneumoniae Infections and Outbreaks by Whole-Genome Analysis: Small-Scale Italian Scenario within a Single Hospital. J Clin Microbiol. 2015;53(9):2861–8.

88. Mollers M, Lutgens SP, Schoffelen AF, Schneeberger PM, Suijkerbuijk AWM. Cost of Nosocomial Outbreak Caused by NDM-1–Containing Klebsiella pneumoniae in the Netherlands, October 2015–January 2016. Emerg Infect Dis. 2017;23(9):1574–6.

89. Wyres KL, Holt KE. Klebsiella pneumoniae as a key trafficker of drug resistance genes from environmental to clinically important bacteria. Curr Opin Microbiol. 2018;45:131–9.

90. Vila J, Sáez-López E, Johnson JR, Römling U, Dobrindt U, Cantón R, et al. Escherichia coli: an old friend with new tidings. FEMS Microbiol Rev. 2016;40(4):437–63.

91. Nordmann P, Poirel L, Toleman MA, Walsh TR. Does broad-spectrum β-lactam resistance due to NDM-1 herald the end of the antibiotic era

for treatment of infections caused by Gram-negative bacteria? J Antimicrob Chemother. 2011;66(4):689–92.

92. Franz E, Veenman C, van Hoek AHAM, Husman A de R, Blaak H. Pathogenic Escherichia coli producing Extended-Spectrum β-Lactamases isolated from surface water and wastewater. Sci Rep. 2015;5(14372):1–9.
93. Espinosa L, Gray A, Duffy G, Fanning S, McMahon BJ. A scoping review on the prevalence of Shiga-toxigenic Escherichia coli in wild animal species. Zoonoses Public Health. 2018;65:911–20.
94. Hu YY, Cai JC, Zhou HW, Chi D, Zhang XF, Chen WL, et al. Molecular Typing of CTX-M-Producing Escherichia coli Isolates from Environmental Water, Swine Feces, Specimens from Healthy Humans, and Human Patients. Appl Environ Microbiol. 2013;79(19):5988–96.
95. Mohd Sazlly Lim S, Wong PL, Sulaiman H, Atiya N, Hisham Shunmugam R, Liew SM. Clinical prediction models for ESBL-Enterobacteriaceae colonization or infection: a systematic review. J Hosp Infect. 2019;102:8–16.
96. Drawz SM, Bonomo RA. Three Decades of Beta-Lactamase Inhibitors. Clin Microbiol Rev. 2010;23(1):160–201.
97. Bush K. Past and Present Perspectives on Beta-Lactamases. Antimicrob Agents Chemother. 2018;62(10):1–20.
98. Jacoby GA. AmpC B-Lactamases. Clin Microbiol Rev. 2009;22(1):161–82.
99. Egawa R, Sawai T, Mitsuhashi S. Drug Resistance of Enteric Bacteria: XII. Unique Substrate Specificity of Penicillinase Produced by R Factor. Jpn J Microbiol. 1967;11(3):173–8.
100. Datta N, Kontomichalou P. Penicillinase Synthesis Controlled By Infectious R Factors In Enterobacteriaceae. Nature. 1965;208:239–41.
101. Fair RJ, Tor Y. Antibiotics and Bacterial Resistance in the 21st Century. Perspect Medicin Chem. 2014;6:25–64.
102. Paterson DL, Bonomo RA. Extended-Spectrum Beta-Lactamases : a

Clinical Update. Clin Microbiol Rev. 2005;18(4):657–86.

103. Rawat D, Nair D. Extended-spectrum β-lactamases in Gram Negative Bacteria. J Glob Infect Dis. 2010;2(3):263–74.

104. Bernard H, Tancrede C, Livrelli V, Morand A, Barthelemy M, Labia R. A novel plasmid-mediated extended-spectrum beta-lactamase not derived from TEM- or SHV-type enzymes. J Antimicrob Chemother. 1992;29(5):590–2.

105. Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, et al. CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother. 2007;59:165–74.

106. Ramadan AA, Abdelaziz NA, Amin MA, Aziz RK. Novel blaCTX-M variants and genotype-phenotype correlations among clinical isolates of extended spectrum beta lactamase-producing Escherichia coli. Sci Rep. 2019;9(4224):1–12.

107. Klein EY, Van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci USA. 2018;115(15):E3463–70.

108. Codjoe FS, Donkor ES. Carbapenem Resistance: A Review. Med Sci. 2018;6(1):1–28.

109. Wilson H, Török ME. Extended-spectrum β-lactamase-producing and carbapenemase-producing Enterobacteriaceae. Microb Genomics. 2018;4:1–14.

110. Naas T, Nordmann P. Analysis of a carbapenem-hydrolyzing class A β-lactamase from Enterobacter cloacae and of its LysR-type regulatory protein. Proc Natl Acad Sci USA. 1994;91:7693–7.

111. Queenan AM, Bush K. Carbapenemases: The Versatile β-Lactamases. Clin Microbiol Rev. 2007;20(3):440–58.

112. Nordmann P, Naas T, Poirel L. Global spread of Carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 2011;17(10):1791–8.

113. Poirel L, Héritier C, Tolun V, Nordmann P. Emergence of Oxacillinanse-

Mediated Resistance to Imipenem in Klebsiella pneumoniae. Antimicrob Agents Chemother. 2004;48(1):15–22.

114. Nordmann P, Poirel L. The difficult-to-control spread of carbapenemase producers among Enterobacteriaceae worldwide. Clin Microbiol Infect. 2014;20(9):821–30.

115. Morosini MI, García-Castillo M, Coque TM, Valverde A, Novais Â, Loza E, et al. Antibiotic Coresistance in Extended-Spectrum-ᴺʟ-Lactamase-Producing Enterobacteriaceae and In Vitro Activity of Tigecycline. Antimicrob Agents Chemother. 2006;50(8):2695–9.

116. Ferranti M, Schiaroli E, Palmieri MI, Repetto A, Vecchiarelli A, Francisci D, et al. Carbapenemase-producing Enterobacteriaceae isolates resistant to last-line antibiotics in an Italian general hospital. New Microbiol. 2018;41(4):274–81.

117. Otter JA, Doumith M, Davies F, Mookerjee S, Dyakova E, Gilchrist M, et al. Emergence and clonal spread of colistin resistance due to multiple mutational mechanisms in carbapenemase-producing Klebsiella pneumoniae in London. Sci Rep. 2017;7(12711):1–8.

118. ECDC (European Centre for Disease Prevention and Control). Surveillance of antimicrobial resistance in Europe. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017 [Internet]. 2018. Available from: https://ecdc.europa.eu/sites/portal/files/documents/EARS-Net-report-2017-update-jan-2019.pdf%0Ahttps://ecdc.europa.eu/en/publications-data/antimicrobial-resistance-surveillance-europe-2016

119. Cantón R, Ruiz-Garbajosa P. Co-resistance: an opportunity for the bacteria and resistance genes. Curr Opin Pharmacol. 2011;11:477–85.

120. Tzouvelekis LS, Markogiannakis A, Psichogiou M, Tassios PT, Daikos GL. Carbapenemases in Klebsiella pneumoniae and Other Enterobacteriaceae: an Evolving Crisis of Global Dimensions. Clin

Microbiol Rev. 2012;25(4):682–707.

121. Banerjee R, Johnson JR. A New Clone Sweeps Clean: the Enigmatic Emergence of Escherichia coli Sequence Type 131. Antimicrob Agents Chemother. 2014;58(9):4997–5004.

122. Micenková L, Šišková P, Bosák J, Jamborová I, Cernohorská L, Šmajs D. Characterization of Human Uropathogenic ESBL-producing Escherichia coli in the Czech Republic: Spread of CTX-M-27-Producing Strains in a University Hospital. Microb Drug Resist. 2014;20(6):610–7.

123. Rodríguez-Baño J, Navarro MD, Romero L, Martínez-Martínez L, Muniain MA, Perea EJ, et al. Epidemiology and Clinical Features of Infections Caused by Extended-Spectrum Beta-Lactamase-Producing Escherichia coli in Nonhospitalized Patients. J Clin Microbiol. 2004;42(3):1089–94.

124. Chong Y, Shimoda S, Yakushiji H, Ito Y, Miyamoto T, Kamimura T, et al. Community spread of extended-spectrum β-lactamase-producing Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis: A long-term study in Japan. J Med Microbiol. 2013;62:1038–43.

125. Moor CT, Roberts SA, Simmons G, Briggs S, Morris AJ, Smith J, et al. Extended-spectrum β-lactamase (ESBL)-producing enterobacteria: factors associated with infection in the community setting, Auckland, New Zealand. J Hosp Infect. 2008;68:355–62.

126. Doi Y, Park YS, Rivera JI, Adams-Haduch JM, Hingwe A, Sordillo EM, et al. Community-Associated Extended-Spectrum β-Lactamase–Producing Escherichia coli Infection in the United States. Clin Infect Dis. 2013;56(5):641–8.

127. Fennell J, Vellinga A, Hanahoe B, Morris D, Boyle F, Higgins F, et al. Increasing prevalence of ESBL production among Irish clinical Enterobacteriaceae from 2004 to 2008: an observational study. BMC Infect Dis. 2012;12:1–8.

128. Ludden C, Cormican M, Vellinga A, Johnson JR, Austin B, Morris D. Colonisation with ESBL-producing and carbapenemase-producing

Enterobacteriaceae, vancomycin-resistant enterococci, and meticillin-resistant Staphylococcus aureus in a long-term care facility over one year. BMC Infect Dis. 2015;15(168):1–12.

129. O'Connor C, Philip RK, Kelleher J, Powell J, O'Gorman A, Slevin B, et al. The first occurrence of a CTX-M ESBL-producing Escherichia coli outbreak mediated by mother to neonate transmission in an Irish neonatal intensive care unit. BMC Infect Dis. 2017;17(16):1–7.
130. Burke L, Humphreys H, Fitzgerald-Hughes D. The Molecular Epidemiology of Resistance in Cefotaximase-Producing Escherichia coli Clinical Isolates from Dublin, Ireland. Microb Drug Resist. 2016;22(7):552–8.
131. Public Health England. English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) [Internet]. 2018. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/759975/ESPAUR_2018_report.pdf
132. Johnson JR, Johnston B, Clabots C, Kuskowski MA, Castanheira M. *Escherichia coli* Sequence Type ST131 as the Major Cause of Serious Multidrug-Resistant *E. coli* Infections in the United States. Clin Infect Dis. 2010;51(3):286–94.
133. Quan J, Zhao D, Liu L, Chen Y, Zhou J, Jiang Y, et al. High prevalence of ESBL-producing Escherichia coli and Klebsiella pneumoniae in community-onset bloodstream infections in China. J Antimicrob Chemother. 2017;72:273–80.
134. Kim S, Sung JY, Cho HH, Kwon KC, Koo SH. Characteristics of the Molecular Epidemiology of CTX-M-Producing Escherichia coli Isolated from a Tertiary Hospital in Daejeon, Korea. J Microbiol Biotechnol. 2016;26(9):1643–9.
135. Moremi N, Manda E V., Falgenhauer L, Ghosh H, Imirzalioglu C, Matee M, et al. Predominance of CTX-M-15 among ESBL Producers from Environment and Fish Gut from the Shores of Lake Victoria in Mwanza,

Tanzania. Front Microbiol. 2016;7:1–11.

136. Huizinga P, Schrauwen E, García-Cobos S, Willemsen I, Verhulst C, Friedrich AW, et al. Extended-spectrum beta-lactamase producing Enterobacteriaceae (ESBL-E) isolated from bean sprouts in the Netherlands. PLoS One. 2018;13(8):1–13.

137. Ye Q, Wu Q, Zhang S, Zhang J, Yang G, Wang J, et al. Characterization of Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae From Retail Food in China. Front Microbiol. 2018;9:1–12.

138. Yaici L, Haenni M, Métayer V, Saras E, Mesbah Zekar F, Ayad M, et al. Spread of ESBL/AmpC-producing Escherichia coli and Klebsiella pneumoniae in the community through ready-to-eat sandwiches in Algeria. Int J Food Microbiol. 2017;245:66–72.

139. Kola A, Kohler C, Pfeifer Y, Schwab F, Kühn K, Schulz K, et al. High prevalence of extended-spectrum-β-lactamase-producing Enterobacteriaceae in organic and conventional retail chicken meat, Germany. J Antimicrob Chemother. 2012;67:2631–4.

140. Tansawai U, Sanguansermsri D, Na-udom A, Walsh TR, Niumsup PR. Occurrence of extended spectrum β-lactamase and AmpC genes among multidrug-resistant Escherichia coli and emergence of ST131 from poultry meat in Thailand. Food Control. 2018;84:159–64.

141. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011;17:873–80.

142. SafeFood. Assessment of antibiotic resistant Escherichia coli in meat production systems [Internet]. 2016. Available from: http://www.safefood.eu/Publications/Research-reports/Assessment-of-antibiotic-resistant-Escherichia-col.aspx

143. Hammerum AM, Jakobsen L, Olsen SS, Agersø Y. Characterization of CTX-M-14- and CTX-M-15-producing Escherichia coli of porcine origin. J Antimicrob Chemother. 2012;67:2047–54.

144. Shiraki Y, Shibata N, Doi Y, Arakawa Y. Escherichia coli producing CTX-M-2 beta-lactamase in cattle, Japan. Emerg Infect Dis. 2004;10(1):69–75.

145. Garcia-Cobos S, Kock R, Mellmann A, Frenzel J, Friedrich AW, Rossen JWA. Molecular Typing of Enterobacteriaceae from Pig Holdings in North-Western Germany Reveals Extended- Spectrum and AmpC β-Lactamases Producing but no Carbapenem Resistant Ones. PLoS One. 2015;10(7):1–12.

146. Maamar E, Hammami S, Alonso CA, Dakhli N, Abbassi MS, Ferjani S, et al. High prevalence of extended-spectrum and plasmidic AmpC beta-lactamase-producing Escherichia coli from poultry in Tunisia. Int J Food Microbiol. 2016;231:69–75.

147. Reich F, Atanassova V, Klein G. Extended-spectrum β-lactamase- and AmpC-producing enterobacteria in healthy broiler chickens, Germany. Emerg Infect Dis. 2013;19(8):1253–9.

148. Dierikx CM, van Duijkeren E, Schoormans AHW, van Essen-Zandbergen A, Veldman K, Kant A, et al. Occurrence and characteristics of extended-spectrum-β-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. J Antimicrob Chemother. 2012;67:1368–74.

149. Hasan B, Laurell K, Rakib MM, Ahlstedt E, Hernandez J, Caceres M, et al. Fecal Carriage of Extended-Spectrum β-Lactamases in Healthy Humans, Poultry, and Wild Birds in León, Nicaragua-A Shared Pool of blaCTX-M Genes and Possible Interspecies Clonal Spread of Extended-Spectrum β-Lactamases-Producing Escherichia coli. Microb drug Resist. 2016;22(8):682–7.

150. Sandegren L, Stedt J, Lustig U, Bonnedahl J, Andersson DI, Järhult JD. Long-term carriage and rapid transmission of extended spectrum beta-lactamase-producing E. coli within a flock of Mallards in the absence of antibiotic selection. Environ Microbiol Rep. 2018;10(5):576–82.

151. Khan FA, Söderquist B, Jass J. Prevalence and Diversity of Antibiotic

Resistance Genes in Swedish Aquatic Environments Impacted by Household and Hospital Wastewater. Front Microbiol. 2019;10:1–12.

152. Egervärn M, Englund S, Ljunge M, Wiberg C, Finn M, Lindblad M, et al. Unexpected common occurrence of transferable extended spectrum cephalosporinase-producing Escherichia coli in Swedish surface waters used for drinking water supply. Sci Total Environ. 2017;587–588:466–72.
153. Maravić A, Skočibušić M, Cvjetan S, Šamanić I, Fredotović Ž, Puizina J. Prevalence and diversity of extended-spectrum-β-lactamase-producing Enterobacteriaceae from marine beach waters. Mar Pollut Bull. 2015;90:60–7.
154. Blaak H, Lynch G, Italiaander R, Hamidjaja RA, Schets FM, De Husman AMR. Multidrug-Resistant and Extended Spectrum Beta-Lactamase-Producing Escherichia coli in Dutch Surface Water and Wastewater. PLoS One. 2015;10(6):1–16.
155. Kürekci C, Aydin M, Yipel M, Katouli M, Gündogdu A. Characterization of extended spectrum β-lactamase (ESBL)-producing Escherichia coli in Asi (Orontes) River in Turkey1. Kürekci C, Aydin M, Yipel M, Katouli M, Gündogdu A. Characterization of extended spectrum β-lactamase (ESBL)-producing Escherichia coli . J Water Health. 2017;15(5):788–98.
156. Bollache L, Bardet E, Depret G, Motreuil S, Neuwirth C, Moreau J, et al. Dissemination of CTX-M-Producing Escherichia coli in Freshwater Fishes From a French Watershed (Burgundy). Front Microbiol. 2019;9(3239):1–11.
157. de Boeck H, Miwanda B, Lunguya-Metila O, Muyembe-Tamfum J-J, Stobberingh E, Glupczynski Y, et al. ESBL-Positive Enterobacteria Isolates in Drinking Water. Emerg Infect Dis. 2012;18(6):1019–20.
158. Korzeniewska E, Harnisz M. Extended-spectrum beta-lactamase (ESBL)-positive Enterobacteriaceae in municipal sewage and their emission to the environment. J Environ Manage. 2013;128:904–11.
159. Tokajian S, Moghnieh R, Salloum T, Arabaghian H, Alousi S, Moussa J,

et al. Extended-spectrum β -lactamase-producing Escherichia coli in wastewaters and refugee camp in Lebanon. 2018;13(1):81–95.

160. Dolejska M, Frolkova P, Florek M, Jamborova I, Purgertova M, Kutilova I, et al. CTX-M-15-producing Escherichia coli clone B2-O25b-ST131 and Klebsiella spp. isolates in municipal wastewater treatment plant effluents. J Antimicrob Chemother. 2011;66:2784–90.
161. Day MJ, Rodríguez I, van Essen-Zandbergen A, Dierikx C, Kadlec K, Schink AK, et al. Diversity of STs, plasmids and ESBL genes among Escherichia coli from humans, animals and food in Germany, the Netherlands and the UK. J Antimicrob Chemother. 2016;71:1178–82.
162. Logan LK, Weinstein RA. The Epidemiology of Carbapenem-Resistant Enterobacteriaceae: The Impact and Evolution of a Global Menace. J Infect Dis. 2017;215(Suppl 1):S28–36.
163. Yigit H, Queenan AM, Anderson GJ, Domenech-sanchez A, Biddle JW, Steward CD, et al. Novel Carbapenem-Hydrolyzing Beta-Lactamase, KPC-1, from a Carbapenem-Resistant Strain of Klebsiella pneumoniae. Antimicrob Agents Chemother. 2001;45(4):1151–61.
164. Albiger B, Glasner C, Struelens MJ, Grundmann H, Monnet DL. Carbapenemase-producing Enterobacteriaceae in Europe: assessment by national experts from 38 countries, May 2015. Eurosurveillance. 2015;20(45):1–18.
165. Kitchel B, Rasheed JK, Patel JB, Srinivasan A, Navon-venezia S, Carmeli Y, et al. Molecular Epidemiology of KPC-Producing Klebsiella pneumoniae Isolates in the United States : Clonal Expansion of Multilocus Sequence Type 258. Antimicrob Agents Chemother. 2009;53(8):3365–70.
166. Cuzon G, Naas T, Correa A, Quinn JP, Villegas M, Nordmann P. Dissemination of the KPC-2 carbapenemase in non- Klebsiella pneumoniae enterobacterial isolates from Colombia. Int J Antimicrob Agents. 2013;42:59–62.
167. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al.

Characterization of a New Metallo-beta-Lactamase Gene, bla NDM-1, and a Novel Erythromycin Esterase Gene Carried on a Unique Genetic Structure in Klebsiella pneumoniae Sequence Type 14 from India. 2009;53(12):5046–54.

168. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis. 2010;10(9):597–602.
169. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. Lancet Infect Dis. 2011;11:355–62.
170. Health Protection Surveillance Centre. Monthly report on May 2019 data for the National Public Health Emergency Team [Internet]. 2019. Available from: https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/strategyforthecontrolofantimicrobialresistanceinirelandsari/carbapenemresistantenterobacteriaceaecre/surveillanceofcpeinireland/cpemonthlysurveillancereports/NPHET_CPE_May Data_2019_v1.0_Final.pd
171. Deparment of Health. Public Health Emergency Plan to tackle CPE [Internet]. 2017. Available from: https://health.gov.ie/national-patient-safety-office/patient-safety-surveillance/antimicrobial-resistance-amr-2/public-health-emergency-plan-to-tackle-cpe/
172. Kelly AM, Mathema B, Larson EL. Carbapenem-resistant Enterobacteriaceae in the community: a scoping review. Int J Antimicrob Agents. 2017;50:127–34.
173. Woodford N, Wareham DW, Guerra B, Teale C. Carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae from animals and the environment: an emerging public health risk of our own making? J Antimicrob Chemother. 2014;69:287–91.
174. Abdallah HM, Reuland EA, Wintermans BB, Al Naiemi N, Koek A,

Abdelwahab AM, et al. Extended-Spectrum β-Lactamases and/or Carbapenemases-Producing Enterobacteriaceae Isolated from Retail Chicken Meat in Zagazig, Egypt. PLoS One. 2015;10(8):1–8.

175. Roschanski N, Guenther S, Vu TTT, Fischer J, Semmler T, Huehn S, et al. VIM-1 carbapenemase-producing Escherichia coli isolated from retail seafood, Germany 2016. Eurosurveillance. 2017;22(43):1–7.
176. Grönthal T, Österblad M, Eklund M, Jalava J, Nykäsenoja S, Pekkanen K, et al. Sharing more than friendship – transmission of NDM-5 ST167 and CTX-M-9 ST69 Escherichia coli between dogs and humans in a family, Finland, 2015. Eurosurveillance. 2018;23(27):1–10.
177. Köck R, Daniels-Haardt I, Becker K, Mellmann A, Friedrich AW, Mevius D, et al. Carbapenem-resistant Enterobacteriaceae in wildlife, food-producing and companion animals – a systematic review. Clin Microbiol Infect. 2018;24(12):1241–50.
178. Piedra-Carrasco N, Fàbrega A, Calero-Cáceres W, Cornejo-Sánchez T, Brown-Jaque M, Mir-Cros A, et al. Carbapenemase-producing Enterobacteriaceae recovered from a Spanish river ecosystem. PLoS One. 2017;12(4):1–11.
179. Machado de Araujo CF, Silva DM, Carneiro MT, Ribeiro S, Fontana-Maurell M, Alvarez P, et al. Detection of Carbapenemase Genes in Aquatic Environments in Rio de Janeiro, Brazil. Antimicrob Agents Chemother. 2016;60(7):4380–3.
180. Paschoal RP, Campana EH, Corrêa LL, Montezzi LF, Barrueto LRL, da Silva IR, et al. Concentration and Variety of Carbapenemase Producers in Recreational Coastal Waters Showing Distinct Levels of Pollution. Antimicrob Agents Chemother. 2017;61(12):1–6.
181. Campana EH, Montezzi LF, Paschoal RP, Picão RC. NDM-producing Klebsiella pneumoniae ST11 goes to the beach. Int J Antimicrob Agents. 2017;49:119–21.
182. Fernando DM, Tun HM, Poole J, Patidar R, Li R, Mi R, et al. Detection of Antibiotic Resistance Genes in Source and Drinking Water Samples

from a First Nations Community in Canada. Appl Environ Microbiol. 2016;82:4767–75.

183. Picão RC, Cardoso JP, Campana EH, Nicoletti AG, Petrolini FVB, Assis DM, et al. The route of antimicrobial resistance from the hospital effluent to the environment: Focus on the occurrence of KPC-producing Aeromonas spp. and Enterobacteriaceae in sewage. Diagn Microbiol Infect Dis. 2013;76:80–5.

184. Health Protection Surveillance Centre. Guidance Relating to Laboratory Testing for Carbapenemase Producing Enterobacterales (CPE) and the Interpretation and Clinical Application of Results [Internet]. 2019. Available from: https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/strategyforthecontrolofantimicrobialresistanceinirelandsari/carbapenemresistantenterobacteriaceaecre/guidanceandpublications/Guidance Relating to Laboratory Testing for CPE and Interpretation and

185. Simner PJ, Gilmour MW, DeGagne P, Nichol K, Karlowsky JA. Evaluation of Five Chromogenic Agar Media and the Rosco Rapid Carb Screen Kit for Detection and Confirmation of Carbapenemase Production in Gram-Negative Bacilli. J Clin Microbiol. 2015;53(1):105–12.

186. Girlich D, Poirel L, Nordmann P. Comparison of the SUPERCARBA, CHROMagar KPC, and Brilliance CRE screening media for detection of Enterobacteriaceae with reduced susceptibility to carbapenems. Diagn Microbiol Infect Dis. 2013;75:214–7.

187. Drieux L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. Clin Microbiol Infect. 2008;14(SUPPL. 1):90–103.

188. Birgy A, Bidet P, Genel N, Doit C, Decré D, Arlet G, et al. Phenotypic Screening of Carbapenemases and Associated Beta-Lactamases in Carbapenem-Resistant Enterobacteriaceae André. J Clin Microbiol. 2012;50(4):1295–302.

189. Pasteran F, Tijet N, Melano RG, Corso A. Simplified Protocol for Carba NP Test for Enhanced Detection of Carbapenemase Producers Directly from Bacterial Cultures. J Clin Microbiol. 2015;53(12):3908–11.

190. Glupczynski Y, Evrard S, Ote I, Mertens P, Huang TD, Leclipteux T, et al. Evaluation of two new commercial immunochromatographic assays for the rapid detection of OXA-48 and KPC carbapenemases from cultured bacteria. J Antimicrob Chemother. 2016;71:1217–22.

191. Wareham DW, Momin MHFA. Rapid Detection of Carbapenemases in Enterobacteriaceae: Evaluation of the RESIST-3 O.K.N (OXA-48, KPC, NDM) Multiplexed Lateral Flow Assay. J Clin Microbiol. 2017;55(4):1223–5.

192. Papagiannitsis CC, Študentová V, Izdebski R, Oikonomou O, Pfeifer Y, Petinaki E, et al. Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry Meropenem Hydrolysis Assay with NH4 HCO3, a Reliable Tool for Direct Detection of Carbapenemase Activity. J Clin Microbiol. 2015;53(5):1731–5.

193. Lutgring JD, Limbago Brandi M. The Problem of Carbapenemase-Producing-Carbapenem-Resistant-Enterobacteriaceae Detection. J Clin Microbiol. 2016;54(3):529–34.

194. Birkett CI, Ludlam HA, Woodford N, Brown DFJ, Brown NM, Roberts MTM, et al. Real-time TaqMan PCR for rapid detection and typing of genes encoding CTX-M extended-spectrum beta-lactamases. J Med Microbiol. 2007;56:52–5.

195. Monteiro J, Widen RH, Pignatari ACC, Kubasek C, Silbert S. Rapid detection of carbapenemase genes by multiplex real-time PCR. J Antimicrob Chemother. 2012;67(4):906–9.

196. Van der Zee A, Roorda L, Bosman G, Fluit AC, Hermans M, Smits PHM, et al. Multi-centre evaluation of real-time multiplex PCR for detection of carbapenemase genes OXA-48, VIM, IMP, NDM and KPC. BMC Infect Dis. 2014;14(27):1–5.

197. Solanki R, Vanjari L, Ede N, Gungi A, Soory A, Vemu L. Evaluation of

LAMP assay using phenotypic tests and conventional PCR for detection of blaNDM-1 and blaKPC genes among carbapenem-resistant clinical Gram-negative isolates. J Med Microbiol. 2013;62:1540–4.

198. Wong YP, Othman S, Lau YL, Radu S, Chee HY. Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. J Appl Microbiol. 2018;124(3):628–43.
199. Hrabák J, Chudáčková E, Papagiannitsis CC. Detection of carbapenemases in Enterobacteriaceae: a challenge for diagnostic microbiological laboratories. Clin Microbiol Infect. 2014;20(9):839–53.
200. Cuzon G, Naas T, Bogaerts P, Glupczynski Y, Nordmann P. Evaluation of a DNA microarray for the rapid detection of extended-spectrum β-lactamases (TEM, SHV and CTX-M), plasmid-mediated cephalosporinases (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR and CMY-1-like/MOX) and carbapenemases (KPC, OXA-48, VIM, IMP and NDM). J Antimicrob Chemother. 2012;67(8):1865–9.
201. Braun SD, Monecke S, Thürmer A, Ruppelt A, Makarewicz O, Pletz M, et al. Rapid Identification of Carbapenemase Genes in Gram-Negative Bacteria with an Oligonucleotide Microarray-Based Assay. PLoS One. 2014;9(7).
202. Mathers AJ, Stoesser N, Sheppard AE, Pankhurst L, Giess A, Yeh AJ, et al. Klebsiella pneumoniae Carbapenemase (KPC)-Producing K. pneumoniae at a Single Institution: Insights into Endemicity from Whole-Genome Sequencing. Antimicrob Agents Chemother. 2015;59(3):1656–63.
203. Besser J, Carleton HA, Gerner-smidt P, Lindsey RL, Trees E. Next-Generation Sequencing Technologies and their Application to the Study and Control of Bacterial Infections. Clin Microbiol Infect. 2018;24(4):335–41.
204. Oniciuc EA, Likotrafiti E, Alvarez-Molina A, Prieto M, Santos JA, Alvarez-Ordóñez A. The Present and Future of Whole Genome Sequencing (WGS) and Whole Metagenome Sequencing (WMS) for Surveillance of

Antimicrobial Resistant Microorganisms and Antimicrobial Resistance Genes across the Food Chain. Genes (Basel). 2018;9(268):1–28.

205. Sabat AJ, Budimir A, Nashev D, Sá-Leão R, van Dijl JM, Laurent F, et al. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Eurosurveillance. 2013;18(4):1–15.
206. United Nations. Political Declaration of the High-level Meeting of the General Assembly on Antimicrobial Resistance [Internet]. 2016. Available from: https://digitallibrary.un.org/record/842813?ln=en
207. Food Safety Authority of Ireland. Potential for Transmission of Antimicrobial Resistance in the Food Chain [Internet]. 2015. Available from: https://www.fsai.ie/resources_publications.html
208. ECDC, EFSA, EMA. ECDC / EFSA / EMA first joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals 1 Joint Interagency Antimicrobial Consumption and Resi. EFSA J. 2015;13(1):1–114.
209. HM Government. UK One Health Report Joint report on antibiotic use and antibiotic resistance, 2013–2017 [Internet]. 2019. Available from: www.gov.uk/organisations/veterinary-medicines-directorate
210. EPA (Envionmental Protection Agency). Enchancing Human Health through Improved Water Quality [Internet]. 2013. Available from: http://erc.epa.ie/safer/reports%0APrepared
211. European Marine Board. Linking Oceans & Human Health: A Strategic Research Priority for Europe [Internet]. 2013. Available from: http://marineboard.eu/publication/linking-oceans-and-human-health-strategic-research-priority-europe
212. Boelee E, Geerling G, van der Zaan B, Blauw A, Vethaak AD. Water and health: From environmental pressures to integrated responses. Acta Trop. 2019;193:217–26.
213. Prüss-Üstün A, Bos R, Gore F, Bartram J. Safer water, better health: costs, benefits and sustainability of interventions to protect and

promote health. World Heal Organ. 2008;Geneva.

214. Leonard AFC, Zhang L, Balfour AJ, Garside R, Gaze WH. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ Int. 2015;82:92–100.

215. Leonard AFC, Zhang L, Balfour AJ, Garside R, Hawkey PM, Murray AK, et al. Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environ Int. 2018;114:1–8.

216. Laurens C, Jean-Pierre H, Licznar-Fajardo P, Hantova S, Godreuil S, Martinez O, et al. Transmission of IMI-2 carbapenemase-producing Enterobacteriaceae from river water to human. J Glob Antimicrob Resist. 2018;15:88–92.

217. UNESCO. The United Nations World Water Development Report 2017. [Internet]. 2017. Available from: https://reliefweb.int/sites/reliefweb.int/files/resources/247153e.pdf

218. Morris D, Harris S, Morris C, Commins E, Cormican M. EPA Report No. 162 Hospital effluent: impact on the microbial environment and risk to human health. EPA Res. 2015;(162).

219. Khan FA, Hellmark B, Ehricht R, Söderquist B, Jass J. Related carbapenemase-producing Klebsiella isolates detected in both a hospital and associated aquatic environment in Sweden. Eur J Clin Microbiol Infect Dis. 2018;37(12):2241–51.

220. EPA (Environmental Protection Agency). Urban Waste Water Treatment in 2017 [Internet]. 2018. Available from: http://www.epa.ie/pubs/reports/water/wastewater/2015 urban waste water report_Web Version.pdf

221. Montezzi LF, Campana EH, Corrêa LL, Justo LH, Paschoal RP, Da Silva ILVD, et al. Occurrence of carbapenemase-producing bacteria in coastal recreational waters. Int J Antimicrob Agents. 2015;45:174–7.

222. Madec JY, Haenni M, Nordmann P, Poirel L. Extended-spectrum β-

lactamase/AmpC- and carbapenemase-producing Enterobacteriaceae in animals: a threat for humans? Clin Microbiol Infect. 2017;23:826–33.

223. Muloi D, Ward MJ, Pedersen AB, Fèvre EM, Woolhouse MEJ, van Bunnik BAD. Are Food Animals Responsible for Transfer of Antimicrobial-Resistant Escherichia coli or Their Resistance Determinants to Human Populations? A Systematic Review. Foodborne Pathog Dis. 2018;15(8):467–74.
224. Ludden C, Raven KE, Jamrozy D, Gouliouris T, Blane B, Coll F, et al. One Health Genomic Surveillance of Escherichia coli Demonstrates Distinct Lineages and Mobile Genetic Elements in Isolates from Humans versus Livestock. MBio. 2019;10:e02693-18.
225. Dorado-García A, Smid JH, van Pelt W, Bonten MJMM, Fluit AC, van den Bunt G, et al. Molecular relatedness of ESBL/AmpC-producing Escherichia coli from humans, animals, food and the environment: a pooled analysis. J Antimicrob Chemother. 2018;73:339–47.
226. Benveniste R, Davies J. Aminoglycoside Antibiotic-Inactivating Enzymes in Actinomycetes Similar to Those Present in Clinical Isolates of Antibiotic-Resistant Bacteria. Proc Natl Acad Sci. 1973;70(8):2276–80.
227. National Ocean Service website. How much water is in the ocean? [Internet]. 2018. Available from: https://oceanservice.noaa.gov/facts/oceanwater.html
228. Locey KJ, Lennon JT. Scaling laws predict global microbial diversity. Proc Natl Acad Sci U S A. 2016;113(21):5970–5.
229. Poirel L, Bonnin RA, Nordmann P. Genetic Features of the Widespread Plasmid Coding for the Carbapenemase OXA-48. Antimicrob Agents Chemother. 2012;56(1):559–62.
230. Mahon BM, Brehony C, McGrath E, Killeen J, Cormican M, Hickey P, et al. Indistinguishable NDM-producing Escherichia coli isolated from recreational waters, sewage, and a clinical specimen in Ireland, 2016

to 2017. Eurosurveillance. 2017;22(15):1–5.

231. National Carbapenemase Producing Enterobacteriaceae (CPE) Reference Laboratory Service. CPEaRLS ANNUAL REPORT 2015 [Internet]. 2015. Available from: https://www.researchgate.net/publication/307601382_Annual_Report_2015_Carbapenemase_Producing_Enterobacteriaceae_Reference_Laboratory_of_Ireland
232. Bushnell G, Mitrani-Gold F, Mundy LM. Emergence of New Delhi metallo-beta-lactamase type 1-producing Enterobacteriaceae and non-Enterobacteriaceae: Global case detection and bacterial surveillance. Int J Infect Dis. 2013;17:e325–33.
233. Berrazeg M, Diene S, L M, Parola P, Drissi M, Raoult D, et al. New Dehli Metallo-beta-lactamase around the world: An eReview using Google Maps. Eurosurveillance. 2014;19(20).
234. O'Connor C, Cormican M, Boo TW, McGrath E, Slevin B, O'Gorman A, et al. An Irish outbreak of New Delhi metallo-beta-lactamase (NDM)-1 carbapenemase-producing Enterobacteriaceae: increasing but unrecognized prevalence. J Hosp Infect. 2016;94:351–7.
235. Zurfluh K, Hächler H, Nüesch-Inderbinen M, Stephan R. Characteristics of extended-spectrum β-lactamase- and carbapenemase-producing Enterobacteriaceae isolates from rivers and lakes in Switzerland. Appl Environ Microbiol. 2013;79(9):3021–6.
236. Poirel L, Barbosa-Vasconcelos A, Simoes RR, Da Costa PM, Liu W, Nordmann P. Environmental KPC-Producing Escherichia coli Isolates in Portugal. Antimicrob Agents Chemother. 2012;56(3):1662–3.
237. Kieffer N, Poirel L, Bessa LJ, Barbosa-vasconcelos A, Martins da Costa P, Nordmann P. VIM-1, VIM-34, and IMP-8 Carbapenemase-Producing Escherichia coli Strains Recovered from a Portuguese River. Antimicrob Agents Chemother. 2016;60(4):2585–6.
238. Kittinger C, Lipp M, Folli B, Kirschner A, Baumert R, Galler H, et al. Enterobacteriaceae Isolated from the River Danube: Antibiotic

Resistances, with a Focus on the Presence of ESBL and Carbapenemases. PLoS One. 2016;11(11):1–17.

239. Morris D, Kavanagh S, Carney K, MacDomhnaill B, Cormican M. CapE (capture, amplify, extract): A rapid method for detection of low level contamination of water with Verocytotoxigenic Escherichia coli (VTEC). Sci Total Environ. 2016;563–564:267–72.
240. EUCAST (European Committee on Antimicrobial Susceptibility Testing). Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0, 2017. [Internet]. 2017. Available from: http://www.eucast.org
241. Swayne RL, Ludlam HA, Shet VG, Woodford N, Curran MD. Real-time TaqMan PCR for rapid detection of genes encoding five types of non-metallo- (class A and D) carbapenemases in Enterobacteriaceae. Int J Antimicrob Agents. 2011;38(1):35–8.
242. CDC (Centers for Disease Control and Prevention). Multiplex Real-Time PCR Detection of Klebsiella pneumoniae Carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM-1) [Internet]. 2011. Available from: https://www.cdc.gov/hai/pdfs/labsettings/KPC-NDM-protocol-2011.pdf
243. Geyer CN, Reisbig MD, Hanson ND. Development of a TaqMan Multiplex PCR Assay for Detection of Plasmid-Mediated AmpC Beta-Lactamase Genes. J Clin Microbiol. 2012;50(11):3722–5.
244. Swaminathan B, Barrett TJ, Hunter SB, Tauxe R V. PulseNet: The Molecular Subtyping Network for Foodborne Bacterial Disease Surveillance, United States. Emerg Infect Dis. 2001;7(3):382–9.
245. EPA (Environmental Protection Agency). Bathing Water Quality in Ireland 2016 [Internet]. 2017. Available from: http://www.epa.ie/pubs/reports/water/bathing/BW Report 2016_web.pdf
246. Edberg SC, Rice EW, Karlin RJ, Allen MJ. Escherichia coli: the best biological drinking water indicator for public health protection. J Appl

Microbiol. 2000;88:106S-116S.

247. Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. Recent Advances in Understanding Enteric Pathogenic Escherichia coli. Clin Microbiol Rev. 2013;26(4):822–80.

248. Mahon BM, Brehony C, Cahill N, McGrath E, Connor LO, Varley A, et al. Detection of OXA-48-like-producing Enterobacterales in Irish recreational water. Sci Total Environ. 2019;690:1–6.

249. Falagas ME, Tansarli GS, Karageorgopoulos DE, Vardakas KZ. Deaths Attributable to Carbapenem-Resistant Enterobacteriaceae Infections. Emerg Infect Dis. 2014;20(7):1170–5.

250. Borer A, Saidel-Odes L, Riesenberg K, Eskira S, Peled N, Nativ R, et al. Attributable Mortality Rate for Carbapenem-Resistant Klebsiella pneumoniae Bacteremia. Infect Control Hosp Epidemiol. 2009 Oct 2;30(10):972–6.

251. Brolund A, Lagerqvist N, Byfors S, Struelens MJ, Monnet DL, Albiger B, et al. Worsening epidemiological situation of carbapenemase-producing Enterobacteriaceae in Europe, assessment by national experts from 37 countries, July 2018. Eurosurveillance. 2019;24(9):1–8.

252. Health Protection Surveillance Centre. Carbapenemase producing Enterobacterales (CPE) in HSE acute hospitals. Monthly report on January 2019 data for the National Public Health Emergency Team [Internet]. 2019. Available from: https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/strategyforthecontrolofantimicrobialresistanceinirelandsari/carbapenemresistantenterobacteriaceaecre/surveillanceofcpeinireland/cpemonthlysurveillancereports/NPHET_CPE_Jan Data_2019_v1.0_Final.pd

253. Skalova A, Chudejova K, Rotova V, Medvecky M, Studentova V, Chudackova E, et al. Molecular Characterization of OXA-48- Like-Producing Enterobacteriaceae in the Czech Republic and Evidence for Horizontal Transfer of pOXA-48-Like Plasmids. Antimicrob Agents

Chemother. 2017;61(2):e01889-16.

254. Beyrouthy R, Robin F, Delmas J, Gibold L, Dalmasso G, Dabboussi F, et al. IS1R-Mediated Plasticity of IncL/M Plasmids Leads to the Insertion of blaOXA-48 into the Escherichia coli Chromosome. Antimicrob Agents Chemother. 2014;58(7):3785–90.

255. Turton JF, Doumith M, Hopkins KL, Perry C, Meunier D, Woodford N, et al. Clonal expansion of Escherichia coli ST38 carrying a chromosomally integrated OXA-48 carbapenemase gene. J Med Microbiol. 2016;65(6):538–46.

256. Essack SY. Environment: the neglected component of the One Health triad. Lancet Planet Heal. 2018;2(6):e238–9.

257. Zarfel G, Lipp M, Gürtl E, Folli B, Baumert R, Kittinger C. Troubled water under the bridge: Screening of River Mur water reveals dominance of CTX-M harboring Escherichia coli and for the first time an environmental VIM-1 producer in Austria. Sci Total Environ. 2017;593–594:399–405.

258. Zerbino DR. Using the Velvet de novo assembler for short-read sequencing technologies. Curr Protoc Bioinforma. 2010;31(11):1–13.

259. Brehony C, Mcgrath E, Brennan W, Tuohy A, Whyte T, Brisse S, et al. An MLST approach to support tracking of plasmids carrying OXA-48-like carbapenemase. J Antimicrob Chemother. 2019;74:1856–1862.

260. Zhou Z, Alikhan N-F, Sergeant MJ, Luhmann N, Vaz C, Francisco AP, et al. GrapeTree: visualization of core genomic relationships among 100 ,000 bacterial pathogens. Genome Res. 2018;28:1395–404.

261. Poirel L, Heritier C, Nordmann P. Chromosome-Encoded Ambler Class D Beta-Lactamase of Shewanella oneidensis as a Progenitor of Carbapenem-Hydrolyzing Oxacillinase. Antimicrob Agents Chemother. 2004;48(1):348–51.

262. Cantón R, González-Alba JM, Galán JC. CTX-M enzymes: Origin and Diffusion. Front Microbiol. 2012;3(110).

263. Cahill N, O'Connor L, Mahon B, Varley Á, McGrath E, Ryan P, et al.

Hospital effluent: A reservoir for carbapenemase-producing Enterobacterales? Sci Total Environ. 2019;672:618–24.

264. Bürgmann H, Frigon D, Gaze WH, Manaia CM, Pruden A, Singer AC, et al. Water and sanitation: an essential battlefront in the war on antimicrobial resistance. FEMS Microbiol Ecol. 2018;94(9):1–14.

265. Müller H, Sib E, Gajdiss M, Klanke U, Lenz-Plet F, Barabasch V, et al. Dissemination of multi-resistant Gram-negative bacteria into German wastewater and surface waters. FEMS Microbiol Ecol. 2018;94(5):1–11.

266. Price LB, Johnson JR, Aziz M, Clabots C, Johnston B, Tchesnokova V, et al. The Epidemic of Extended-Spectrum- β -Lactamase-Producing Escherichia coli ST131 Is Driven by a Single Highly Pathogenic Subclone, H 30-Rx. MBio. 2013;4(6):e00377-13.

267. Agabou A, Pantel A, Ouchenane Z, Lezzar N, Khemissi S, Satta D, et al. First description of OXA-48-producing Escherichia coli and the pandemic clone ST131 from patients hospitalised at a military hospital in Algeria. Eur J Clin Microbiol Infect Dis. 2014;33:1641–6.

268. Dimou V, Dhanji H, Pike R, Livermore DM, Woodford N. Characterization of Enterobacteriaceae producing OXA-48-like carbapenemases in the UK. J Antimicrob Chemother. 2012;67:1660–5.

269. Roe CC, Vazquez AJ, Esposito EP, Zarrilli R, Sahl JW. Diversity, Virulence, and Antimicrobial Resistance in Isolates From the Newly Emerging Klebsiella pneumoniae ST101 Lineage. Front Microbiol. 2019;10:1–13.

270. Loucif L, Kassah Laouar A, Saidi M, Messala A, Chelaghma W, Rolain J-M. Outbreak of OXA-48-producing Klebsiella pneumoniae involving an ST 101 clone in Batna University Hospital, Algeria. Antimicrob Agents Chemother. 2016;60(12):7494–7.

271. Cubero M, Cuervo G, Dominguez MÁ, Tubau F, Martí S, Sevillano E, et al. Carbapenem-resistant and carbapenem-susceptible isogenic isolates of Klebsiella pneumoniae ST101 causing infection in a tertiary hospital. BMC Microbiol. 2015;15(177):1–8.

272. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis. 2019;19:56–66.

273. Ashbolt NJ, Amézquita A, Backhaus T, Borriello P, Brandt KK, Collignon P, et al. Review Human Health Risk Assessment (HHRA) for Environmental Development and Transfer of Antibiotic Resistance. Environ Health Perspect. 2013;121(9):993–1002.

274. Prestinaci F, Pezzotti P, Pantosti A. Antimicrobial resistance: a global multifaceted phenomenon. Pathog Glob Health. 2015;109(7):309–18.

275. Larsson DGJ, Andremont A, Bengtsson-Palme J, Brandt KK, de Roda Husman AM, Fagerstedt P, et al. Critical knowledge gaps and research needs related to the environmental dimensions of antibiotic resistance. Environ Int. 2018;117:132–8.

276. EPA (Environmental Protection Agency). Bathing Water Quality in Ireland 2017 [Internet]. 2018. Available from: http://www.epa.ie/pubs/reports/water/bathing/Bathing Water Quality in Ireland - 2017.pdf

277. Doumith M, Day M, Ciesielczuk H, Hope R, Underwood A, Reynolds R, et al. Rapid Identification of Major Escherichia coli Sequence Types Causing Urinary Tract and Bloodstream Infections. J Clin Microbiol. 2015;53(1):160–6.

278. Health Protection Surveillance Centre. EARS-Net data on antimicrobial resistance in Ireland [Internet]. 2019. Available from: https://www.hpsc.ie/a-z/microbiologyantimicrobialresistance/europeanantimicrobialresistancesurveillancesystemearss/ears-netdataandreports/

279. Health Protection Surveillance Centre. Monthly report on April 2019 data for the National Public Health Emergency Team [Internet]. 2019. Available from: http://www.hpsc.ie/a-

z/microbiologyantimicrobialresistance/strategyforthecontrolofantimicrobialresistanceinirelandsari/carbapenemresistantenterobacteriaceaecre/surveillanceofcpeinireland/cpemonthlysurveillancereports/NPHET_CPE_Jan Data_2019_v1.0_Final.pdf

280. SMALT Version 0.7.4 [Internet]. 2013. Available from: ftp://ftp.sanger.ac.uk/pub/resources/software/smalt/smalt-manual-0.7.4.pdf.

281. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078–9.

282. Carver T, Simon R. Harris, Berriman M, Parkhill J, McQuillan JA. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. Bioinformatics. 2012;28(4):464–9.

283. Liu Y, Zhang H, Zhang X, Jiang N, Zhang Z, Zhang J, et al. Characterization of a NDM-19-producing Klebsiella pneumoniae strain harboring two resistance plasmids from China. Diagn Microbiol Infect Dis. 2019;93(4):355–61.

284. Mancini S, Keller PM, Greiner M, Bruderer V, Imkamp F. Detection of NDM-19, a novel variant of the New Delhi metallo-β-lactamase with increased carbapenemase activity under zinc-limited conditions, in Switzerland. Diagn Microbiol Infect Dis. 2019;In Press.

285. Wang Y, Tong MK, Chow KH, Cheng VCC, Tse CWS, Wu AKL, et al. Occurrence of highly conjugative IncX3 epidemic plasmid carrying bla NDM in enterobacteriaceaeisolates in geographically widespread areas. Front Microbiol. 2018;9(SEP):1–8.

286. Wailan AM, Paterson DL, Kennedy K, Ingram PR, Bursle E, Sidjabat HE. Genomic Characteristics of NDM-Producing Enterobacteriaceae Isolates in Australia and Their blaNDM Genetic Contexts. Antimicrob Agents Chemother. 2016;60(1):136–41.

287. Dortet L, Nordmann P, Poirel L. Association of the Emerging

Carbapenemase NDM-1 with a Bleomycin Resistance Protein in Enterobacteriaceae and Acinetobacter baumannii. Antimicrob Agents Chemother. 2012;56(4):1693–7.

288. Hao Y, Shao C, Bai J, Jin Y. Genotypic and Phenotypic Characterization of IncX3 Plasmid Carrying blaNDM-7 in Escherichia coli Sequence Type 167 Isolated From a Patient With Urinary Tract Infection. Front Microbiol. 2018;9:1–6.
289. Huang Y, Yu X, Xie M, Wang X, Liao K, Xue W, et al. Widespread Dissemination of Carbapenem-Resistant Escherichia coli Sequence Type 167 Strains Harboring blaNDM-5 in Clinical Settings in China. Antimicrob Agents Chemother. 2016;60(7):4364–8.
290. Shen P, Yi M, Fu Y, Ruan Z, Du X, Yu Y, et al. Detection of an Escherichia coli Tandem Copies of bla NDM-1 in the Chromosome. J Clin Microbiol. 2017;55(1):199–205.
291. Markovska R, Stoeva T, Boyanova L, Stankova P, Schneider I, Keuleyan E, et al. Multicentre investigation of carbapenemase-producing Klebsiella pneumoniae and Escherichia coli in Bulgarian hospitals – Interregional spread of ST11 NDM-1-producing K. pneumoniae. Infect Genet Evol. 2019;69:61–7.
292. Baraniak A, Izdebski R, Fiett J, Gawryszewska I, Bojarska K, Herda M, et al. NDM-producing Enterobacteriaceae in Poland, 2012-14: inter-regional outbreak of Klebsiella pneumoniae ST11 and sporadic cases. J Antimicrob Chemother. 2016;71:85–91.
293. Voulgari E, Gartzonika C, Vrioni G, Politi L, Priavali E, Levidiotou-Stefanou S, et al. The Balkan region: NDM-1-producing Klebsiella pneumoniae ST11 clonal strain causing outbreaks in Greece. J Antimicrob Chemother. 2014;69:2091–7.
294. Nicolas-Chanoine MH, Bertrand X, Madec JY. Escherichia coli ST131, an intriguing clonal group. Clin Microbiol Rev. 2014;27(3):543–74.
295. Matsumura Y, Pitout JDD, Gomi R, Matsuda T, Noguchi T, Yamamoto M, et al. Global Escherichia coli sequence type 131 clade with blaCTX-

M-27 gene. Emerg Infect Dis. 2016;22(11):1900–7.

296. Birgy A, Bidet P, Levy C, Sobral E, Cohen R, Bonacorsi S. CTX-M-27–Producing Escherichia coli of Sequence Type 131 and Clade C1-M27, France. Emerg Infect Dis. 2017;23(5):885.

297. Ghosh H, Doijad S, Falgenhauer L, Fritzenwanker M, Imirzalioglu C, Chakraborty T. blaCTX-M-27 –Encoding Escherichia coli Sequence Type 131 Lineage C1-M27 Clone in Clinical Isolates, Germany. Emerg Infect Dis. 2017;10(3):1754–6.

298. Wang J, Gibbons JF, Mcgrath K, Bai L, Li F, Leonard FC, et al. Molecular characterization of blaESBL-producing Escherichia coli cultured from pig farms in Ireland. J Antimicrob Chemother. 2016;71:3062–5.

299. Falgenhauer L, Imirzalioglu C, Oppong K, Akenten CW, Hogan B, Krumkamp R, et al. Detection and Characterization of ESBL-Producing Escherichia coli From Humans and Poultry in Ghana. Front Microbiol. 2019;9:1–8.

300. Cortes P, Blanc V, Mora A, Dahbi G, Blanco EJ, Blanco M, et al. Isolation and Characterization of Potentially Pathogenic Antimicrobial-Resistant Escherichia coli Strains from Chicken and Pig Farms in Spain. Appl Environ Microbiol. 2010;76(9):2799–805.

301. Hammerum AM, Larsen J, Andersen VD, Lester CH, Skytte TSS, Hansen F, et al. Characterization of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third-or fourth-generation cephalosporins. J Antimicrob Chemother. 2014;69:2650–7.

302. Blaak H, De Kruijf P, Hamidjaja RA, Van Hoek AHAM, De Roda Husman AM, Schets FM. Prevalence and characteristics of ESBL-producing E. coli in Dutch recreational waters influenced by wastewater treatment plants. Vet Microbiol. 2014;171:448–59.

303. Lothigius A, Sjoling A, Svennerholm A., Bolin I. Survival and gene expression of enterotoxigenic Escherichia coli during long-term

incubation in sea water and freshwater. J Appl Microbiol. 2010;108:1441–9.

304. Dirk van Elsas J, Semenov A V, Costa R, Trevors JT. Survival of Escherichia coli in the environment: fundamental and public health aspects. ISME J. 2011;5:173–83.
305. Davies CM, Long JAH, Donald M, Ashbolt NJ. Survival of Fecal Microorganisms in Marine and Freshwater Sediments. Appl Environ Microbiol. 1995;61(5):1888–96.
306. Williams AP, Avery LM, Killham K, Jones DL. Persistence, dissipation, and activity of Escherichia coli O157:H7 within sand and seawater environments. FEMS Microbiol Ecol. 2007;60:24–32.
307. Davies-Colley RJ, Bell RG, Donnison AM. Sunlight Inactivation of Enterococci and Fecal Coliforms in Sewage Effluent Diluted in Seawater. Appl Environ Microbiol. 1994;60(6):2049–58.
308. Fakruddin M, Shahnewaj KBM, Andrews S. Viable but Nonculturable Bacteria: Food Safety and Public Health Perspective. ISRN Microbiol. 2013;(703813):1–6.
309. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. Front Microbiol. 2014;5(258):1–20.
310. Garza DR, Dutilh BE. From cultured to uncultured genome sequences: metagenomics and modeling microbial ecosystems. Cell Mol Life Sci. 2015;72:4287–308.
311. Handelsman J. Metagenomics and Microbial Communities. Encyclopedia of Life Sciences. John Wiley & Sons; 2007.

-

CHAPTER 8: APPENDICES

Appendix 1 Antimicrobial Concentrations (Antimicrobial Susceptibility Testing)

Antimicrobial discs

Ampicillin (10µg)	Oxoid
Cefotaxime (5µg)	Oxoid
Cefoxitin (30µg)	Oxoid
Cefpodoxime* (10µg)	Mast Diagnostics
Cefpodoxime* (10µg) and Clavulanic acid (1µg)	Mast Diagnostics
Ceftazidime (30µg)	Oxoid
Choramphenicol (30µg)	Oxoid
Ciprofloxacin (5µg)	Oxoid
Ertapenem (10µg)	Oxoid
Gentamicin (10µg)	Oxoid
Kanamycin (30µg)	Oxoid
Meropenem (10µg)	Oxoid
Nalidixic Acid (30µg)	Oxoid
Tetracycine (30µg)	Oxoid
Trimethoprim (5µg)	Oxoid

*Cefpodoxime and clavulanic acid combination disc was used to screen for extended-spectrum β-lactamase (ESBL) production. If the zone of clearance for the cefpodoxime and clavulanic acid combination was > 5mm than the zone of clearance for cefpodoxime the isolate was a presumptive ESBL producer (confirmed via real-time PCR). *Klebsiella pneumonia* ATCC 700603 was used as a control when testing for ESBL production.

Appendix 2 VIM and IMP Real-Time PCR Assay

This duplex VIM and IMP real-time PCR assay was developed in-house by the National Carbapenemase-Producing *Enterobacterales* Reference Laboratory Service, University Hospital Galway.

Table S1 Primer and probe sequences

Primer/probe	Sequence
Forward *bla*$_{VIM}$ primer	5'-GATTGCYGATGGTGTTTGG-3'
Reverse *bla*$_{VIM}$ primer	5'-TGAGACCATTGGACGGGTAGA-3'
bla$_{VIM}$ probe	5'-JOE-CGCATATCGCAACGCRGTCGTTTG-3'
Forward *bla*$_{IMP}$ primer 1	5'-GGTTTGTDGAGCGYGGCTAT-3'
Forward *bla*$_{IMP}$ primer 2	5'-GGTTTGTDGARCGYGGCTAT-3'
Reverse *bla*$_{IMP}$ primer 1	5'-TTCATTTGTTAATWCAGATGCATAYG-3'
bla$_{IMP}$ probe 1	5'-FAM-AAGGCAGCATTTCCTCTCATTTTCATAGCG-BHQ1-3'
bla$_{IMP}$ probe 1	5'-FAM-AAGGCASYATTTCCTCWCATTTYCATAGYG-BHQ1-3'

Table S2 Mastermix

Reagent	Concentration of reagent (µM)	Volume per reaction (µl)
All primers (x5)	10	1 (x5)
bla$_{VIM}$ probe	10	1
bla$_{IMP}$ probes (x2)	10	0.4 (x2)
ABI mastermix		12.5
Sterile Water		0.7
Total		20

5µl DNA template was added per reaction

Table S3 Cycling conditions on ABI 7500 Fast Real-time PCR machine

Temperature and Time	No. of Cycles
50°C for 2 minutes	1
95°C for 10 minutes	1
95°C for 15 sec 60°C for 1 min	35

Appendix 3 Control Strains

Antimicrobial susceptibility testing control strains

Escherichia coli ATCC 25922
*Klebsiella pneumoniae A*TCC 700603

Table S4 Real-time PCR control strains

Control	Target
E. coli ATCC 25922	Negative Control
E. coli NCTC 13441	CTX-M Group-1
E. coli NCTC 13462	CTX-M Group-2
E. coli U51624	CTX-M Group-9
K. pneumoniae NCTC 13442	OXA-48
K. pneumoniae, NCTC 13438	KPC-3
K. pneumoniae NCTC 13443	NDM-1
K. pneumoniae, NCTC 13439	VIM-1
E. coli NCTC 13476	IMP

Pulsed-field gel electrophoresis control strain

Salmonella braenderup H9812

Appendix 4 Search Settings for ResFinder, PlasmidFinder and FimTyper

All available at http://www.genomicepidemiology.org/

ResFinder 3.1.0

Chromosomal mutations

- Select species - *E. coli*
- Show unknown mutations – show only known mutations
- Select threshold for %ID – 90%
- Select minimum length – 60%

Acquired antimicrobial resistance genes

- Select antimicrobial configuration – all (aminoglycoside, beta-lactam, colistin, fluoroquinolone, Fosfomycin, fusidic acid, glycopeptide, MLS-macrolide, lincosamide and streptogram, nitroimidazole, oxazolidinone, phenicol, rifampicin, sulphonamide, tetracycline, trimethoprim)
- Select threshold for %ID – 90%
- Select minimum length – 60%

Select type of your reads-Assembled Genome/Contigs

PlasmidFinder 2.0.2

- Select database- *Enterobacteriaceae*
- Select threshold for %ID – 95%
- Select minimum length – 60%
- Select type of your reads-Assembled or Draft Genome/Contigs*(fasta)

FimTyper 1.0

- Select database – FimH *E. coli*
- Select threshold for %ID – 95%
- Select type of your reads-Assembled Genome/Contigs

Appendix 5 Supplementary Table S5

Table S5 Overview of seawater and sewage sample sites, sampling dates, and detection of carbapenemase-producing *Enterobacterales* from Chapter 4.

Sample Site	Sample Type	Sampling Dates	Detection of carbapenemase-producing *Enterobacterales*
Bathing Site	Seawater	29/05/2017	OXA-48-like-producing *E. coli* (Ec_BM707)
Bathing Site	Seawater	19/06/2017	Not detected
Bathing Site	Seawater	10/07/2017	Not detected
Bathing Site	Seawater	17/07/2017	Not detected
Bathing Site	Seawater	14/08/2017	OXA-48-like-producing-*Klebsiella pneumoniae* (Kp_BM758)
Bathing Site	Seawater	11/09/2017	Not detected
SWWTP	Influent	12/06/2017	Not detected
SWWTP	Influent	26/06/2017	Not detected
SWWTP	Influent	10/07/2017	Not detected
SWWTP	Effluent	12/06/2017	Not detected
SWWTP	Effluent	26/06/2017	Not detected
SWWTP	Effluent	10/07/2017	Not detected

Appendix 6 Supplementary Table S6

Table S6 Overview of seawater and sewage sample sites, sampling dates, and detection of carbapenemase-producing *Enterobacterales* and extended-spectrum β-lactamase-producing *Enterobacterales* from Chapter 5.

Sample Site	Sampling Dates	Detection of carbapenemase-producing *Enterobacterales*	Detection of extended-spectrum β-lactamase-producing *Enterobacterales*
Stream A	25/05/2016	Not detected	Not detected
Stream A	22/06/2016	Not detected	ESBL-producing *E. coli*
Stream A	13/07/2016	Not detected	Not detected
Stream A	10/08/2016	Not detected	Not detected
Stream A	24/08/2016	Not detected	Not detected
Stream A	07/09/2016	Not detected	Not detected
Stream A	18/01/2017	Not detected	Not detected
Stream A	16/02/2017	Not detected	Not detected
Stream A	02/03/2017	Not detected	Not detected
Stream B.1	25/05/2016	Not detected	ESBL-producing *E. coli*
Stream B.1	22/06/2016	Not detected	ESBL-producing *E. coli*
Stream B.1	13/07/2016	NDM-producing *E. coli*	Not detected
Stream B.1	10/08/2016	Not detected	ESBL-producing *E. coli*
Stream B.1	24/08/2016	NDM-producing *E. coli*	ESBL-producing *E. coli*
Stream B.1	07/09/2016	Not detected	ESBL-producing *E. coli*
Stream B.1	15/09/2016	Not detected	ESBL-producing *E. coli*
Stream B.1	18/01/2017	Not detected	Not detected
Stream B.1	16/02/2017	Not detected	Not detected

Stream B.1	02/03/2017	Not detected	Not detected
Stream B.2	15/09/2016	Not detected	ESBL-producing *E. coli*
Stream B.3	15/09/2016	Not detected	ESBL-producing *E. coli*
Stream B.4	15/09/2016	Not detected	Not detected
River A.5	16/02/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
River A.5	02/03/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
River A.4	02/03/2017	Not detected	ESBL-producing *E. coli*
River A.3	02/03/2017	Not detected	ESBL-producing *E. coli*
River A.2	02/03/2017	Not detected	ESBL-producing *E. coli*
River A.1	15/09/2016	Not detected	ESBL-producing *E. coli*
River A.1	02/03/2017	Not detected	ESBL-producing *E. coli*
Seawater A	18/01/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Seawater A	16/02/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Seawater A	02/03/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Seawater A	24/05/2017	NDM-producing *E. coli*, NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Seawater A	21/06/2017	NDM-producing *E. coli*	Not detected
Seawater A	12/07/2017	Not detected	ESBL-producing *E. coli*
Seawater A	09/08/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Seawater A	06/09/2017	NDM-producing *E. coli*	ESBL-producing *E. coli*
Seawater B	18/01/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Seawater B	16/02/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Seawater B	02/03/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*

Sewage A.1	16/02/2017	Not detected	Not detected
Sewage A.1	02/03/2017	Not detected	Not detected
Sewage A.2	15/09/2016	NDM-producing *E. coli*, NDM-producing *K. pneumoniae*	Not detected
Sewage A.2	18/01/2017	NDM-producing *K. pneumoniae*	Not detected
Sewage A.2	16/02/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Sewage A.2	02/03/2017	NDM-producing *K. pneumoniae*	ESBL-producing *E. coli*
Sewage A.3	16/02/2017	Not detected	Not detected
Sewage A.3	02/03/2017	Not detected	ESBL-producing *E. coli*
Sewage A.4	15/09/2016	NDM-producing *E. coli*, NDM-producing *K. pneumoniae*	Not detected
Sewage A.4	18/01/2017	NDM-producing *K. pneumoniae*	Not detected
Sewage A.4	16/02/2017	Not detected	ESBL-producing *E. coli*
Sewage A.4	02/03/2017	Not detected	Not detected
Sewage A.5	15/09/2016	NDM-producing *E. coli*	ESBL-producing *E. coli*

Appendix 7 Awards and Honours Received During PhD

- 1st place at the Annual Scientific Meeting of the One Health European Joint Program, on Foodborne Zoonoses, Antimicrobial Resistance and Emerging Threats Oral Presentation Award Ireland, Dublin, May 2019.
- 1st place at College of Medicine, Nursing & Health Sciences Postgraduate Research Day Oral Presentation Award, NUI, Galway, Ireland, May 2018.
- 1st place at Irish Society for Clinical Microbiology Oral Presentation Award, Limerick, Ireland, November 2017.
- 3rd place at Antimicrobial Resistance and One Health Conference Poster Presentation Award, NUI Maynooth, Ireland, August 2017.
- 1st place at Irish Society for Clinical Microbiology Poster Presentation Award, Dublin, Ireland, March 2017.
- Travel Award from the American Society for Microbiology to present at the American Society for Microbiology, Annual Scientific Meeting, San Francisco, USA, June 2019.
- Travel Award from the Microbiology Society (UK) to present at the American Society for Microbiology, Annual Scientific Meeting, San Francisco, USA, June 2019.
- Travel Award from Ryan Institute, NUI Galway, to present at the American Society for Microbiology, Annual Scientific Meeting, San Francisco, USA, June 2019.
- Recipient of an Education and Outreach Grant from the Microbiology Society, UK, March 2018.
- Recipient of the European Centre for Disease Prevention and Control (ECDC) 'FUNDING INITIATIVE ESCAIDE 2018' to present at ESCAIDE, Malta, November 2018.
- Short-Term Mission Travel Award from the Med-Vet-Net association to complete a research placement in the Institut Pasteur, Paris, France, June 2018.

- Travel Award from ECDC to present at the European Society of Clinical Microbiology and Infectious Diseases Annual Scientific Meeting, Madrid, Spain, April 2018.
- Travel Award from British Society for Antimicrobial Chemotherapy (BSAC) to present at BSAC Antibiotic Resistance and Mechanisms Workshop for Researchers', Birmingham, UK, December 2017.
- Travel Award to attend Summer School in Bioinformatics, Cambridge, UK, June 2017.
- Travel Award from BSAC to present at the BSAC Antibiotic Resistance and Mechanisms Workshop for Researchers', Birmingham, UK November 2016.
- Travel Award from Infectious Disease Society of Ireland (IDSI) to present at IDSI ASM, Dublin, Ireland May 2016.

Appendix 8 Dissemination of Research

Peer Reviewed Publications:

Mahon, B.M., Brehony, C., McGrath, E., Cormican, M., Rodrigues, C., Brisse, S., Jolley, K.A., Maiden, M.C., and Morris, D. High-resolution genomic analysis of carbapenemase-producing *Enterobacterales* and extended spectrum-β lactamase-producing *Enterobacterales* isolated from the aquatic environment, sewage and clinical specimens. *To be submitted.*

Mahon, B.M., Brehony, C., Cahill, N., McGrath, E., O'Connor, L., Varley, A., Cormican, M., Ryan, S., Hickey, P., Keane, S., Mulligan, M., Ruane, B., Jolley, K.A., Maiden, M.C., Brisse, S. and Morris, D. 2019. Detection of OXA-48-like-producing *Enterobacterales* in Irish recreational water. Science of the Total Environment, 690, 1-6. https://doi.org/10.1016/j.scitotenv.2019.06.480

Cahill, N., O'Connor, L., **Mahon, B.,** Varley, A., McGrath, E., Ryan, P., Cormican, M., Brehony, C., Jolley, K.A., Maiden, M.C., Brisse, S., Morris, D. 2019. Hospital effluent: a reservoir for carbapenemase-producing Enterobacterales? Science of the Total Environment, 672, 618–624. https://doi.org/10.1016/j.scitotenv.2019.03.428

Mahon, B.M., Brehony, C., McGrath, E., Killeen, J., Cormican, M., Hickey, P., Keane, S., Hanahoe, B., Dolan, A., Morris, D. 2017. Indistinguishable NDM-producing *Escherichia coli* isolated from recreational waters, sewage, and a clinical specimen in Ireland, 2016 to 2017. Euro Surveill, 22(15):pii=30513. https://doi.org/10.2807/1560-7917.ES.2017.22.15.30513

Conference Presentations

International conferences

- Whole Genome Sequence Analyses of NDM-19-Producing *Enterobacterales* Isolated from Recreational Waters and Sewage in Ireland. American Society for Microbiology, Annual Scientific Meeting, San Francisco, USA, June 2019 (poster and flash oral presentation).
- Detection of Carbapenemase-Producing Enterobacterales and Extended-Spectrum β-Lactamase-Producing *Enterobacterales* in Irish Recreational Waters, 2016-2017. 1st annual scientific meeting of the One Health European Joint Program on Foodborne Zoonoses, Antimicrobial Resistance and Emerging Threats, Dublin, Ireland, May 2019 (oral presentation).
- Carbapenemase-Producing *Enterobacterales* and Extended-Spectrum β-Lactamase-Producing *Enterobacterales* Detected in Irish Recreational Waters, 2016-2017. Microbiology Society (UK), Annual Scientific Meeting, Belfast, Northern Ireland, April 2019 (poster presentation).
- OXA-48-Producing *Enterobacteriaceae* Detected in Irish Seawater, 2017. European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE), Saint Julian's, Malta, November 2018 (oral presentation).
- Whole Genome Sequencing Analysis of Extended Spectrum Beta-Lactamase-Producing *Escherichia coli* Isolated from Recreational Water and Sewage. European Congress of Clinical Microbiology and Infectious Disease, Madrid, Spain, April 2018 (Eposter with mini-oral)
- Consistent Contamination of Recreational Water with Carbapenemase-Producing *Enterobacteriaceae*, Ireland, 2016-2017. European Congress of Clinical Microbiology and Infectious Disease, Madrid, Spain, April 2018 (poster presentation).

- Characterisation of New Delhi Metallo-Beta-Lactamase-Producing *Enterobacteriaceae* isolated from Recreational Water, Sewage, and Clinical Specimens in Ireland, 2016 to 2017. Antibiotic Resistance and Mechanisms: Workshop for Researchers', The British Society for Antimicrobial Chemotherapy, Birmingham, UK, December 2017 (poster presentation).
- Whole Genome Sequence Analyses Identifies Diversity Among Antimicrobial Resistant *Escherichia coli* which Originated from Irish Retail Meats. Antibiotic Resistance and Mechanisms: Workshop for Researchers', The British Society for Antimicrobial Chemotherapy, Birmingham, UK, November 2016 (poster presentation).

National conferences/NUI Galway postgraduate research days

- Consistent Contamination of Recreational Water with Carbapenemase-Producing *Enterobacteriaceae*, Ireland, 2016-2017. College of Medicine, Nursing and Health Sciences Postgraduate Research Day, NUI Galway, Ireland, May 2018 (oral presentation).
- Characterisation of Extended Spectrum Beta-Lactamase-Producing *Escherichia coli* Isolated from Recreational Water, using Whole Genome Sequencing Analysis. College of Medicine, Nursing and Health Sciences Postgraduate Research Day, NUI Galway, Ireland, May 2018 (poster presentation).
- Irish Recreational Water Consistently Contaminated with Carbapenemase-Producing *Enterobacteriaceae*. Infectious Diseases Society of Ireland ASM, Galway, Ireland, May 2018 (oral presentation).
- Whole Genome Sequencing Analysis of Extended Spectrum Beta-Lactamase-Producing *Escherichia coli* Isolated from Recreational Water and Sewage. Infectious Diseases Society of Ireland ASM, Galway, Ireland, May 2018 (poster presentation).

- Use of Whole Genome Sequencing to Characterise and Compare ESBL Producing-*Escherichia Coli* Isolated from Recreational Water and Sewage. Environ, Cork Institute of Technology, Ireland, March 2018 (oral presentation).
- Recreational Water Consistently Contaminated with Carbapenemase-Producing *Enterobacteriaceae*, Ireland, 2016-2017. Environ, Cork Institute of Technology, Ireland, March 2018 (oral presentation).
- Similar Strains of New Delhi Metallo-Beta-Lactamase-Producing *Enterobacteriaceae* Isolated from Recreational Water, Sewage, and Clinical Specimens in Ireland, 2016-2017. Irish Society of Clinical Microbiology Autumn Meeting, Limerick, Ireland, November 2017 (oral presentation).
- The Agri-Food Chain as a Reservoir for Antimicrobial Resistant *Escherichia coli.* Antimicrobial Resistance & One Health Conference, Microbiology Society, NUI Maynooth, Ireland, August 2017 (poster presentation).
- Indistinguishable NDM-Producing *Escherichia coli* Isolated from Recreational Waters, Sewage, and a Clinical Specimen in Ireland. Antimicrobial Resistance & One Health Conference, Microbiology Society, NUI Maynooth, Ireland, August 2017 (poster with mini oral presentation).
- Similar Strains of New Delhi Metallo-Beta-Lactamase-Producing *Enterobacteriaceae* Isolated from Recreational Water, Sewage, and Clinical Specimens in Ireland. Faculty of Public Health Medicine Summer Scientific Meeting, Dublin, Ireland, May 2017 (oral presentation).
- The Agri-Food Chain as a Reservoir for Antimicrobial Resistant *Escherichia coli.* Environ, Athlone Institute of Technology, Ireland, April 2017 (oral presentation).

- Food Production Systems as a Reservoir for Antimicrobial Resistant *Escherichia coli.* Irish Society for Clinical Microbiology Spring Meeting, Dublin, Ireland, March 2017 (**poster presentation**).
- Whole Genome Sequence Analysis of Antimicrobial Resistant *Escherichia Coli* Isolated from Irish Retail Meats. College of Medicine, Nursing and Health Sciences Postgraduate Research Day, NUI Galway, Ireland, May 2016 (**oral presentation**).
- Whole Genome Sequence Analysis Reveals Diversity Among Antimicrobial Resistant *Escherichia coli* Isolated from Meats in the Irish Market Place. Environ, Limerick, Ireland, April 2016 (**oral presentation**).

National Reports

- Environmental Protection Agency: Bathing Water Quality Report for 2016, added in a new section (2.4 Emerging Issue - Antibiotic resistant bacteria) which referred to the early findings of this thesis (Chapter 3). Available from https://www.epa.ie/pubs/reports/water/bathing/BW%20Report%202016_web.pdf

Appendix 9 Media Coverage of Thesis Findings

- Featured on RTE's Eco Eye Series 17, Episode 6 'The Living Beach' Aired (on RTE1) on 12th February 2019 https://www.youtube.com/watch?v=6xvnLgw-82c
- http://www.irishtimes.com/opinion/editorial/untreated-sewage-1.3062565
- http://www.irishexaminer.com/ireland/powerfulsuperbug-detected-at-two-beaches-in-galway-448621.html
- https://www.irishtimes.com/news/ireland/irish-news/ndm-superbug-found-in-co-galway-a-first-on-european-beaches-1.3061199
- https://www.mummypages.ie/stay-safe-superbug-found-in-galway-waters-blamed-on-sewage
- http://connachttribune.ie/spiddal-beaches-superbug-sewage-flowing-sea-422/
- http://coastmonkey.ie/superbugs-bathing-waste-water-infrastructure-eu-epa/
- http://coastmonkey.ie/irish-beaches-good-bad-blue-flag/
- http://www.independent.ie/irish-news/health/superbug-that-can-cause-major-health-problems-found-out-at-two-irish-beaches-35652495.html
- http://www.irishmirror.ie/news/irish-news/powerful-superbug-detected-two-irish-10290178
- https://www.joe.ie/news/powerful-superbug-detected-two-irish-beaches-586267
- https://galwaybayfm.ie/galway-bay-fm-news-desk/superbug-detected-two-galway-beaches/
- https://galwaybayfm.ie/galway-bay-fm-news-desk/spiddal-revealed-location-beach-superbug/

- https://www.thesun.ie/news/909413/powerful-superbug-resistant-to-the-most-powerful-antibiotics-detected-at-two-irish-beaches/
- https://www.thetimes.co.uk/article/superbug-discovered-at-irish-beaches-0lnsc86c8
- http://extra.ie/news/irish-news/deadly-superbug-discovered-two-irish-beaches
- https://lovin.ie/counties/galway/powerful-superbug-detected-at-two-irish-beaches
- http://www.irishsun.com/news/252941367/researchers-from-galway-find-powerful-superbug-at-two-irish-beaches
- https://www.yourdaysout.ie/news/powerful-superbug-found-on-irish-beaches-due-to-untreated-human-sewage-2398
- http://theliberal.ie/warning-issured-a-rare-superbug-has-been-found-on-two-irish-beaches/
- http://www.breakingnews.ie/ireland/raw-sewage-blamed-as-superbug-found-on-two-irish-beaches-787006.html
- http://www.tv3.ie/xpose/article/entertainment-news/237970/Powerful-superbug-detected-at-TWO-Irish-beaches
- http://tuairisc.ie/superbug-aimsithe-ag-tranna-an-spideil-an-chead-uair-riamh-a-thangthas-ar-a-leitheid-san-eoraip/