THE UNIVERSITY OF HULL

Isolation and characterisation of imipenem-resistant bacteria from natural environments and clinical settings

being a Thesis submitted for the Degree of
Doctor of Philosophy in Biological Sciences (Molecular Microbiology)
in the University of Hull

by

Fahd Alkhaleefah BSc. MSc.

March 2015

Dedication

I would like to dedicate this work to my father, who sadly passed away during my postgraduate studies, and also to my mother, for all the unconditional love and support they have given me throughout my life.

To my wife, Abeer, for her constant love and companionship, and for sharing the good times as well as the bad during our time away from home.

To Yara and Asil, my children, for bringing so much joy and happiness into my life.

To my brothers and sisters, for always being there for me and cheerfully giving freely of their love and encouragement.

To all my friends in Hull, for their warmth and kindness during the years I have been here, and for all the great times we have had together.

Abstract

The development and spread of bacterial resistance to antimicrobials is now recognised as a key threat to public health and society. A small number of antimicrobials, including imipenem and vancomycin, are now considered to be the drugs of 'last resort' for treating antibiotic resistant bacteria. This study investigates and characterises antibiotic (imipenem) resistant bacteria in environmental and clinical samples from the U.K. Imipenem resistant (ImR) bacteria were isolated and characterised from river water samples from East Yorkshire and soil samples from Lincolnshire. ImR clinical isolates from different hospitals (York, Sheffield and Hull) were also characterised. Phenotypic resistance to imipenem was observed in 11.2% (75/670 CFU ml^{-1}), 13.3% (145.35 x 10^5/ 109.1 x 10^6 CFU g^{-1}) and 38.5% (42/109) of water, soil and clinical bacterial isolates, respectively.The minimum inhibitory concentrations (MICs) of the clinical isolates were generally higher (> 32 mg L^{-1} in 71.4% of isolates) than those of the environmental isolates, which were around 4 mg L^{-1} in 63.4% of water isolates and in 42.7% of soil isolates. β-lactamase activity studies showed that the most common β-lactamases among the environmental isolates were class B metallo β-lactamases (MBLs) (84.2%), while class A *Klebsiella pneumoniae* carbapenemases (KPCs) (40.5%) were the most common β-lactamases observed in the clinical isolates. Higher frequencies of multi-drug resistant (MDR) patterns were detected among the environmental isolates than among the clinical strains. Sequencing of 16S rRNA genes identified 30 (17 species), 96 (27 species), and 42 (11 species) ImR bacteria in water, soil and clinical samples, respectively. The most abundant genera identified were *Caulobacter* (36.7%), *Stenotrophomonas* (44.8%) and *Stenotrophomonas* (40.5%) from water, soil and clinical environments, respectively. PCR products were generated from ImR clinical isolates and some of the environmental isolates using primers targeting β-lactamase genes. Sequence analysis of these products from clinical isolates showed that they were specific and related to β-lactamase genes. However, the products from environmental isolates were not related to known genes characterised from antibiotic resistant clinically important bacteria. This suggests that there is a potentially large and divergent gene pool encoding for imipenem ressitance within natural environments, and that river water and agricultural soil are important as reservoirs of novel antibiotic resistance. Genome sequencing was used to characterise 8 MDR *Stenotrophomonas* spp. isolates from water, soil and clinical samples. This analysis showed the detection of β-lactamases genes (between 8 and 15 genes per isolate) including class A (L2), B (L1) and C (AmpC), fluoroquinolones resistance genes (between 4-8 genes per isolate), and genes encoding MDR efflux pumps (between 23-32 genes per isolate). Antibiotic resistance genes for other antimicrobials were also observed in small numbers; these represented aminoglycoside, sulphonamide and tetracycline resistance. Genes encoding resistance to heavy metal resistance (between 13-27 genes per isolate) were also observed. Overall, this research has demonstrated the widespread presence of imipenem resistant bacteria in environmental and clinical settings, carrying multiple resistances to other antibiotics. In particular, imipenem resistant *Stenotrophomonas* spp. were present in all of the environments studied and these bacteria were found to harbour multiple and diverse antibiotic resistance genes, that differed between isolates from environmental and clinical origins.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Professor Mark Osborn, for all he has done to help this study come to fruition. I would like to thank Mark especially for his inspiration and guidance during each stage of the project and for patiently and meticulously reading through my work. He has always been more than generous with his experience and knowledge.

Special thanks are also due to Mrs Christine Murphy for her constant support and encouragement throughout this study. Christine has always been there for me whenever I have needed advice and nothing was ever too much trouble for her.

I would also like to thank Dr Jennifer Waby for her advice and encouragement and for engaging me in various academic activities. Tremendous thanks also go to Professor John Greenman, who has been a major help in making practical arrangements and ensuring that everything ran smoothly for me. I would also like to thank Dr Gavin Paterson for taking an interest in my work and for reassuring me in some difficult moments.

I am greatly indebted to my employer and sponsor, the Ministry of Health in Saudi Arabia, for giving me this wonderful opportunity to study at a UK university and for financially supporting me during the whole process. Thanks also go to the Ministry of Education in Saudi Arabia and the Cultural Bureau of Saudi Arabia in London for all their help in managing the practical matters involved in living and studying abroad.

Finally, I would like to thank the University of Hull and the School of Biological, Biomedical and Environmental Sciences for the many ways in which they have helped me and made me feel at home.

Abbreviations

Abbreviation	Description
AMR	Antimicrobial drug resistance
AR	Antibiotic resistant
ARG	Antibiotic resistance gene
ART	Anti-retroviral therapy
ATM	Aztreonam
ATP	Adenosine tri-phosphate
BA	Boronic acid
BLAST	Basic Local Alignment Search Tool
CAZ	Ceftazidime
CDST	Combined disc synergy test
CF	Cystic fibrosis
CFU	Colony-forming unit
CGE	Centre for Genomic Epidemiology
CIP	Ciprofloxacin
CN	Gentamicin
CPE	Carbapenemase-producing enterobacteria
CRAB	Carbapenem-resistant *Acinetobacter baumanii*
CRKP	Carbapenem-resistant *Klebsiella pneumoniae*
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum β-lactamase
GC-content	Guanine-cytosine content
gDNA	Genomic DNA
GI	Genomic island
GISA	Glycopeptide-intermediate resistant *Staphylococcus aureus*
GNSA	Gram-negative selective agar

Abbreviation	Description
HAI	Hospital-acquired infection
HGT	Horizontal gene transfer
Im/IMP/IPM	Imipenem
ImR	Imipenem-resistant
IS	Insertion sequence
KPC	*Klebsiella pneumoniae* carbapenemase
LEV	Levofloxacin
MBL	Metallo-β-lactamase
MCA	MacConkey agar
MDR	Multi-drug resistant
MEM/MER	Meropenem
MGE	Mobile genetic element
MH	Minocycline
MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
MLST	Multi-Locus Sequence Typing
MRG	Heavy metal resistance gene
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NDM	New Delhi metallo-β-lactamase
NGS	Next-generation sequencing
NPK	Nitrogen, phosphorus and potassium
OD	Optical density
ORF	Open reading frame
OXA	Oxacillin
PABA	Para-aminobenzoic acid
PBP	Penicillin-binding protein
PCA	Plate count agar
PCR	Polymerase chain reaction

Abbreviation	Description
PEG	Protein encoding gene
PIA	*Pseudomonas* isolation agar
RAST	Rapid Annotation using Subsystem Technology
RNA	Ribonucleic acid
RND	Resistance nodulation-cell division
SCV	Small colony variant
SHV	Sulfhdryl variable
SXT	Co-trimoxazole
TBDR	TonB-dependent receptor
TE	Tetracycline
TIM	Ticarcillin-clavulanate
TSA	Tryptone soya agar
TTC	Tergitol-7-agar
TVC	Total viable bacterial count
UTI	Urinary tract infection
VIM	Verona integron–encoded metallo-β-lactamase
VISA	Vancomycin- intermediate resistant *Staphylococcus aureus*
Vm	Vancomycin
VmR	Vancomycin-resistant
VRE	Vancomycin-resistant *Enterococci*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
W	Trimethoprim
XDR	Extensively drug-resistant

Table of Contents

List of Tables

Appendix

List of Figures

Appendix

Chapter 1: Introduction

1.1 Overview

Before the discovery of antimicrobial agents, bacteria were known to cause severe morbidity and high rates of mortality (Medeiros, 1997). Antimicrobial agents were known and used for the control of bacterial infections in the form of mercury, bismuth and other heavy metals and were used in treatment of syphilis caused by *Treponema pallidum* from the early 1400s (Shlaes, 2010). However, the use of such agents, despite being somewhat effective in treating the infection, was associated with toxic effects on the patients (Hawkey, 2008; Shlaes, 2010). In 1908, Paul Ehrlich and colleagues discovered Salvarsan, which is an arsenic compound used in the treatment of syphilis, again with toxic effects on the host (Shlaes, 2010).

Efforts were continuous in order to discover agents which would treat infection but not harm the host. This was achieved by the discovery of the first conventional antimicrobial drug sulfonamide by Bayer in 1932 (Figure 1.1). This was followed in the early 1940s by the purification and application of penicillin compounds by Florey and his colleagues in Oxford, based upon the discovery of Alexander Fleming's natural penicillin (Fleming, 1929) which was considered the first natural antibiotic to be discovered and opened the gate for isolation and use of other antibiotics and antimicrobials (Greenwood, 2000). Discovery of other antimicrobials followed later e.g., streptomycin in 1944, chloramphenicol in 1947 and chlortetracycline in 1948 (Martel *et al.*, 2001).

In contrast to the common belief which considers antimicrobial resistance to be a new discovery, it is actually older. The concept of drug resistance had already been considered in 1907 during work by Ehrlich in trying to discover an agent for the drug-resistant *Trypanosoma brucei*, which was known as "drug-fast". Resistance was explained, at that time, by the adaptation of the organism to the action of a drug and was noticed to be dose-dependent (Ehrlich, 1907). Many research groups were subsequently established to study, in detail, the mechanisms associated in the development of bacterial antimicrobial drug resistance based upon the study of microbial metabolism and enzymatic production; mutation was the genetic mechanism implicated in such resistance (Lederberg and Lederberg, 1952).

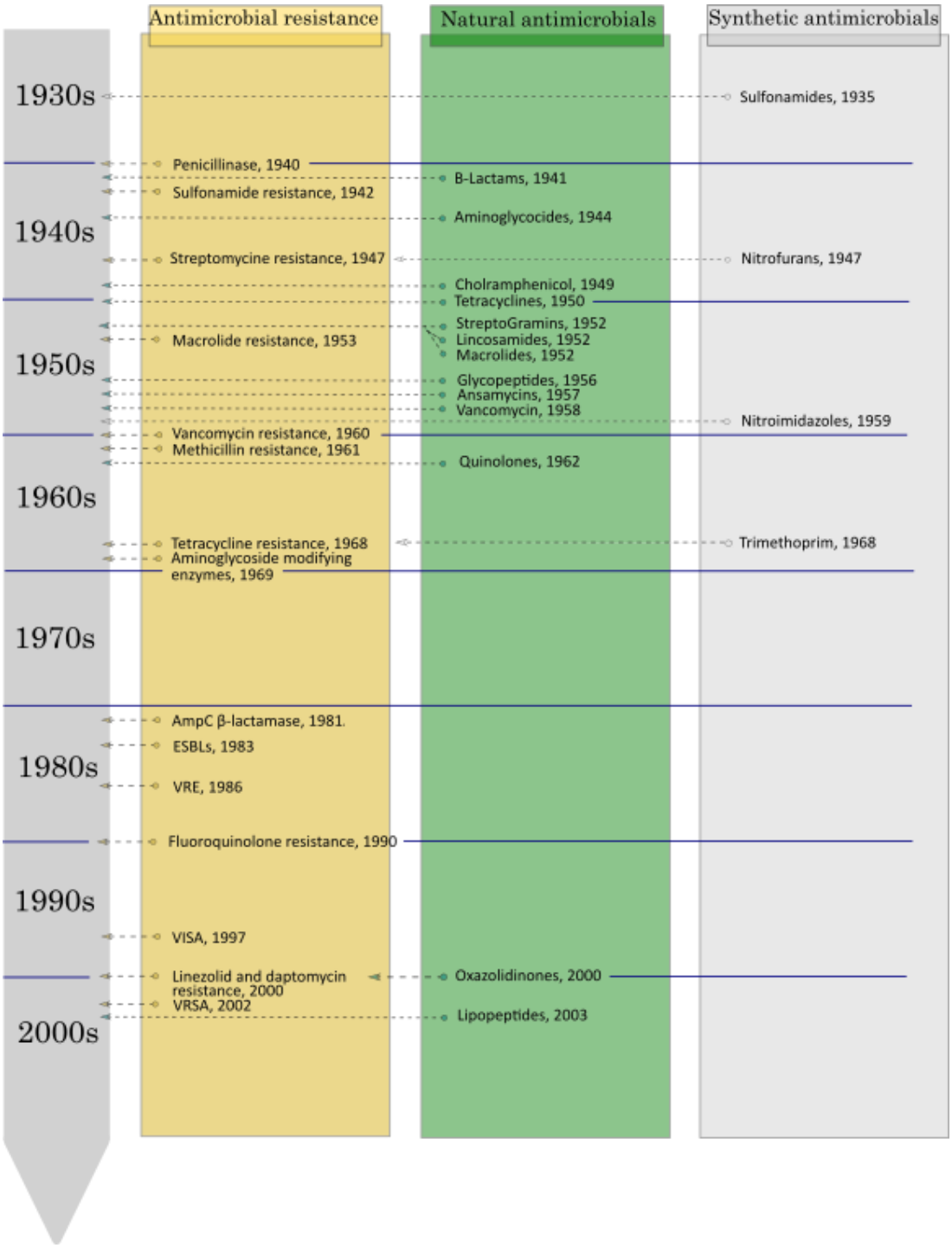


Figure 1.1: Timeline of antimicrobial discoveries and the appearance of drug resistance

The first antimicrobial to be discovered was sulphonamide, then penicillins. Resistance to these were developed after a few years (adapted from information in Greenwood (2000); Walsh (2003); Silver (2011) and Lewis (2013)). Green boxes = natural antimicrobials; gray boxes = synthetic antimicrobials; yellow boxes = antimicrobial resistance. Abbreviations: ESBLs = extended spectrum β-lactamases; VRE = vancomycin-resistant enterococci; VISA = vancomycin-intermediate resistant *Staphylococcus aureus*; VRSA = vancomycin-resistant *Staphylococcus aureus.*

Bacterial antimicrobial drug resistance increased and became more complicated via a number of resistance mechanisms with the introduction of further antimicrobial drugs in the 1950s. Resistance to many antimicrobials developed further and was selected for at the same time in the form of single microorganisms known as multidrug-resistant (MDR) bacteria. Mutation alone did not provide a sufficient explanation for multidrug resistance, as multiple mutations mostly affects one or more genes at a time due to the proofreading mechanisms in bacteria. Gene transfer of resistance genes from one bacterial strain to another was therefore suggested to play a role through the transfer of plasmids (Ochiai *et al.*, 1959; Watanabe, 1963). A few years later, transposons, integrons and gene cassettes were identified as having roles in antimicrobial resistance gene transfer (Hedges and Jacob, 1974; Stokes and Hall, 1989).

Antimicrobial resistance is clinically, economically and socially important. It leads to an increase in the rates of morbidity and mortality related to microbial infections and consequently considerable financial resources are directed towards the discovery of new agents (Hawkey, 2008). Antimicrobial resistance also leads to an increase in the number of hospital admissions and higher incidences of complications associated with infections due to multidrug-resistant microbial strains. This leads to increased costs due to the isolation procedures for infection control which have to be undertaken by the hospitals, and can also result in delays and cancellations of some operative interventions (Hawkey, 2008).

The observation that the emergence of antimicrobial-resistant organisms is due to the selective pressure of antimicrobials used in clinical practice, agriculture and animal feeding is reported by many researchers. It relates the emergence of antimicrobial drug resistance in the community to the use of such antimicrobials (Levy, 2002; Goossens *et al.*, 2005). However, surprisingly, resistant bacteria have been detected in communities with remote or minimum usage of antibiotic selective pressure, adding further complications to the matter (Pallecchi *et al.*, 2007).

In both aquatic and soil environments, antibiotics are produced by many microorganisms. These environments are also the sites where antibiotics are deposited (i.e. as a sink) and where antimicrobial-resistance genes evolve and are transferred between different bacterial species. These factors have led to the transfer of antimicrobial-resistant genes to clinical isolates of bacteria by horizontal gene

transfer (HGT) and via the food chain, with the consequent increasing loss of efficacy of most of the available antimicrobials currently in use (Kümmerer, 2009).

1.2 Antimicrobial agents

Antimicrobial agents are substances that kill or inhibit the growth of microorganisms and are suitable for systemic use, i.e. they do not harm the host. The term "antibiotic" (Table 1.1) refers to a substance produced as a secondary metabolite by a bacterium (e.g. *Streptomyces orientalis* producing vancomycin, and *Bacillus subtilis* producing bacitracin) or by a fungus (e.g. *Penicillium chrysogenum* producing penicillin) which inhibits or kills other microorganisms. Since several of these antibiotics are now chemically synthesized, the term "antibiotic" is generally used for antimicrobial agent, whether naturally or synthetically produced (Hogg, 2005a; Kayser, 2005; Davies, 2006).

Table 1.1: Some naturally produced antibiotics in soil and aquatic environments

	Microorganism	Antibiotic produced
Bacillus group	*Bacillus subtilis*	Bacitracin
	Bacillus polymixa	Polymixin
Actinomycetes	*Micromonos porapurpurea*	Gentamycin
	Verrucosispora (Actinomycetes)	Abyssomicin C*
Streptomyces	*Streptomyces erythreus*	Erythromycin
	Streptomyces griseus	Streptomycin
	Streptomyces rimosus	Tetracycline
	Streptomyces orientalis	Vancomycin
	Streptomyces azureus and others	Thiostrepton*
Fungi	*Penicillium chrysogenum*	Penicillin
	Cephalosporium acremonium	Cephalosporins
Miscellaneous	*Amycolatopsis* spp. (related to Gram-negative bacteria)	Amythiamicin D*
	Pseudomonas fluorescens	Pseudomonic acids*
	Nocardia	Thiolactomycin*

(Source: Hogg, 2005a; Nicolaou *et al.*, 2009)

* = natural antibiotics under investigation and clinical trial

1.3 Classification and mechanism of action of antimicrobials

There are different classifications of antimicrobials according to the spectrum of activity, the mechanism of action, the killing or inhibitory effect upon microorganisms, and their chemical structure. Regarding their killing or inhibitory effect (Table 1.2), antibiotics are often referred to as “bactericidal”, for those causing the killing of the intended microorganism, e.g. penicillins and cephalosporins, or “bacteriostatic” for those causing a temporary state of inhibition of growth of the organism of interest (e.g. sulfonamides, tetracyclines, and chloramphenicol); the organism can be then killed by the host’s immune mechanism or grow again once the antibiotic is removed. However, in the case of a bactericidal agent multiplication cannot be resumed again (Calderon and Sabundayo, 2007). However, it is to be

noted that the classification into bacteriostatic or bactericidal is variable and is dependent upon the relative concentration of the antimicrobial used against the target microorganism (Forbes *et al*., 2007).

Table 1.2: Classification of antimicrobials according to their bactericidal (killing) or bacteriostatic (inactivation) activity

Generally bactericidal	Generally bacteriostatic
β-lactams	Macrolides
Aminoglycosides	Chloramphenicol
Quinolones	Sulfonamides
Vancomycin	Trimethoprim
Daptomycin	Tetracyclines
Teicoplanin	Tigecyclines
Metronidazole	Linezolid
Rifampin	Clindamycin
	Quinupristin/dafloprístin

(Source: Forbes *et al*., 2007)

The spectrum of activity which expresses the categories of microorganisms affected by certain antimicrobials is described as either narrow or broad (Table 1.3). Narrow-spectrum antimicrobials are those which inhibit a small number of organisms e.g. benzyl penicillin, which is active against many Gram-negative and -positive cocci but has little action against Gram-negative bacilli. Broad-spectrum antimicrobials, e.g. cephalosporins, tetracyclines, and chloramphenicol, affect a wide range of microorganisms including those which are Gram-positive and -negative. The spectrum of activity includes the initial susceptibility, and the organism is still considered susceptible even if most strains of a particular species develop acquired resistance (Ryan and Drew, 2004).

Antimicrobial agents can also be described as antiseptic or disinfectant if they destroy bacteria but are too toxic for host tissues and cells to be used systemically, and so are used instead for environmental and surface decontamination (Calderon and Sabundayo, 2007).

The classification of antibiotics according to their mechanism of action depends on the inhibition of a cellular structure or a metabolic channel that is present

in the bacterium but not in the host cell (selective toxicity). Several mechanisms are known (Table 1.3), such as the inhibition of bacterial cell wall synthesis, interference with cell membrane functions, inhibition of bacterial protein synthesis, and inhibition of nucleic acid synthesis (Levinson, 2008).

Table 1.3: Antimicrobial classification by mechanism of action and spectrum of activity

Class of antimicrobial	Mechanism of action	Spectrum
β-lactams (including carbapenems)	Inhibit the synthesis of **cell walls** by binding to enzymes involved in peptidoglycan production.	Both Gram-positive and Gram-negative bacteria with variation of spectrum according to individual antimicrobial.
Aminoglycosides	Inhibit the synthesis of bacterial **proteins** by binding to 30S ribosomal subunit.	Gram-positive and Gram-negative bacteria but not acting upon anaerobic bacteria.
Chloramphenicol	Inhibit the synthesis of bacterial **proteins** by binding to 50S ribosomal subunit.	Gram-positive and Gram-negative bacteria.
Glycyclines (Tigecycline)	Inhibit the synthesis of bacterial **proteins** by binding to 30S ribosomal subunit.	Broad spectrum, both Gram-positive and Gram-negative bacteria, even those resistant to tetracyclines.
Fluoroquinolones	Inhibit DNA synthesis by binding to DNA gyrases.	Gram-positive and Gram-negative bacteria with a variation in spectrum according to the individual antimicrobial.
Ketolides (telithromycin)	Inhibit the synthesis of bacterial proteins by binding to the 50S ribosomal subunit.	Gram-positive cocci and some Gram-negative bacilli e.g., *Hemophilus* and *Moraxilla*.
Oxazolidinone (Linezolid)	Bind to the 50S ribosomal subunit, inhibiting the initiation of protein synthesis.	Gram-positive bacteria
Lipopeptides (Daptomycin)	Binding and disrupting the cell membrane	Gram-positive bacteria including those resistant to β-lactams.
Rifampin	Binding to DNA-dependent RNA polymerase leading to inhibition of RNA synthesis.	Gram-positive and Gram-negative bacteria.
Tetracyclines	Inhibit the synthesis of bacterial proteins by binding to the 30S ribosomal subunit.	Gram-positive and Gram-negative bacteria and intracellular microorganisms such as *Chlamydia*.
Sulfonamides	Interferes with folic acid synthesis.	Gram-positive and many Gram-negative bacteria.
Trimethoprim	Interferes with folic acid synthesis.	Gram-positive and many Gram-negative bacteria.
Streptogramins (quinupristin/dafloprístin)	Inhibit the synthesis by binding to separate sites on 30S ribosomal subunits.	Gram-positive bacteria.
Glycopeptides (vancomycin and teicoplanin)	Inhibit cell wall synthesis but not via the PBP receptors.	Gram-positive bacteria, as glycopeptides are large molecules, so do not pass through Gram-negative bacterial cell walls.

(Source: Forbes *et al.*, 2007)

1.3.1 Inhibitors of bacterial cell wall synthesis

This group of drugs inhibits the synthesis of peptidoglycan, which is found only in the cell walls of bacteria. There is no impact on the patient's cells (as the drugs are bacteria-specific). Both penicillins and cephalosporins (Table 1.4) exert their actions after binding to a protein called the penicillin-binding protein (PBP). There are several types of PBP, and they act as enzymes catalyzing the synthesis of peptidoglycan. There is structural similarity between β-lactams and PBPs (Llarrull *et al*., 2009). After inhibition of cell wall synthesis, a bacterium will rapidly take fluids inside due to the higher internal osmotic pressure and then burst, i.e. bacteriolysis occurs, and thus all of these antimicrobials are bactericidal in action. This group of drugs includes β-lactam drugs, which have the β-lactam ring within their chemical structure (penicillins, cephalosporins, carbapenems and monobactams) (Figure 1.2). The β-lactam ring is essential for the antimicrobial activity of this group, as it damages the integral cell wall by irreversible acylation of the active serine site of the transpeptidase, which is essential for the synthesis of peptidoglycan in the cell wall (Konaklieva, 2014). The second group of cell wall inhibitors is the non-β-lactams (glycopeptides; vancomycin), containing no β-lactam rings (Figure 1.3) (Ryan and Drew, 2004; Levinson, 2008).

Table 1.4: Penicillins, cephalosporins and carbapenems as examples of cell wall-inhibiting antimicrobials

Antimicrobial group	Examples	Spectrum of action
Penicillins		
1. Natural Pencillins	Penicillin G (injection) Penicillin V (oral)	Active mainly against Gram-positive bacteria.
2. Penicillinase-resistant penicillins	Cloxacillin, dicloxacillin, methicillin, nafcillin, oxacillin	Anti-Staphylococcal action.
3. Aminopenicillins	Amoxicillin, ampicillin, amoxicillin/clavulanate, ampicillin/sulbactam, bacampicillin	Active against Gram-positive and Gram-negative bacteria.
4. Carboxypenicillins	Carbenicillin, ticarcillin, ticracillin/clavulante	Greater activity against Gram-negative organisms.
5. Ureidopenicillins and piperazinepenicillins	Mezlocillin, piperacillin piperacillin/tazobactam	They have the broadest spectrum of all penicillins, especially with *Pseudomonas aeruginosa.*
Cephalosporins		
1. 1st generation	Cefadroxil, cefazolin, cephalexin, cephalothin, cephradine	Effective against Gram-positive bacteria and a few Gram-negative bacteria, such as *E. coli, Klebsiella pneumoniae* and *Proteus mirabilis.*
2. 2nd generation	Cefaclor, cefamandole, cefmetazole, cefoxitin, cefoprzil, cefuroxime	More effective against some Gram-negative bacteria, e.g. *Klebsiella, Proteus, Enterobacter, Hemophilus* spp. Has less effect upon Gram-positive bacteria.
3. 3rd generation	Cefixime, cefoprazone, cefotaxime, ceftazidime, ceftriaxone	Better effect upon *Pseudomonas* and *Enterobacter* spp. Have a role in the treatment of nosocomial infections.
4. 4th generation	Cefepime	Have better action upon Gram-positive and Gram-negative organisms, including *P. aeruginosa.*
Carbapenems		
Carbapenems	Imipenem, Meropenem, Doripenem, Ertapenem.	Broad spectrum of activity against both Gram-positive and Gram-negative microorganisms

(Source: Kayser, 2005; Levinson, 2008)

One of the most important groups of β-lactam antibiotics are the carbapenems, which are of particular interest because of their broad spectrum activity against Gram-positive methicillin-sensitive *Staphylococcus aureus* (MSSA) and Gram-

negative bacteria such as *Pseudomonas aeruginosa*, and their use against other antibiotic-resistant bacteria, e.g. *Acinetobacter baumannii*. Carbapenems include imipenem, meropenem and ertapenem. However, hydrolysis of carbapenems (i.e. resistance) is caused by several classes of β-lactamases, including classes A and B (metallo-β-lactamases) and D (carbapenem-hydrolysing oxacillinases), which have been reported in a number of *Enterobacteriaceae* (Carrer *et al.*, 2010). The Class A *Klebsiella pneumoniae* carbapenemase (KPC) makes resistance to carbapenems more threatening due to their widespread distribution. Class D is the most commonly reported mechanism of resistance to carbapenems within *Acinetobacter* species (Gur *et al.*, 2008). The spread and dissemination of carbapenem-resistant *Acinetobacter baumanii* (CRAB) due to the production of these enzymes was a sentinel event in drug treatment, as it was previously the drug of choice for treating infections caused by this bacterium (Xu *et al.*, 2010; Higgins *et al.*, 2010).

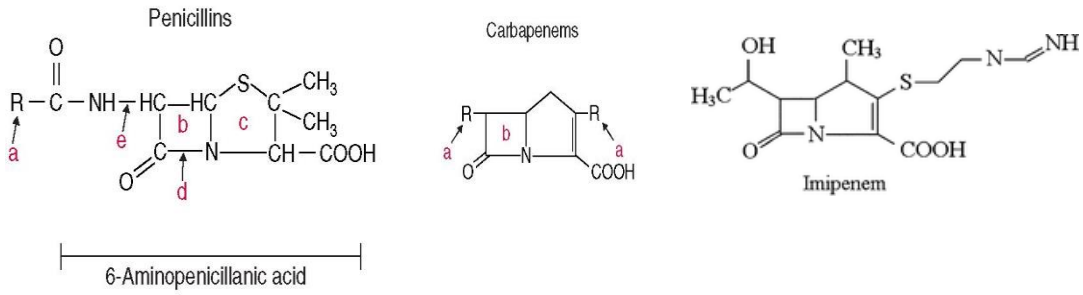


Figure 1.2: Chemical structures of basic penicillins and carbapenems

(a) the site for attachment of different side chains, (b) the β-lactam ring, (c) thiazolidine ring, (d) the site of action of β-lactamase enzymes, and (e) the site of action of amidases (Ryan and Drew, 2004). The chemical structure of imipenem with the attached different side chains are shown to the right (Wright *et al*., 2014).

Another important group of non-β-lactam antibiotics are the glycopeptides, which are a group of non-β-lactam antimicrobials that inhibit cell wall synthesis of bacteria (Figure 1.3) (Ryan and Drew, 2004; Levinson, 2008). The glycopeptide group of antibiotics includes vancomycin, bacitracin and teicoplanin, and has special importance, as vancomycin, which is an example of a natural antibiotic produced by *Nocardia orientalis*, is the drug of last resort for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) (Forbes, 2007; Perichon and Courvalin, 2009).

Vancomycin inhibits cell wall synthesis via a different mechanism from that of the β-lactams. It binds to the D-alanyl-D-alanine (D-Ala–D-Ala) terminus of the intermediates in peptidoglycan formation, inhibiting cell wall cross-linking. Strains resistant to the action of vancomycin are now present, including vancomycin-resistant *Enterococcus* (VRE) and vancomycin-resistant *Staphylococcus aureus* (VRSA) (Perichon and Courvalin, 2009; Xu *et al.*, 2010). The limiting factor for the administration of vancomycin is its nephrotoxicity (Bush, 2012).

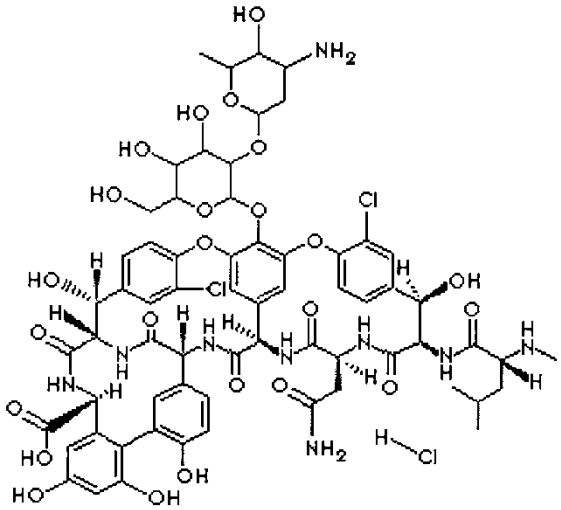


Figure 1.3: Molecular structure of vancomycin hydrochloride

This has a basic structure of a heptapeptide (having seven amino acids) with an additional vancosaminyl side chain and extra HCl (Jalilian *et al*., 2006)

1.3.2 Inhibitors of cell membrane function

This group includes antibacterial agents, e.g. polymyxins, and antifungal agents e.g. amphotericin B, imidazoles and nystatins. The polymixins comprise five groups of polypeptide compounds (A, B, C, D, and E) secreted by some *Bacillus* spp. and act as cationic detergents for bacterial cell membranes, causing leakage of cytoplasmic components. There are only two of these in clinical use: polymixin B and polymixin E (known as colistin); they are associated with considerable toxicity when used systemically and so are mostly used topically. They are active against Gram-negative bacteria, including *Pseudomonas aeruginosa* (Greenwood, 2000; Levinson, 2008).

Other agents active against bacterial cell membranes include Gramicidin and tyrocidine which are used in topical preparations due to their systemic toxicity. A number of disinfectants (including biguanides, quaternary ammonium compounds, and phenols) are also active against cell membranes, but are too toxic to be used systemically and so are again used as skin disinfectants only (Greenwood, 2000).

1.3.3 Inhibitors of bacterial protein synthesis

The process of protein synthesis inside bacterial cells occurs in structures called ribosomes, which are formed from protein and RNA. This type of ribosome differs from that of the eukaryotic ribosome (Table 1.3) in size (70S) as each is formed from two subunits: a small subunit (30S protein and 16S rRNA) and a large subunit (50S protein and a 23S rRNA subunit) (Hogg, 2005). The process of protein synthesis is inhibited at certain stages by certain antimicrobials (Table 1.5) with different effects on the bacteria (Kayser, 2005; Calderon and Sabundayo, 2007).

Table 1.5: Antimicrobial drugs causing inhibition of protein synthesis

Antimicrobial Group	Examples	Mechanism of action	Effect on bacteria	Comments
Aminoglycosides	Amikacin Kanamycin Neomycin Netilimicin Paromomycin Streptomycin Tobramycin Gentamycin	Irreversible binding to the 30S ribosomal subunit.	Bactericidal	Mostly natural antibiotics from *Streptomyces* species.
Tetracyclines	Demeclocycline Doxycycline Minocycline Oxytetracycline Tetracycline	Reversibly binds to the 30S subunit.	Bacteriostatic	Effective in cases of *Chlamydia trachomatis, Rickettsia*.
Glycocyclines	Tigecycline	Binds to the 30S ribosomal subunit.	Bacteriostatic (mostly)	Used for MRSA, GISA, ESBL and VRE*.
Chloramphenicol	Chloramphenicol	Binds to 50S and inhibits peptidyl transferase enzyme.	Bacteriostatic	Can cause bone marrow suppression, aplastic anaemia. Broad-spectrum.
Clindamycin	Clindamycin	Binds to 50S	Bacteriostatic	Effective against anaerobes.
Macrolides	Azithromycin Clarithromycin Dirthromycin Erythromycin Troleandomycin	Binds to the 50S subunit inhibiting RNA-dependent protein synthesis.	Bacteriostatic or bactericidal	Treatment of pneumonia, *Legionella*, and other respiratory tract infections.
Ketolides	Telithromycin	Binds to the 50S subunit.	Bacteriostatic or bactericidal	Similar to macrolides but with a ketone group.
Oxazolidinones	Linezolid	Binds to the 50S subunit.	Bacteriostatic or bactericidal	Approved by the FDA in 2000 for VRE, MSSA and MRSA.
StreptaGramins	Quinupristin-dalfopristin (Synercid)	Binds to the 50S ribosomal subunit of Gram-positive bacteria.	Bactericidal	Approved in 1999 by the FDA for resistant Gram-positive bacteria.
Cyclic lipopeptides	Daptomycin	Not completely understood; alters cell membrane activity.	Bactericidal	Only against Gram-positive bacteria, first used in 2003.

(Source: Kayser, 2005; Calderon and Sabundayo, 2007)

*MRSA = methicillin-resistant *Staphylococcus aureus*; VRE = vancomycin-resistant *Enterococci*; ESBL = extended-spectrum β-lactamase producing bacteria; GISA = glycopeptide-intermediate resistant *S. aureus;* MSSA = methicillin-sensitive *Staphylococcus aureus*.

Most aminoglycosides, apart from amikacin and netilimicin, are natural antibiotics (i.e. produced by living organisms). They are used most commonly in the situation of hospital infections due to their relatively low cost; especially gentamycin, which is usually used in combination with cell wall-inhibitor antimicrobials such as β-lactams for the treatment of serious Gram-negative bacterial infections. However, aminoglycosides are associated with ototoxicity (toxicity to hearing) and nephrotoxicity (Levinson, 2008).

Quinupristin-dalfopristin (Synercid) is a newly-introduced drug (1999) approved for the treatment of resistant Gram-positive bacteria e.g. VRE and MRSA. The two drug components act via the inhibition of protein synthesis by binding to the 50S ribosomal subunit of Gram-positive bacteria, so inhibiting the binding of aminoacyl-tRNA to the A site and peptidyl-tRNA to the ribosomal sites (Ahmed and Donaldson, 2007).

Tigecycline is another newly introduced antimicrobial which acts via the inhibition of protein synthesis by binding to the 30S ribosomal subunit. It is used in places where there is resistance against tetracyclines as it escapes tetracycline resistance mechanisms, namely the efflux pump and the ribosomal protection proteins. Clinically it is useful in the treatment of infections due to MRSA, glycopeptide-intermediate resistant *Staphylococcus aureus* (GISA), VRE, and penicillin-resistant *Streptococcus pneumoniae*. It is also effective against *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, most *Enterobacteriaceae*, including some extended-spectrum β-lactamase (ESBL) producers, and is also effective against atypical and anaerobic bacteria, including *Bacteroides fragilis*, *Clostridium difficile*, and *Clostridium perfringes* (Šeputienė *et al.*, 2010).

1.3.4 Inhibitors of nucleic acid synthesis

Antimicrobial drugs hinder either DNA or RNA synthesis by inhibiting one of the steps involved in their formation (Table 1.6). Most of these antimicrobial agents have well-understood mechanisms of action, while some of them, such as daptomycin, do not. Daptomycin has skeletal muscle toxicity in large doses, so is only approved for use in small doses, i.e. < 4 mg/L (Streit *et al.*, 2004).

Table 1.6: Antimicrobial drugs causing inhibition of nucleic acid synthesis

Antimicrobial group	Examples	Mechanism of action	Effect on bacteria	Comments
Fluoroquinolone	1st Nalidixic acid 2nd Ciprofloxacin Levofloxacin Norfloxacin Ofloxacin 3rd Gatifloxacin 4th Trovafloxacin	Inhibits DNA gyrase.	Bactericidal	1st, 2nd, 3rd, and 4th are the generations of quinolones
Sulfonamides	Sulfisoxazole Sulfamethoxazole Sulfadiazine Sulfadoxine Sulfasalazine Sulfapyridine	Blocks folic acid (FA) production by inhibiting the incorporation of PABA to FA precursors.	Bacteriostatic	When sulfonamides combine with trimethoprim they act as bactericidals.
Trimethoprim	Trimethoprim	Inhibits the dihydrofolate reductase enzyme	Bacteriostatic	
Cyclic lipopeptides	Daptomycin	Not clearly understood, disruption of DNA, RNA and protein synthesis.	Bactericidal	• Natural antibiotic. • Toxic in high doses. • Used for MRSA and VRE.
Rifamycins	Rifampin Rifabutin Rifapentine	Inhibits RNA synthesis by binding to DNA-dependent RNA polymerase.	Bactericidal	• Antibiotics produced by *Streptomyces mediterranei.* • Used in the treatment of tuberculosis.

(Source: Calderon and Sabundayo, 2007)

MRSA = methicillin-resistant *Staphylococcus aureus;* VRE = vancomycin-resistant *Enterococci*.

Fluoroquinolones are classified into four generations based on their antimicrobial activity (Table 1.6), with the narrowest spectrum in the first generation against Gram-negative bacteria, while the fourth generation has an extended spectrum to include Gram-positive bacteria, e.g. *Staphylococcus* and some anaerobes, e.g. *Bacteroides*. The mechanism of action of fluoroquinolones is not

clearly understood. However, it is known that they inhibit the DNA gyrase enzyme used in bacterial replication (Park-Wyllie *et al.*, 2006).

As inhibitors of DNA, both sulfonamides and trimethoprim inhibit the production of purine, which is essential for DNA synthesis, via their interference with folic acid. Sulfonamides inhibit the incorporation of para-aminobenzoic acid (PABA) into folic acid precursors by competitive similarity, while trimethoprim inhibits the dihydrofolate reductase enzyme and consequently interferes with the conversion of folic acid to folinic acid; bacteria cannot form the folic acid which is essential for their growth while the host (human) takes folic acid from dietary sources. Inhibition of PABA leads to interference with many enzymes needed for purine synthesis and other metabolites, e.g. methionine, glycine, and proteins. Both sulfonamide (particularly sulfamethoxazole) and trimethoprim are separately baceriostatic; however, when combined together, they are bactericidal due to the sequential blocking effects upon folate synthesis leading to death of bacterial cells (Calderon and Sabundayo, 2007).

1.4 Antimicrobial drug resistance

As already mentioned, resistance to antimicrobials is an ancient process which has been recognised with the introduction of nearly all of the antimicrobials introduced for the therapeutic purposes of treating bacterial infections. These resistance mechanisms have led to the appearance of MDR bacteria. These MDR bacteria, via multiple mutations, coupled with the acquisition of antibiotic resistance genes by HGT, have earned the name of 'superbugs' and have been responsible for many human infections, characterised by increased hospital admissions and increased mortality e.g. MDR *Mycobacterium tuberculosis*. Other 'superbugs' include, but are not limited to, some strains of *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, and *Clostridium difficile* (Davies and Davies, 2010).

1.4.1 Classification of antimicrobial drug resistance mechanisms

The resistance of microorganisms to antimicrobials is classified as being either natural or acquired.

1.4.1.1 Natural resistance

An organism is termed as having natural or intrinsic resistance when it has an inherent resistance to the action of an antibiotic; this pattern of resistance is common to all isolates of the species, e.g. the resistance of *E.coli* to macrolides and the resistance of *Pseudomonas aeruginosa* to most drugs. The intrinsic resistance of a microorganism to an antimicrobial is explained by the absence or inaccessibility of the target of the drug action, e.g. Gram-negative bacteria are naturally resistant to some antibiotics e.g. erythromycin, due to the non-permeability of the outer membrane (Towner, 2000; Summers, 2008).

1.4.1.2 Acquired resistance

Acquired resistance to an antibiotic is that which is developed by a previously susceptible microorganism; it develops within one or more isolates of the species, i.e. not all strains of a species are resistant. Acquired resistance was initially attributed to the adaptation of a bacterium to the action of an antibiotic, which was later proved not to be true as resistance can develop to an antibiotic to which the bacterium had not previously been exposed. The genetic mechanisms underlying the emergence of acquired bacterial resistance are either mutation and/or gene transfer (Towner, 2000; Summers, 2008).

1.4.2 Functional mechanisms of acquired resistance

For an antimicrobial to produce its intended action, it has to have a target for its action (e.g. in the form of an enzyme or protein) within the bacterial cell, to be able to reach this target, and also to reach the target in its active form, i.e. not having been destroyed. Thus, in their attempts to combat the action of antimicrobials, bacteria take one of the following steps (see Table 1.7 and Figure 1.4): 1) they may destroy or inactivate the antibiotic; 2) bacteria can use an efflux system to transport the drug from the interior; 3) bacteria can produce alterations in the target site used by antimicrobials to act or they may completely prevent this binding; 4) bacteria can

reduce their cell surface permeability or even completely block the entrance of the antimicrobial to the cell, so that the antimicrobial can no longer act; and 5) bacteria can produce a bypass mechanism by using alternative pathways which are different from those inhibited by the antibiotic (Towner, 2000).

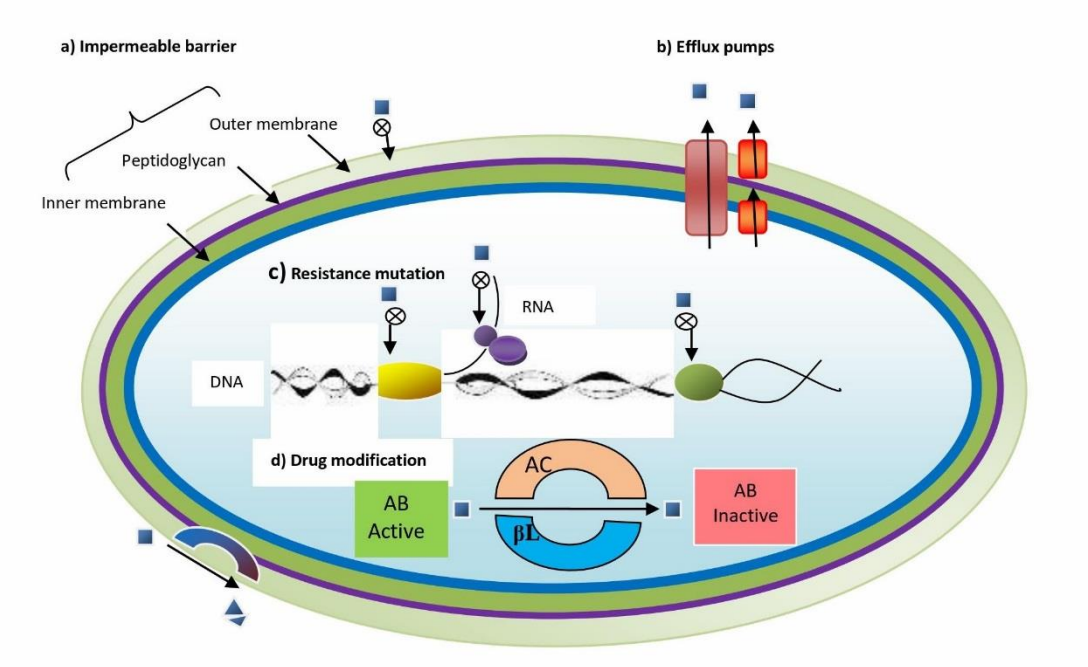


Figure 1.4: Mechanisms of antimicrobial resistance in Gram-negative bacteria

a) the intrinsic (natural) resistance due to lack of target or impermeability to a drug by some bacteria is shown in the blue squares; b) shows the multidrug resistant efflux pumps which exclude the drugs from the bacteria (pink) or the periplasmic space (red); c) indicates resistance due to mutations e.g. in the gyrase (green), RNA polymerase (yellow) or in the ribosomal subunits (purple); d) shows examples of antibiotic (AB) inactivation by covalent modification of aminoglycosides by acetyltransferase (AC) or by the β-lactamase (βL) enzyme (adapted from Allen *et al*., 2010).

Table 1.7: Different mechanisms of antimicrobial resistance used by bacteria

Mechanism	Examples of affected antimicrobials
Destruction, modification, or inactivation of the antimicrobial.	β-lactam antibiotics Chloramphenicol Aminoglycosides
Multidrug efflux pumps.	Tetracycline
Target site alteration.	β-lactam antibiotics Chloramphenicol Streptomycin Quinolones Fusidic acid Erythromycin Glycopeptides Rifampicin
Reduction in the cell surface permeability or access of the antimicrobial to the cell interior.	Retracyclines Quinolones β-lactam antibiotics Aminoglycosides Chloramphenicol
New metabolic bypass mechanism.	Trimethoprim Sulphonamides

(Source: Towner, 2000)

NB: Some bacteria have more than one mechanism of resistance to antibiotics.

1.4.2.1 Destruction, modification, or inactivation of the antimicrobial

β-lactam antimicrobials are examples of drugs which are inactivated by bacteria via the production of enzymes called β-lactamases, which destroy most penicillins and cephalosporins. The production of β-lactamases is typically chromosomally-mediated and produced by a number of different bacteria, e.g. *Staphylococcus*, *Acinetobacter* and *Citrobacter* spp. The most important and clinically dangerous is the carriage of these enzymes upon plasmids in Gram-negative bacteria, facilitating their spread among bacterial isolates; some of these enzymes are in turn also carried upon transposons, and so can move from plasmid to chromosome and vice versa (Mabilat *et al*., 1992; Stokes and Hall, 1992). There are many molecular types of β-lactamases, with the metalloenzymes (carbapenemases) being responsible for the inactivation of imipenem and other carbapenems (Cha *et al*., 2008).

1.4.2.2 Target site alteration

Target site alteration occurs by selection of mutant strains which have changed the site of the antimicrobial binding target. This is evident in the case of β-lactam antimicrobials by the production of altered penicillin-binding proteins (PBPs), which can bind the antimicrobial drug but do not lead to its bactericidal action. Vancomycin and teicoplanin resistance is due in some instances to modification of the peptidoglycan lattice to which they bind, and is also reported in enterococci and staphylococci (Reynolds *et al*., 1994; Towner, 2000).

1.4.2.3 Reduction in cell surface permeability or in access of an antimicrobial to the cell interior

The outer membrane of Gram-negative bacteria has porin proteins which have a role in its permeability to low molecular weight, water-soluble molecules including penicillins and cephalosporins. Changes in the size or function of these porins due to alterations in the *micF* gene which controls the outer membrane functions in *E. coli* can result in resistance to these antimicrobials (Chou *et al*., 1993).

1.4.2.4 Efflux

An active efflux pump acts as a mechanism for antimicrobial resistance by excluding the antimicrobial molecules from the interior of the cell. This efflux pump works in synergy with the reduced permeability of the outer membrane of Gram-negative bacteria to antimicrobials (Poole and Srikumar, 2001). There are five known families of efflux pump: 1) resistance-nodulation-division (RND), which is part of a tripartite (antiports, symports, and uniports) permease family that expels drugs outside the cytoplasm. The genes for this include MexAB-OprM and MexCD-OprJ; 2) a small multi-drug resistant family which is found in *E. coli* (*EmrE*) and *Mycobacterium tuberculosis* (*Mmr*); 3) an ATP-binding cassette family which is the primary active transport system for the entry of many drugs into cells; 4) a major facilitator family present in many Gram-negative bacterial isolates; and, lastly, 5) the multi-drug and toxic compound exclusion family (Bambeke *et al*., 2000; Schweizer, 2003).

1.5 The basis/origin of antimicrobial resistance

The mechanism underlying antimicrobial resistance is either natural (intrinsic) or acquired (see 1.4.1). Acquired resistance is due to mutation (vertical gene transmission) and/or gene transfer. Horizontal gene transfer mechanisms include transformation, conjugation, transduction or transposition (Dantas and Sommer, 2014).

Mutation is a heritable change in the structure of genes which may arise spontaneously as an error of replication and as a fundamental evolutionary process for the vertical origin of antimicrobial resistance by various bacteria (Dantas and Sommer 2014). This natural occurrence of mutation is affected by some biological and environmental factors including exposure to UV light, radiation, or alkylating agents (Neidhardt, 2004). It is sometimes termed chromosomal resistance, as it usually originates in a chromosome as a spontaneous mutation in a locus responsible for the antimicrobial drug action. A mutation may arise as a result of changes in nucleotide sequence by insertion, deletion, inversion, duplication or replacement of single or multiple nucleotide(s) during multiplication (Kumar and Chatterji, 1992). A mutation producing a single amino acid change in the PBPs is one of the mechanisms responsible for low level resistance to penicillins and cephalosporins (Chambers, 1999). Mutations in the 23S ribosomal RNA gene also lead to linezolid-resistant strains of VRE and MRSA, linezolid being a newly-introduced antimicrobial for VRE and MRSA (Herrero *et al*., 2002).

1.6 Mobile genetic elements and horizontal gene transfer

The spread and exchange of antimicrobial resistance genes among different bacterial isolates everywhere are mediated via a group called mobile genetic elements, sometimes termed the “mobilome”, which can spread among bacteria, even if they are not closely related, and across different phyla (Wellington *et al*., 2013). These mobile genetic elements (MGEs) include conjugative plasmids, gene cassettes within integrons, transposons, and insertion sequence (IS) elements (Carattoli, 2001).

1.6.1 Mobile genetic elements (MGEs)

Conjugative plasmids are those having the ability to transfer themselves (and other plasmids and/or chromosomal genes) from one bacterial cell to another (Bennett, 2008). Conjugative plasmids commonly harbour antimicrobial resistance genes in both Gram-positive and -negative bacteria for antimicrobials such as cephalosporins, fluoroquinolones and aminoglycosides (Bennett, 2008).

Integrons, which are present naturally as gene expression elements as they contain open reading frames (ORFs) enabling them to express the genes they contain, are formed from two conserved flanking regions which incorporate one or more resistance gene(s) in between (Stokes and Hall, 1989). The mobile genetic elements within integrons are known as gene cassettes. Many gene cassettes are known and have been identified as mediating resistance to several antimicrobials e.g., penicillins, cephalosporins, aminoglycosides, chloramphenicol, and trimethoprim. Some of these genes encode extended-spectrum β-lactamases (ESBLs) or carbapenemases (e.g., bla_{IMP}, $bla_{\text{VEB-1}}$, bla_{VIM}), as well as genes for other antimicrobial resistance such as *aacA4* for aminoglycosides and *cmlA2* for chloramphenicol (Lauretti *et al.*, 1999).

Insertion sequence (IS) elements are the simplest transposable DNA elements (Figure 1.5a). However, they mostly do not carry antimicrobial resistance genes (Mahillon *et al.*, 1999; Kayser, 2005). Transposons (Figure 1.5b and c) are MGEs that contain self-transmissible elements, including transposase and recombination DNA segments (Kleckner, 1981). Some transposons have been shown to mediate the transfer of integrons both horizontally and vertically. Examples of transposons include Tn*5*, which encodes kanamycin, streptomycin and bleomycin resistance (Reznikoff, 2002), Tn*9* for chloramphenicol resistance, and Tn*10*, which encodes tetracycline resistance (Haniford, 2002).

Conjugative transposons (Figure 1.5d), which can be present either in bacterial plasmids or chromosomes, may have a broad host range and/or may mediate transfer of many antimicrobial resistant genes in Gram-positive bacteria, mostly in *enterococci*. They are also less frequently found in *Bacteroides*. They have the ability to be excised from their sites on a plasmid or chromosome to form a non-replicative circle, which can be transferred to other bacteria by conjugation

(Churchward, 2002). Examples of these conjugative transposons include Tn*916* which encodes tetracycline resistance (Franke and Clewell, 1981) and Tn*5382* (also termed Tn*1549*) carrying the *vanB* gene for vancomycin resistance in *Enterococcus faecium* (Garnier *et al*., 2000).

Genomic islands (GIs) insert into the chromosome using bacteriophage-related integrases and are MGEs that are present in some bacterial strains and absent in very closely related strains (Osborn and Boltner, 2002). Some of these GIs are conjugative and contain plasmid-derived conjugation gene operons, for example the *clc* element which encodes chlorocatechol degradation was detected as having transferred from *Pseudomonas aeruginosa* to *P. putida* (Ravatn *et al*., 1998). Other examples of conjugative GIs include the integrative and conjugative element (ICE) known as R391, which was previously thought to be a conjugative transposon, and the related sulfamethoxazole and trimethoprim (SXT) element, which carry metal and/or antimicrobial resistance genes; they have the ability of self-excision and can transfer to another bacterial cell. R391 was isolated from *Providencia rettgeri,* while SXT was isolated from *Vibrio cholerae.* They have limited host-integration sites, either to one or two sites within the host chromosome (Osborn and Boltner, 2002).

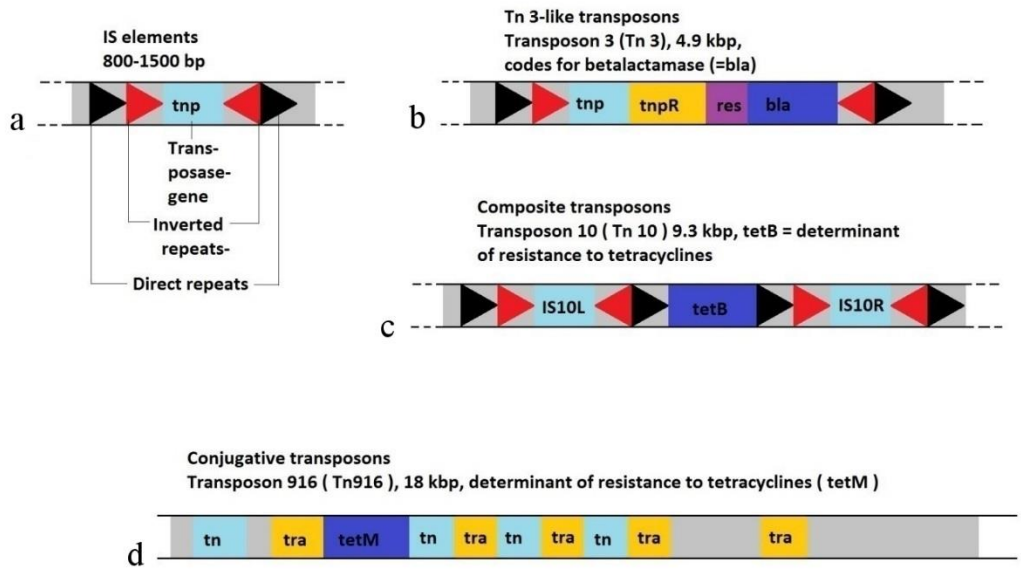


Figure 1.5: Transposable DNA elements

a) Insertion sequence (IS) elements are the simplest transposable DNA sequences, have reversed identical sequences (inverted repeats: IR) of 10-40 nucleotides at both ends flanking the transposase (*tnp*) gene. The direct repeats formed from 5-9 bp at the extremities of the structure are the target for the enzyme transposase during the integration process; b) the simple Tn*3* transposons containing the transposase gene *tnpA*, regulator sequence (*tnpR*) and the (*res*) site to which the resolvase enzyme binds; c) the composite transposons which are formed from two IS elements making a frame for a region which is not essential for transposition e.g. tetracycline resistance gene (*tetB*); d) represents the conjugative transposons which have certain segments encoding factors used in the control of the transfer (Tra) and transposition (Tn) processes, adapted from Kayser *et al*., 2005).

1.6.2 Horizontal (lateral) gene transfer (HGT)

Gene transfer between different bacteria occurs through one of three mechanisms: transformation, transduction or conjugation. Conjugation is the most frequent mechanism mediated in HGT in several of the bacteria that transfer MGEs (plasmids, and conjugative transposons) from donor to recipient bacteria (Muniesa *et al*., 2011) and, in particular, is important in the transfer of antibiotic-resistance genes (ARGs). Gene transfer from one locus to another within the same bacterium is called transposition. When the transferred gene encodes antimicrobial resistance, the recipient bacterium mostly acquire this characteristic (Kayser, 2005). HGT is the dominant mechanism with MGEs for antimicrobial resistance and bacterial conjugation is the most prevalent HGT mechanism involved in the genetic transfer (Dantas and Sommer, 2014).

Genetic analysis has detected that a significant proportion (14%) of bacterial genome ORFs within a bacterial genome can be obtained via horizontally acquired mechanisms (Nakamura *et al*., 2004). The spread of ARGs among different bacterial isolates leads to an increase in the morbidity and mortality of patients due to widespread distribution of multidrug-resistant (MDR) bacteria, in addition to the economic burden related to the cost of medical treatment and prolonged hospital stays (Hawkey and Jones, 2009). Multidrug and extensive drug-resistant (XDR) *Pseudomonas aeruginosa* has been detected in some countries where the carbapenemase gene $bla_{vim\text{-}3}$ is carried upon a transposon (Tn*6001*). Molecular analysis has shown that the transposon was transferred by an unidentified HGT mechanism (Tseng *et al*., 2009).

There are many reservoirs for the horizontal transfer of genes among different bacterial isolates. One of the recognized ways is through the presence of different bacterial isolates in the same environment e.g., the presence of different Gram-negative bacteria inside the intestinal tract, both commensal and pathogenic, whereby HGT has been proven to play a major role in promoting the transfer of genes (*qnrS*) responsible for resistance to quinolones in Vietnam (Vien *et al*., 2009). Another reservoir for the potential horizontal transfer of resistant genes is in bacteria associated with animals, for example, household pets, including cats and dogs, where antimicrobials e.g., penicillins, quinolones and macrolides given for human treatment are also used for such animals (Guardabassi *et al*., 2004).

There are some limitations that control the extent of HGT, including bacterial competence for transformation and the similarity of the DNA to be taken and integrated, as well as the controlling role played by the recipient cell (Thomas and Nielsen, 2005). Another controlling mechanism is the restriction of DNA uptake by restriction endonucleases for foreign DNA segments which excise foreign DNA integrated within host DNA. However, plasmids and small gene sequences can escape such restriction due to their possible possession of restriction sequence homology (Jeltsch, 2003). Many plasmids also have restriction modification systems to modify the DNA so that the recipient bacterium can avoid lysis (Naito *et al*., 1995). This DNA modification makes the recipient bacteria escape degradation by the newly introduced restriction endonuclease (Kobayashi, 2001). Plasmids also have the ability to self-replicate without the need to be integrated into the

chromosome, and this is very important in their maintenance of natural HGT by the expression of the genes they carry after integration into the recipient bacteria (Maestro *et al*., 2002).

1.7 Phenotypes of β-lactamases and modes of dissemination

As already mentioned, β-lactam anitbiotics are those containing a β-lactam ring in their molecular structure, and include penicillins, cephalosporins, monobactams, and carbapenems. β-lactamases, on the other hand, are enzymes produced by various bacteria that destroy the β-lactam ring inhibiting the action of β-lactam antimicrobials. Extended-spectrum β-lactamase (ESBL) enzymes inhibit all β-lactams except cephamycins and carbapenems (Kong *et al.,* 2010; Lakshmi *et al*., 2014).

There are two classifications in current use for β-lactamase enzymes. The first one is the molecular (structural) classification which is based upon the sequences of amino acids within the enzyme. The molecular classification (Table 1.8) is the most commonly used and divides β-lactamase enzymes into class A, B, C, and D. Classes A, C, and D perform the hydrolysis of antimicrobial substrate by the formation of an acyl enzyme. Class B comprises metallo-enzymes that use metal ions e.g. zinc ions to perform the hydrolysis of the β-lactamases (Ambler, 1980; Ambler *et al*., 1991). The second classification is the functional one which relates each group of β-lactamase enzymes to the clinical role played by them i.e. the phenotype of the clinical isolates taking into account the substrate and the inhibitor profiles e.g. cephalosporinases hydrolyze cephalosporins assigned to group I of the Bush and Jacoby classification (Bush and Jacoby, 2010).

Table 1.8: Ambler classifications of β-lactamases

	β-lactamase class	β -lactamases	Important examples	Occurrence	Phenotypic resistance
Serine-β-lactamases	A	Broad-spectrum β-lactamases	TEM-1, TEM-2, SHV-1, SHV-11	*Enterobacteria* and sugar non-fermenters	Ampicillin, cephalothin
		ESBL TEM-type	TEM-3, TEM-52		Penicillins, 3rd-G cephalosporins
		ESBL SHV-type	SHV-5, SHV-12		
		ESBL CTX-M-type	CTX-M-1, CTX-M-15		
		Carbapenemases	KPC, GES, SME		All β-lactams
	C	AmpC cephamycinases (chromosomal-encoded)	AmpC	*Enterobacter* spp. *Citrobacter* spp.	Cephamycins (cefoxitin), 3rd-G cephalosporins
	D	AmpC cephamycinases (plasmid-encoded)	CMY, DHA, MOX, FOX, ACC.	*Enterobacteria*	Cephamycins (cefoxitin), 3rd-G cephalosporins
		Broad-spectrum β-lactamases	OXA-1, OXA-9	*Enterobacteria*; *Acinetobacter baumannii*	Oxacillin, ampicillin, cephalothin
		ESBL OXA-type	OXA-2, OXA-10		Penicillins, 3rd-G cephalosporins
		Carbapenemases	OXA-48; OXA-23,24,-58		Ampicillin, imipenem, all β-lactams
Metallo-β-lactamases	B	Metallo-β-lactamases (Carbapenemases)	VIM, IMP, NDM, IND	*Enterobacteria* and sugar non-fermenters	β-lactams

(Source: Ambler *et al.*, 1991)

1.7.1 Molecular classification

1.7.1.1 TEM β-lactamases

These were the first type of ESBLs to be discovered, and were found in Greece in the 1960s, in Gram-negative bacteria in a patient called Temoniera (Datta and Kontomichalou, 1965). This was followed by the discovery of TEM-2. Both TEM-1 and TEM-2 are narrow-spectrum β-lactamases that are not active against higher generations of cephalosporins, such as ceftriaxone, ceftazidime, cefotaxime, and cefepime. TEM-1 and TEM-2 are the most common plasmid-mediated β-lactamases in Gram-negative bacteria (Bradford, 2001).

1.7.1.2 SHV Sulfhdryl variants

SHVs are less common variants of β-lactamases and are most commonly found in *Klebsiella pneumoniae*, with a narrow spectrum of activity in enterobacteria arising by point mutation of the chromosomal SHV gene (Bradford, 2001; Lakshmi *et al*., 2014; Monuz-Price *et al*., 2014).

1.7.1.3 ESBLs

ESBLs have a wide range of activity, hydrolysing cephalosporins including 3rd- and 4th-generation and monobactams. ESBLs are either classical, derived from TEM and SHV enzymes, or non-classical, derived from other enzymes such as OXA and CTX-M (Bradford, 2001).

1.7.1.4 CTX-M β-lactamases

These are so termed because of their activity against cefotaxime rather than ceftriaxone, ceftazidime and cefepime. They represent plasmid-mediated genes transferred from bacterial chromosomes or mostly arising by mutation of CTX genes giving several variants. More than 130 types of this group have been detected and they represent the most common ESBLs in enterobacteria (Canton and Coque, 2006). There has been a marked increase in the prevalence of ESBLs of the CTX-M type since 2005, with different types of the enzyme according to the geographical locality. The most common are CTX-M enzymes types 1, 2, 8, and 9 worldwide, while CTX-M 14 is prevalent in China and type 15 worldwide and the most common prevalent type of CTX-M (Wellington *et al*., 2013).

1.7.1.5 OXA β-lactamases

These are plasmid-mediated enzymes that deactivate oxacillin and related antimicrobials such as methicillin and cloxacillin. The spectrum of these enzymes may extend to include carbapenems in class D carbapenemases (Lakshmi *et al*., 2014).

1.7.1.6 AmpC β-lactamases

AmpC enzymes are both plasmid- and chromosomally-determined genes giving resistance to cefoxitin and cefotetan, which are not normally inhibited by other ESBLs. They are also not affected by β-lactamase inhibitors such as clavulanic acid, making their isolation and characterisation practically difficult (Peter-Getzlaff *et al*., 2011).

1.7.1.7 Carbapenemases

Carbapenemases are the most powerful type of β-lactamases, hydrolysing penicillins, cephalosporins and carbapenems, and causing carbapenems to reduce their efficiency in treating resistant bacteria. The Ambler classification of carbapenemases places them in four classes: A, B, C and D (Table 1.9) (Nordmann *et al.,* 2012). Class C is very rare clinically.

Table 1.9: Ambler classification of carbapenemases

Classification	Enzyme	Most common bacteria	Inhibitor
Class A	Chromosomal: IMI, SME, NMC Plasmid: KPC, GES	*Enterobacteriaceae*	Boronic acid (clavulanic)
Class B	Metallo-β-lactamases: IMP, GIM, VIM, SPM, NDM-1 L1	*Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Acinetobacter* spp., *Stenotrophomonas maltophilia*	EDTA (Dipicolinic acid)
Class D	OXA β-lactamases: Oxa-23, Oxa-48,	*Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Acinetobacter* spp.	---

(Source: Nordmann *et al.*, 2012; Petridou *et al.*, 2010)

1.7.2 Functional classification

Functional classification places β-lactamases into three main groups (Table 1.10); Group 1 comprises cephalosporinases which belong to class C of the Ambler molecular classification. Group 2 is the largest group of serine β-lactamases and includes both classes A and D of the Ambler classes. Group 3 comprises metallo-β-lactamases (MBLs), which are zinc dependent (Bush and Jacoby, 2010).

Table 1.10: Functional classification schemes for bacterial β-lactamases

Bush-Jacoby group	Ambler Class	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzymes (numbers)
			CA or TZB[a]	EDTA		
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99,ACT-1, CMY-2,FOX-1, MIR-1 (51 enzymes)
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1 (23 enzymes)

Bush-Jacoby group	Ambler Class	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzymes (numbers)
			CA or TZB[a]	EDTA		
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1 (16 enzymes)
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino- β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2,CTX-M-15, PER-1, VEB-1 (200 enzymes)
2br	A	Penicillins	No	No	Resistance to clavulanic acid,sulbactam and tazobactam	TEM-30, SHV-10 (24 enzymes)
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino- β- lactams combined with resistance to clavulanic acid, sulbactam and tazobactam	TEM-50
2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3 (19 enzymes)
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, Cefepime and cefpirome	*RTG-4*
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10 (31 enzymes)
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	Kpc-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
	B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	Unknown					(9 enzymes)

[a] CA, clavulanic acid; TZB, tazobactam.
[b] NI, not included. (quoted from Bush and Jacoby, 2010; Ghafourian *et al.*, 2014).

1.8 Antibiotic resistance in the environment

The term “resistome” was introduced in 2007 and used for the presence and transmission of antimicrobial resistance genes within and between different environments (Wright, 2007) (Figure 1.6). A resistome is defined as the entire suite of genes which confer antimicrobial resistance in a microorganism or microorganisms, including all genes for antimicrobial resistance at any scale in a single or multiple organisms in defined environmental samples (Dantas and Sommer, 2014).

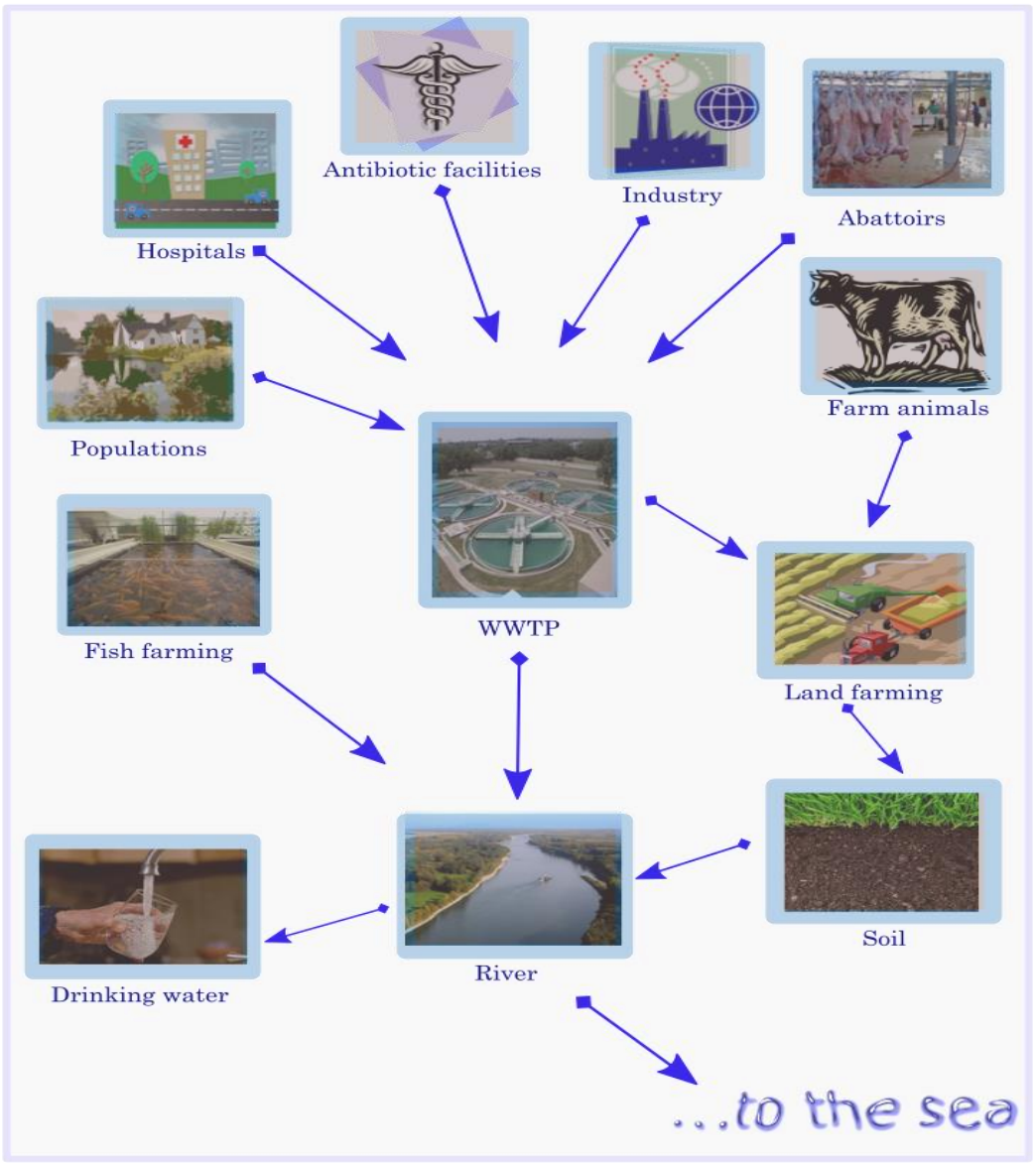


Figure 1.6: Various routes for antimicrobial resistance gene spread from human activity origins to the environment (adapted from Stalder *et al.*, 2012)

The above diagram shows the spread and dissemination routes of antimicrobial-resistant genes between human and various environmental, industrial, animal, and agricultural areas (WWTP = waste water treatment plants).

1.8.1 Soil as a reservoir of antibiotic resistance

Antimicrobial-resistant bacteria are present in soil and represent the evolutionary reservoir of resistance for most bacteria. The soil resistome is the largest and most divergent among other types of environments. Many clinically

resistant bacteria arise from soil bacteria by different mechanisms (D'Costa *et al*., 2006).

The non-clinical environment is affected by tonnes of antimicrobials used daily all over the world, with an accompanying emergence of bacterial resistance due to their injudicious use (Levy and O'Brien, 2005). This has been further complicated by the use of antibiotics for non-medical purposes e.g., in animal feed and agriculture, which are used in the absence of acute infections to promote growth of animals. This has contributed to the greater selection for resistant bacterial strains in the environment. Antimicrobials are also used in fish farms. All these forms of antimicrobial use could select for increased antibiotic resistant bacteria and eventual transmission to human beings (Allen *et al*., 2010).

Another selection pressure for antimicrobial resistance is through the presence of naturally-produced antibiotics which are secreted by some microorganisms and affect surrounding microorganisms. It is estimated that more than 80% of the antibiotics in clinical use either come directly from soil bacteria, like *Actinomycetes* species, or after being semi-synthesized from natural products (Kieser *et al*., 2000). The antibiotic-secreting microorganisms contain genes to protect themselves from the lethal actions of these antibiotics; these genes can then be transmitted to other microorganisms in the surrounding natural environment and consequently to humans. The resistance mechanisms for some antibiotics in clinical use were identical in both soil and clinically isolated bacteria, as in cases of resistance to aminoglycosides (kanamycin and neomycin) by acetylation and phosphorylation (Benveniste and Davies, 1973). This similarity in resistance mechanisms was also detected and identified in vancomycin by modification of its target site of action at the D-Ala-D-Ala termini of cell wall peptidoglycan in Gram-positive bacteria (Bugg *et al*., 1991).

There are millions of bioactive small molecules (parvome) secreted by microorganisms in nature; only a few of them have been isolated and identified. The secretion of such molecules is said to have started 500 million years ago (Baltz, 2008; Davies, 2009). The exposure of microorganisms to environmental factors including pollution or radiation helps in the dissemination of resistant genes, e.g. the spread of the resistance mechanism, via an efflux pump, which inhibits the entry of heavy metals and some antimicrobials (chloramphenicol and tetracycline) as was

noted in *E. coli* and other bacteria living within toxin-containing environments (Poole, 2005; Allen *et al*., 2009).

The linkage between antibiotic resistance and other substances in nature such as heavy metals has also been observed. Mercury is widely distributed in natural environments and has detrimental effects upon the lives of humans and animals (Barkay *et al*., 2003). Bacteria living in mercury-contaminated water are resistant to mercury. Mercury-resistant bacteria also carry genes for other antimicrobial resistance mechanisms due to the fact that the metabolism of mercury is controlled via the *mer* operon, which regulates the expression of the mercuric reductase enzyme (*MerA*). This operon is often part of Tn*21*-like transposons, which can carry class 1 integrons with their related antimicrobial resistance genes (Lee *et al*., 1993). Thus, bacterial resistance to antimicrobials can be co-selected in nature without pre-exposure to antimicrobials, due to the selective presence of mercury in the environment (Skurnik *et al*., 2010). The same mechanism is also implicated for other heavy metals, including iron, copper, manganese, cadmium and zinc (Permina *et al*., 2006). In another study in Scotland, the relationship between the presence of low concentrations of heavy metals in soil sampled before the increased usage of antimicrobials (between 1940 and 1970) and the level of ARGs was examined by quantitative PCR. A significant correlation was detected between the levels of copper, chromium, nickel, lead and iron and a number of antibiotic resistance genes. The genes under study included ESBLs, and tetracycline and erythromycin resistant genes. It was concluded that even low levels of heavy metals may help in the selection of antimicrobial resistance in soil environments (Knapp *et al*., 2011).

Thus, the soil environment, due to the above-mentioned factors (use of antibiotics in agriculture, animal feeding, naturally secreted antibiotics, and heavy metal-resistant bacteria), has a high density of ARGs, which are collectively referred to as the antibiotic resistome, and act as a very important reservoir of this antibiotic resistance (D'Costa *et al*., 2007). The increase in ARGs in soil has been found to be increasing with time as levels of ARGs were found to be 15 times higher in 2009 than in the 1970s in The Netherlands due to waste disposal into the environment and the use of antibiotics in agriculture (Knapp *et al.*, 2010).

However, recent studies have revealed controversial new findings regarding the common belief that antimicrobial resistance has arisen as a result of the

injudicious use or even misuse of antimicrobial agents. One study carried out upon bacteria from a cave which had been isolated for over 4 million years in the USA showed that genes present in these bacteria were resistant to 14 commercially available antimicrobials, including daptomycin, which is used as the last resort in the treatment of resistant Gram-negative bacteria (Bhullar *et al*., 2012). Another study detected the presence of antimicrobial-resistant genes by metagenomic testing of DNA dating back more than 30,000 years in Beringian permafrost sediments encoding resistance to many antimicrobials, including to β-lactam, tetracycline and glycopeptides (D'Costa *et al*., 2011).

Another potential role for antibiotics is in signalling pathways, where these antibiotics bind to their target receptors in the cytoplasm, such as DNA, RNA, cell wall components or ribosomes, leading to changes in the metabolic behaviour of the microorganisms, either inhibiting or initiating the transcriptional activity of bacteria (Davies *et al*., 2006; Ryan and Dow, 2008). Some bacterial properties are changed and affected by the signalling functions of antimicrobials, including the virulence of bacteria and biofilm-formation ability (Yim *et al*., 2007).

1.8.2 Sewage and water environments as a reservoir of antimicrobial resistance

Wastewater can contain considerable amounts of material of human and animal origin, including antimicrobials, pharmaceutical substances and detergents treated to eliminate the detrimental effects of such compounds upon the receiving water. Treatment is carried out by degrading organic substances, removing solid materials and disinfecting the effluent stream in specific areas when necessary (Wellington *et al*., 2013). However, such treatment is not always efficient in removing resistant bacteria and preventing ARGs from being introduced into the receiving water. A study in Brazil showed that *Klebsiella pneumoniae*-producing ESBLs were present at all stages of hospital-treated sewage water (Prado *et al*., 2008). In Ireland, ESBL-producing *E. coli* were detected after the handling of sewage water using modern technology in wastewater treatment (Galvin *et al*., 2010).

It has been proposed that sewage sludge containing ARGs is a major source of soil contamination with resistant bacteria, especially fluoroquinolone-resistant bacteria, as other ARGs are poorly studied in sewage sludge (Kinney *et al.*, 2006).

In another study carried out in the U.K., the contamination of river water by faecal ESBL-producing *E. coli* was demonstrated to be coming from the effluent of a wastewater treatment plant. The *E. coli* detected downstream was carrying highly divergent forms of CTX-M-15. It was also suggested that the downstream contamination of water in the U.K. had originated from the surrounding farms containing resistant bacteria in the period from 2009 to 2011 and that river water is a reservoir for the spread of CTX-M-15 ESBLs (Amos *et al.*, 2014).

Regarding sediment and water environments as sources of ARGs, it has been found that the levels of ARGs were significantly higher in sediments with increased levels of heavy metals, e.g. copper, and levels of ARGs were also found to be higher in sediments near pharmaceutical factories as well as in relation to higher populations in certain localities, as levels are affected by human activity (Graham *et al*., 2011).

1.8.3 Manure and ARG spread

The amount of antibiotics used for non-medical reasons in agricultural is so extensive that it was estimated that around 350-400 tonnes of antibiotics were used in the U.K. in 2006-2011 in food producing animals (Hutchison *et al*., 2004). It was also estimated that 70 million tonnes of animal manure are used in agriculture yearly in the U.K. (Hutchison *et al*., 2004). Many antimicrobial resistant bacteria and genes have been detected in soil where animal manure was used and also contaminated the nearby water supply in many rivers. It is now believed that contaminating coliforms in river water are from agricultural sources (Wellington *et al.*, 2013).

1.8.4 Wildlife as a reservoir of ARGs

The dissemination of ARGs through wildlife despite being of great importance was considered in the 1980s, but has not been thoroughly studied. The source of contamination by resistant bacteria is thought to have originated from contact with animal manure or sewage. The survival of resistant bacteria without exposure to

antibiotics in wildlife is not completely known. Birds thought to be involved include birds of prey (eagles, hawks, falcons, etc.) and waterfowl (Guenther *et al.,* 2011). It has been suggested that wildlife is affected in the vicinity of human activity, as resistant bacteria were nearly absent from the faeces of rodents and ungulates in a remote area of Finland (Osterblad *et al*., 2001).

1.8.5 Social aspects of the problem

The dissemination of antimicrobial resistance has many detrimental effects upon morbidity and mortality, which needs the cooperation of different sectors in the community to be addressed. Much effort has been concentrated upon health care workers, especially physicians and pharmacists, to improve their prescribing habits to limit resistance. Campaigns are also carried out to increase the awareness of the general public about this and it has proved useful in developed countries (Goossens *et al*., 2006). However, these efforts are hampered by the work of some physicians in increasing the loyalty of patients by prescribing antimicrobials and also in part by the promotional activities of commercial companies for the introduction of new antimicrobials (Faber *et al.,* 2010).

Other social aspects of the problem include self-medication in many countries, the ethnic beliefs of populations, personal income, the country of residence, and educational level. These factors affect awareness about the presence and scope of the resistance problem (Deschepper *et al*., 2008; Faber *et al*., 2010).

The behaviour of farmers all over the world in using antibiotics (growth promoters) to increase the stock of breeding animals is also important. Such behaviour is banned in Europe and legalisation is required for it to be banned in other regions and countries (Casewell *et al*., 2003).

1.9 Whole bacterial genome sequencing

1.9.1 History

The first bacterial genome to be sequenced completely was that of *Haemophilus influenzae* in 1995 (Fleischmann *et al*., 1995). Since that time, sequencing has been improving rapidly, and many bacterial genomes have been

sequenced completely (there were 8,500 by 2011) (Pagani *et al*., 2012) or drafted, i.e. sequenced but not completely closed. Most of these sequences were performed after 2008 after the commercial introduction of high-throughput sequencing. Several sequencing techniques were introduced that made whole bacterial genome sequencing easier and cheaper and facilitated a shift from sequencing individual genomes to sequencing multiple strains (Dark, 2013). Sequencing was revolutionised by the introduction of next-generation sequencing (NGS) in 2005, which enabled a drastic reduction in the cost of sequencing, and facilitated performance of sequencing in small and medium-sized laboratories (Barbosa *et al*., 2014). These NGS techniques include the Illumina (Genome Analyzer), the 454 GS FLX platform (Roche), and SOLiD (Life Technologies) (Pareek *et al*., 2011).

1.9.2 Approach

Whatever the technique applied in bacterial genome sequencing, the general steps remain unchanged and include sample preparation, DNA sequencing, sequence assembly, and bioinformatic analysis (Dark, 2013). Advances in sample preparation have made it possible to use a small amount of even degraded original material used for DNA sequencing through the application of isothermal amplification for multiple displacement amplification (MDA). This technique is crucial in the sequencing of unculturable bacteria (Woyke *et al*., 2010).

1.9.2.1 Sequencing techniques

There are a number of DNA sequencing technologies available at the current time. Each technique has its advantages and disadvantages and no one technique has all the benefits (Dark, 2013).

Pryosequencing (454) by Roche uses sequencing by synthesis approach and has the advantage of giving larger-length reads of up to ~400 bp with a short turnaround time, making it until recently the preferred technique in the sequencing of *de novo* projects (Dark, 2013). Sequencing by Oligo Ligation Detection (SOLiD) developed by Life Technology has the advantage of being highly accurate so is used in single nucleotide polymorphism (SNP) analysis, but has the disadvantage of giving short read lengths of ~150 bp. Both IlluminaMiSeq and HiSeq are accurate with low error rates. However, they give short reads. The PacBio RS II Single

Molecule Real-Time Sequencing (SMRT) technique has the advantage of detecting DNA methylation and giving long lengths of reads, with the disadvantage of less accuracy in detecting SNP due to the relative errors in long reads (Koren *et al.,* 2012; Murray *et al*., 2012; Dark, 2013).

1.9.3 Application of DNA sequencing

1.9.3.1 Medical applications

Genome sequencing is now used directly to identify a microorganism and determine the association between phenotype and genotype (Torok *et al*., 2012), helping in the identification of antimicrobial resistance, identifying the source of nosocomial infections (Reuters *et al*., 2013), and can also be used in criminal investigations in forensic medicine (Fierer *et al*., 2010).

1.9.3.2 Genomic archaeology

Sequencing has also been used in identifying the source of ancient and current infections for bacteria such as *Yersinia pestis* and *Mycobacterium leprae* (Bos *et al.,* 2011; Schuenemann *et al*., 2013).

1.10 *Stenotrophomonas maltophilia*

This organism was first isolated in 1943 and was known as *Bacterium bookeri* (Hugh and Ryschenkow, 1961). After that it was known as *Pseudomonas maltophilia*. With further analysis of its rRNA genes, it was then called *Xanthomonas maltophilia* and, with greater advances in PCR-based 16S rRNA, it was classified as *Stenotrophomonas maltophilia* (Brooke, 2012). *Stenotrophomonas maltophilia* is a Gram-negative rod-shaped bacterium which is an obligate aerobe, motile with a few polar flagella, and non-fermentative. An oxidase test is usually negative, although some strains are oxidase-positive (Carmody *et al*., 2011). The bacterium is present in large numbers in hospital environments, as it prefers humid and aquatic environments and can even be present in the drinking water supply. Despite the fact that the organism is not highly pathogenic, it is now suspected in increasing numbers in nosocomial hospital-acquired infections (HAIs), with a mortality rate ranging from 14% to 69% in bacteraemic patients (Brooke, 2012;

Ferrer-Navarro *et al*., 2013). The most common infections associated with *S. maltophilia* are those of the respiratory tract, especially pneumonia, bacteraemia, biliary tract sepsis, bone and joint infections, urinary tract infections, eye infections (endophthalmitis, keratitis, scleritis), endocarditis, and meningitis. Immunocompromised patients are at high risk of infection with this organism and its presence in biofilm plays a role in that infection (Brooke, 2012).

Other underlying risk factors for infections with *S. maltophilia* include malignancy, the presence of chronic lung diseases, indwelling catheters, long-term hospitalization, ICU admission, septic shock and end organ failure (Calza *et al*., 2003).

S. maltophilia is a multidrug-resistant (MDR) bacterium that can be isolated from different environments, especially those that are aqueous, including soil, plants, animals, invertebrates, water distribution and treatment systems, sinks, rivers, wastewater plants, haemodialysis water, and even hand-washing soap and antiseptic solutions (Brooke, 2012).

The MDR pattern of *S. maltophilia* is related to a number of factors, including low membrane permeability to β-lactams, the production of β-lactamase, the presence of chromosomally encoded multi-drug efflux pumps, and the production of antibiotic-modifying enzymes (Sanchez *et al*., 2009). This makes the organism intrinsically resistant to many antimicrobials and this intrinsic resistance is mainly acquired from environmental strains by methods of horizontal gene transfer. There are no worldwide adapted guidelines for testing the antimicrobial susceptibility of *S. maltophilia* (Brooke, 2012).

S. maltophilia is sometimes misidentified as *Pseudomonas cepacia* due to errors in the reading of both oxidase and DNAse tests (Burdge *et al*., 1995). In order to improve the isolation of such an organism from cystic fibrosis (CF), where infection with *S. maltophilia* is more common, it is better to use specific media containing vancomycin, imipenem and amphotericin B (VIA medium) to inhibit the growth of other co-isolated bacteria (Denton *et al*., 2000). The Gram-negative selective agar (GNSA) medium was also created for the better detection of Gram-negative bacteria in the sputum of CF patients. GNSA medium contains novobiocin, cycloheximide, amphotericin, nisin, and crystal violet (Moore *et al*., 2003). It is

difficult to isolate it from culture due to associated polymicrobial infections with *S. maltophilia* and *P. aeroginosa* in CF patients, so molecular testing using PCR-based 16S rRNA identification is recommended (Nakamura *et al*., 2010).

There are molecular and biological differences between environmental and clinical isolates of *S. maltophilia*. K279a isolated from a CF patient and compared with the environmental strain (R551-3) showed that the clinical strain contained nine resistance nodulation-cell division (RND) transporters and other mechanisms implicated in antimicrobial resistance that were not detected in the environmental strain (R551-3) (Denton *et al*., 2000; Brooke, 2012). The two strains also showed differences regarding the presence of genomic islands responsible for the major genomic variability between the two strains. Genomic islands in the R551-3 environmental strain were identified and correlated to type I and IV secretion systems, metal resistance genes, filamentous haemagglutinin genes, and LPS genes (Rocco *et al*., 2009). However, no genomic islands were associated with specific functions related to pathogenicity in the clinical strain. There were shared genes related to specific functions, but no shared common genomic islands (Brooke, 2012).

1.11 Aims and objectives

This study aims to investigate the existence of antibiotic-resistant bacteria in environmental samples (river water from Beverley Beck, and agricultural soil from Riseholme and Lodge Farms) and in clinical settings in the U.K., using the phenotypic method of culture-based isolation followed by MIC determination, phenotypic study of β-lactamase activity and multiple resistance profiling. The resistant bacteria were then identified and the genes encoding resistance to imipenem characterised using the molecular techniques of PCR and sequencing. A whole bacterial genome was sequenced for selected MDR bacteria. This research has led to the identification of mechanisms of resistance and enabled investigation of the links between species/genotype/phenotype and isolate origin.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Media

The media (Oxoid, Hampshire, UK) used in the study are shown in Table 2.1.

Table 2.1: Media used in this study

Medium	Composition	g L^{-1}
Plate count agar (PCA)	Tryptone	5.0
	Yeast extract	2.5
	Glucose	1.0
	Agar	9.0
MacConkey agar (MCA)	Peptone	20.0
	Lactose	10.0
	Bile salts	5.0
	Sodium chloride	5.0
	Neutral red	0.075
	Agar	12.0
Nutrient broth (agar*)	`Lab-Lemco' powder	1.0
	Yeast extract	2.0
	Peptone	5.0
	Sodium chloride	5.0
	(Agar*)	15.0
Iso-Sensitest broth (agar*)	Hydrolysed casein	11.0
	Peptones	3.0
	Glucose	2.0
	Sodium chloride	3.0
	Soluble starch	1.0
	Disodium hydrogen phosphate	2.0
	Sodium acetate	1.0
	Magnesium glycerophosphate	0.2
	Calcium gluconate	0.1
	Cobaltous sulphate	0.001
	Cupric sulphate	0.001
	Zinc sulphate	0.001
	Ferrous sulphate	0.001
	Manganous chloride	0.002
	Menadione	0.001
	Cyanocobalamin	0.001
	L-Cysteine hydrochloride	0.02
	L-Tryptophan	0.02
	Pyridoxine	0.003
	Pantothenate	0.003
	Nicotinamide	0.003
	Biotin	0.0003

Medium	Composition	g L^{-1}
	Thiamine	0.00004
	Adenine	0.01
	Guanine	0.01
	Xanthine	0.01
	Uracil	0.01
	(Agar*)	8.0
Buffered peptone water	Peptone	10.0
	Sodium chloride	5.0
	Disodium phosphate	3.5
	Potassium dihydrogen phosphate	1.5

*either agar or broth

2.1.1.1 Preparation of plate count agar (PCA) and MacConkey agar (MCA) plates with and without antibiotics

These media were supplied in dehydrated form by Oxoid (UK) and prepared according to the manufacturer's recommendations in Duran bottles (equivalent to 17.5 g L^{-1} and 52 g L^{-1}, respectively) then autoclaved at 121 ^{o}C for 15 minutes in order to sterilize the agar. When required to be supplemented by antibiotic, the recommended amount of antibiotic stock solution was added to the media after cooling to 55 ^{o}C and mixed gently, then poured (about 20 ml) into 90 mm diameter sterile Petri dishes making a layer 3-5 mm thick. This was carried out inside a class II microbiological safety cabinet to avoid contamination and the plates were left to solidify before being placed in bags and kept in the cold room at 4 ^{o}C, to be used within one week of preparation.

2.1.1.2 Preparation of nutrient broth and nutrient broth supplemented with antibiotic

The nutrient broth powder (Oxoid, Hampshire, UK) was reconstituted according to the manufacturer's recommendations in Duran bottles (equivalent to 13 g L^{-1}). Bottle lids were loosened prior to being autoclaved at 121 °C for 15 minutes to sterilize the solution. When the antibiotic needed to be supplemented, the recommended amount of antibiotic stock solution was added to the broth after cooling to 55 ^{o}C and mixed gently, then an amount (about 3 ml) of the broth containing the antibiotic was pipetted into 15 ml sterile Falcon tubes. This was carried out inside a class II microbiological safety cabinet to avoid contamination

and the tubes were then placed in racks and kept in the cold room at 4 ^{o}C, to be used within one week of preparation.

2.1.1.3 Preparation of Iso-Sensitest agar plates

The agar was prepared according to the manufacturer's recommendations (equivalent to 31.4 g L^{-1}) and autoclaved at 121 ^{o}C for 15 minutes in order to sterilize the agar. The agar was poured into 90mm diameter sterile Petri dishes after cooling to 55 ^{o}C, making a layer 3-5 mm thick. This was done inside a Microbiological Safety Cabinet Class II to avoid contamination and the plates were left to solidify before being placed in bags and kept in the fridge at 5-8 ^{o}C, to be used within a week of preparation.

2.1.1.4 Preparation of buffered peptone water broth

Buffered peptone water (Oxoid) was prepared according to the manufacturer's recommendations (equivalent to 20 g L^{-1}). The constituents were placed in a glass beaker and then dissolved well. Then 3 ml was added to a glass test tube, stoppered well and autoclaved for 15 minutes at 121 ^{o}C. It was then cooled and kept in the fridge at 5-8 ^{o}C until needed.

2.1.1.5 Preparation of Iso-Sensitest broth

The Iso-Sensitest broth powder (Oxoid**,** Hampshire, U.K.) was reconstituted according to the manufacturer's recommendations (equivalent to 23.4 g L^{-1}). It was then autoclaved at 121 °C for 15 minutes to sterilise the solution. After cooling, the broth was kept in glass bottles in the fridge until needed.

2.1.2 Antibiotics

The antibiotics (Sigma-Aldrich, UK) used in solution are shown in Table 2.2.

Table 2.2: Antibiotic powder used in this study

Antibiotic	Stock solution	Final concentration	Reference
Imipenem (Im)	1 mg ml^{-1}	1 µg ml^{-1}	Girlich *et al*., 2010
Vancomycin (Vm)	2 mg ml^{-1}	8 µg ml^{-1}	Kuhn *et al*., 2005

2.1.2.1 Preparation of antibiotics

The antibiotics imipenem (Im) and vancomycin (Vm) were supplied in powder form by Sigma (Sigma-Aldrich, UK) and kept at the optimum temperature (-20 ^{o}C and 5 ^{o}C, respectively). Stock solutions of Im (1 mg ml^{-1}) and Vm (2 mg ml^{-1}) were prepared by dissolving appropriate amounts of these antibiotics in sterile distilled water using 15 ml sterile Falcon tubes and vortexed until the antibiotic had dissolved. Working concentrations were then achieved using concentrations utilized in previous studies: imipenem 1µg ml^{-1} (Girlich *et al*., 2010) and vancomycin 8 µg ml^{-1} (Kuhn *et al.*, 2005).

The antibiotics (Oxoid, Hampshire, U.K.) used in the disc assays are shown in Table 2.3.

Table 2.3: Antibiotic discs used in this study

Antibiotic class	Antibiotic	Symbol	Disc content (µg)
Penicillins	Ticarcillin-clavulanate	TIM	85
Aminoglycosides	Gentamicin	CN	10
Cephalosporins	Ceftazidime	CAZ	30
Other β-Lactams	Aztreonam	ATM	30
Carbapenems	Imipenem	IPM/IMI	10
	Meropenem	MEM/MER	10
Quinolones	Ciprofloxacin	CIP	1
	Levofloxacin	LEV	5
Sulfonamides	Trimethoprim	W	2.5
	Co-trimoxazole	SXT	25
Tetracyclines	Tetracycline	TE	10
	Minocycline	MH	30

2.1.3 Buffers and solutions

The buffers and solutions used in this study are shown in Table 2.4.

Table 2.4: Buffers used in this study

Buffer	Composition	g L^{-1}
TBE (10x stock solution) (Fermentas, UK)	Tris base	108
	Boric acid	55
	EDTA	7.4
25% Ringer's solution (Fisher Scientific, Leicestershire, UK).	NaCl	2.25
	KCl	0.15
	$CaCl_2$	0.12
	$NaHCO_3$	0.05

2.1.4 Oligonucleotides primers

The oligonucleotide primers used in this study are shown in Table 2.5.

Table 2.5: Oligonucleotides used in this study

Primer name	Sequence (5' to 3')	Amplicon size (bp)	Target gene	Optimum annealing temp	Reference
63-F	CAGGCCTAACACATGCAAGTC	1300	16S rRNA	54 °C	Marchesi *et al.*, 1998
1389-R	ACGGGCGGTGTGTACAAG			54 °C	Osborn *et al.*, 2000
IMP-F	CTACCGCAGCAGAGTCTTTG	587	*Bla* $_{IMP-1}$	55 °C	Lu *et al.*, 2010
IMP-R	AACCAGTTTTGCCTTACCAT			55 °C	Lu *et al.*, 2010
OXA-23-F	GATCGGATTGGAGAACCAGA	501	*Bla*$_{OXA-23}$	53 °C	Lin *et al.*, 2011
OXA-23-R	ATTTCTGACCGCATTTCCAT			53 °C	Lin *et al.*, 2011
L1-F	CACACCTGGCAGATCGGCAC	888	*Bla* $_{L1}$	65 °C	Petridou *et al.*, 2010
L1-R	GCCGCATCCGCGAGGC				
NDM1-F	CAGCACACTTCCTATCTC	350	*Bla*$_{NDM-1}$	56 °C	Liu *et al.*, 2012
NDM1-R	CCGCAACCATCCCCTCTT				
UniIND-F	GCCCAGGTTAAAGATTTTGTAAT	580	*Bla*$_{IND-1}$	53 °C	Hong Lin *et al.*, 2008
UniIND-R	CATGGCCACCGCCTTTCCATTC				

Primer name	Sequence (5' to 3')	Amplicon size (bp)	Target gene	Optimum annealing temp	Reference
B-F	GCTTGATTCTTGCTCTTG	205	*Bla*$_{B-1}$	52 ^{o}C	Woodford *et al.*, 2000
B-R	AATTTGTCTTCTCCCCAC				
CAU-F	TCGCGATTAAGGAGGTCGCCGCA TGAAGCG	870	*Bla*$_{CUA-1}$	60 ^{o}C	Docquier *et al.* 2002
CAU-R	GAGAATGAGAATACGCTCCTTGG				
UniIMP-F	GGAATAGAGTGGCTTAAYTC*	232	*Bla*$_{IMP}$	52 ^{o}C	Nordmann *et al.*, 2011
UniIMP-R	TCGGTTTAAYAAAACAACCACC				
UniVIM-F	GATGGTGTTTGGTCGCATA	390	*Bla*$_{VIM-1}$		
UniVIM-R	CGAATGCGCAGCACCAG				
UniNDM-F	GGTTTGGCGATCTGGTTTTC	621	*Bla*$_{NDM}$		
UniNDM-R	CGGAATGGCTCATCACGATC				
UniKPC-F	CGTCTAGTTCTGCTGTCTTG	798	*Bla*$_{KPC}$		
UniKPC-R	CTTGTCATCCTTGTTAGGCG				
UniOXA-48-F	GCGTGGTTAAGGATGAACAC	438	*Bla*$_{OXA-48}$		
UniOXA-48-R	CATCAAGTTCAACCCAACCG				
Mbl1b-F	ATGAAGCGCCTGATCCTGGC	580	*Bla*$_{Mbl1b}$	60 ^{o}C	Simm *et al.*, 2001
Mbl1b-R	GATCGGTCATCGCTTGGGCC				

F = forward primer; R = reverse primer; bp = base pair, * Y= C or T (Eurofins MWG Operon, Germany)

2.1.4.1 Preparation of primers

Stock primers were prepared according to the manufacturer's recommendations (Eurofins MWG Operon, Germany) by adding the recommended amount of sterile distilled water and mixing gently to obtain a concentration of 100 pmol µl^{-1} and then diluting this to 10 pmol µl^{-1} (the primer concentration used in PCR) by taking 20 µl of stock primer plus 180 µl of sterile distilled water).

2.2 Methods

2.2.1 Isolation and subculturing antibiotic-resistant bacteria

2.2.1.1 Sample types and collection

Water samples were collected from river water in order to identify the existence of antibiotic resistant environmental bacteria contained therein. These were taken from four sites seven metres apart, 15 cm below the surface of the water from the Beverley Beck section of the River Hull [53.839511,-0.410579] (see Figure 2.1) on 14 September 2011. Beverley Beck is a short canal around 0.8 miles in length in the East Riding of Yorkshire, England, which connects the River Hull to the town of Beverley, dating back to 1296. Water is raised into Beverley Beck from the River Hull by means of an engine and there is a drainage tunnel running underneath. Please see:
http://www.canalbarge.co.uk/Canals%20and%20Rivers/Beverley%20Beck.html.

Thus, the water current is not strong and the canal was chosen for sampling primarily due to its proximity to the sewage works, as it contains effluent of sewage after being biologically treated. Sampling was conducted downstream of the sewage effluent.

In addition, soil samples were collected on 26 November 2012 from three different crops (winter wheat [W], sugar beet [S], and spring beans [B]) to a depth of 5 cm from farm at Riseholme (R) [52.856816,-0.188351] where cattle manure was used in fertilisation and from the Lodge Farm (L) [53.240595,-0.348023] where inorganic fertilisers (ammonium nitrate and triple super phosphate) were used. These were collected from six sites (Figure 2.2 and Table 2.6).

Samples were collected in triplicate in 50 ml sterile Falcon tubes from different sites. The tubes were kept on ice to preserve the viability of microorganisms present. Bacteria were isolated on media either with or without antibiotics on the same day of collection.

Isolates were also obtained from a number of clinical samples which were obtained from three different hospitals in Sheffield, York and Hull in 2012 and 2013.

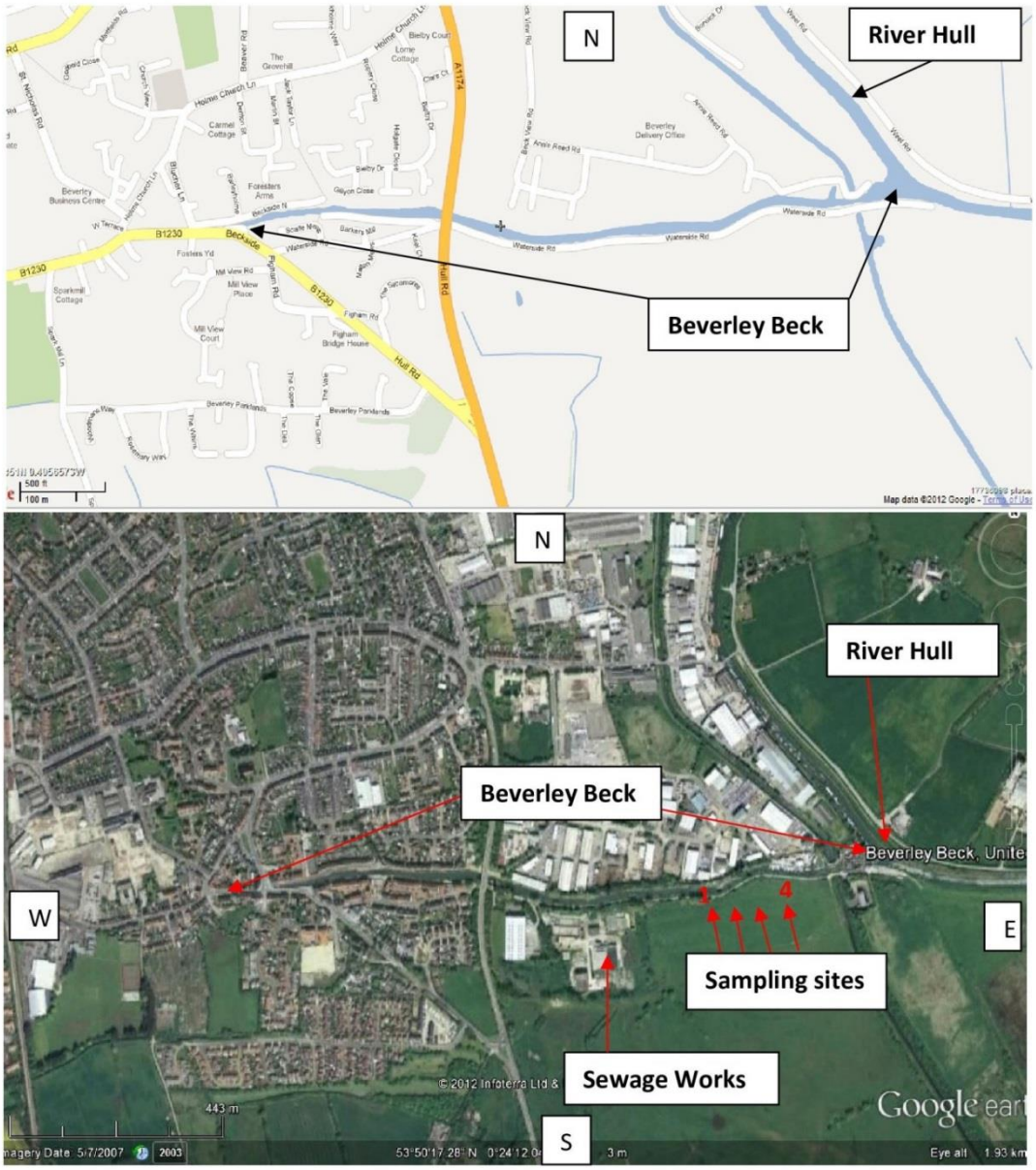
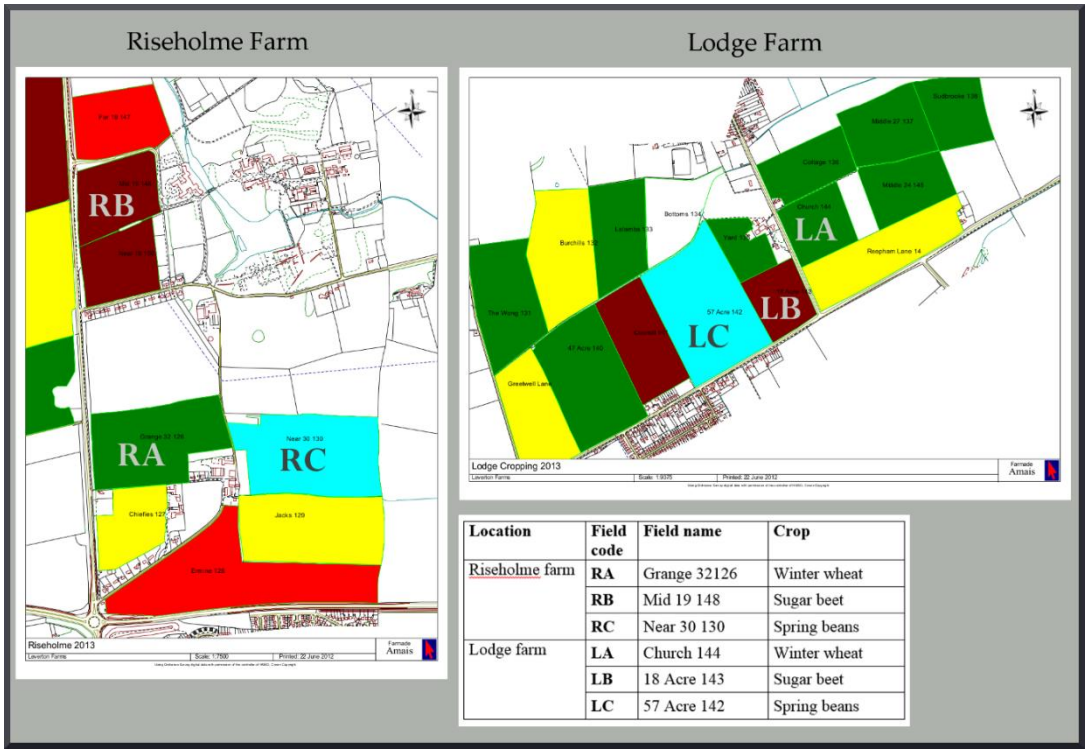


Figure 2.1: A map and photograph showing the location of the origin and termination of Beverley Beck and the sampling sites

Taken with some modification from www.google.com.



Location	Field code	Field name	Crop
Riseholme farm	RA	Grange 32126	Winter wheat
	RB	Mid 19 148	Sugar beet
	RC	Near 30 130	Spring beans
Lodge farm	LA	Church 144	Winter wheat
	LB	18 Acre 143	Sugar beet
	LC	57 Acre 142	Spring beans

Figure 2.2: A map showing the sampling fields for two farms where soil samples were collected

NB: Left: Riseholme Farm; right: Lodge Farm. Soil samples were collected from the fields detailed in Table 2.6 below.

Table 2.6: Fields (crops) in two farms where soil samples were collected

Location	Field code	Field name	Crop
Riseholme Farm	**RA**	Grange 32126	Winter wheat
	RB	Mid 19 148	Sugar beet
	RC	Near 30 130	Spring beans
Lodge Farm	**LA**	Church 144	Winter wheat
	LB	18 Acre 143	Sugar beet
	LC	57 Acre 142	Spring beans

2.2.1.2 Isolation of bacteria from environmental samples

The samples were manipulated under aseptic conditions inside a Class II Microbiological Safety Cabinet. Prior to inoculation of the samples onto different culture media, water samples were 10-fold serially diluted using ¼-strength Ringer's solution starting from the undiluted specimen 10^0 down to 10^{-5}. Then 100 µl from each dilution was inoculated onto solid media (plate count agar [PCA] and MacConkey agar [MCA] plates) both with and without antibiotics using a spread-plating technique. The antibiotics added to both media were either imipenem (1 µg ml^{-1}) or vancomycin (8 µg ml^{-1}) and were added to each of the PCA or MCA plates. This isolation technique was performed to enable the isolation of the antibiotic-resistant colonies (grown on antibiotic-containing media) and relate them to the total viable number of microorganisms (grown on antibiotic-free media) of the same type. Cultured plates were then incubated at 25 ^oC for PCA or 37 ^oC for MCA for 72 hours or 24 hours, respectively. The resulting isolated colonies were then counted for each dilution on each type of media.

2.2.1.3 Subculture and isolation of microorganisms from plates

After counting all of the colonies obtained over all media types (with or without antibiotic), each single colony obtained on the plates containing antibiotic was subcultured onto the same type of plate (media and antibiotic) using the same culture conditions used in the original isolation for further characterisation and study. The subculture plates were then stored in the cold room at 4 ^oC and used within a week of subculturing.

2.2.1.4 Subculturing of microorganisms using nutrient broth

In order to confirm the resistance of the microorganisms to the antibiotic, microorganisms were re-subcultured by taking two to three colonies from media containing an antibiotic and inoculated into a 15 ml sterile Falcon tube containing 3 ml nutrient broth (supplemented with antibiotics: either imipenem or vancomycin). Each isolate was inoculated into two nutrient broth tubes; one tube with antibiotic and the other without antibiotic to be used as a positive control. These tubes were placed in a shaking incubator (Innova®44 Incubator shaker series) at 100 rpm at 25 ^oC for PCA or 37 ^oC for MCA for 24 hours, respectively. Turbid (positive) tubes were then used to store antibiotic-resistant strains at -80 ^oC.

2.2.1.5 Storage of the strains

Antibiotic-resistant isolates from the broth cultures above were stored for use over longer periods at -80 ^{o}C using 2 ml sterile Eppendorf tubes containing 15% sterile (autoclaved) glycerol suitable for long-term storage of bacterial isolates (Ash *et al.*, 2002). Viability was checked after three days of storing by scraping the frozen culture surface with a sterile plastic inoculation loop and inoculating into the appropriate media at the optimum temperature and incubation period. Thawing of the stock was always avoided.

2.2.2 Determination of minimum inhibitory concentration (MIC)

As the screening concentration for imipenem resistance in this study was 1µg ml^{-1}, it was important to determine the resistance levels of isolated bacteria. The MIC of the isolated strains was determined, to a range of 0.004-32 mg L^{-1} imipenem, by the quantitative microdilution method using microtiter plates, following the EUCAST 2012 guidelines. Negative (plain ISO broth) and positive (ISO broth culture) controls for each organism were included. In addition, positive reference strains *Klebsiella pneumoniae*-producing metallo-β-lactamase (MBL) 'NCTC 13439', and *Klebsiella pneumoniae*-producing *Klebsiella pneumoniae* carbapenemase (KPC) 'NCTC 13438' were included. The negative control *Escherichia coli* 'NCTC 10418' was also included. The microtiter plates were prepared and incubated for 24 hours at 37 ˚C (clinical isolates) and at 25 ˚C (environmental isolates) using a shaking incubator. The MIC was determined as the lowest concentration of antimicrobial agent that completely inhibited growth of the organism in the tubes or microdilution wells, as detected by the unaided eye (CLSI, 2012a).

2.2.3 Study of β-lactamase activity

2.2.3.1 Modified Hodge Test

The modified Hodge test (MHT) is used to confirm carbapenemase production in bacterial isolates (Figure 2.3). It is based upon the formation of a characteristic clover leaf shape around the edge of a carbapenem disc (i.e. attacking the disc) by the carbapenemase-positive tested bacterial isolate which is inhibiting a known

carbapenemase negative control strain (e.g. *E. coli* ATCC 25922 'NCTC 10418') on a Muller-Hinton (iso-sensitest) agar plate (Carvalhaes *et al.*, 2010; Seah *et al*, 2011; Nordmann *et al*., 2012).

Following the BSAC guidelines, the imipenem resistant (ImR) strains were tested using the MHT upon iso-sensitest agar to confirm β-lactamase (carbapenamase) production (BSAC, 2013; CLSI, 2012b).

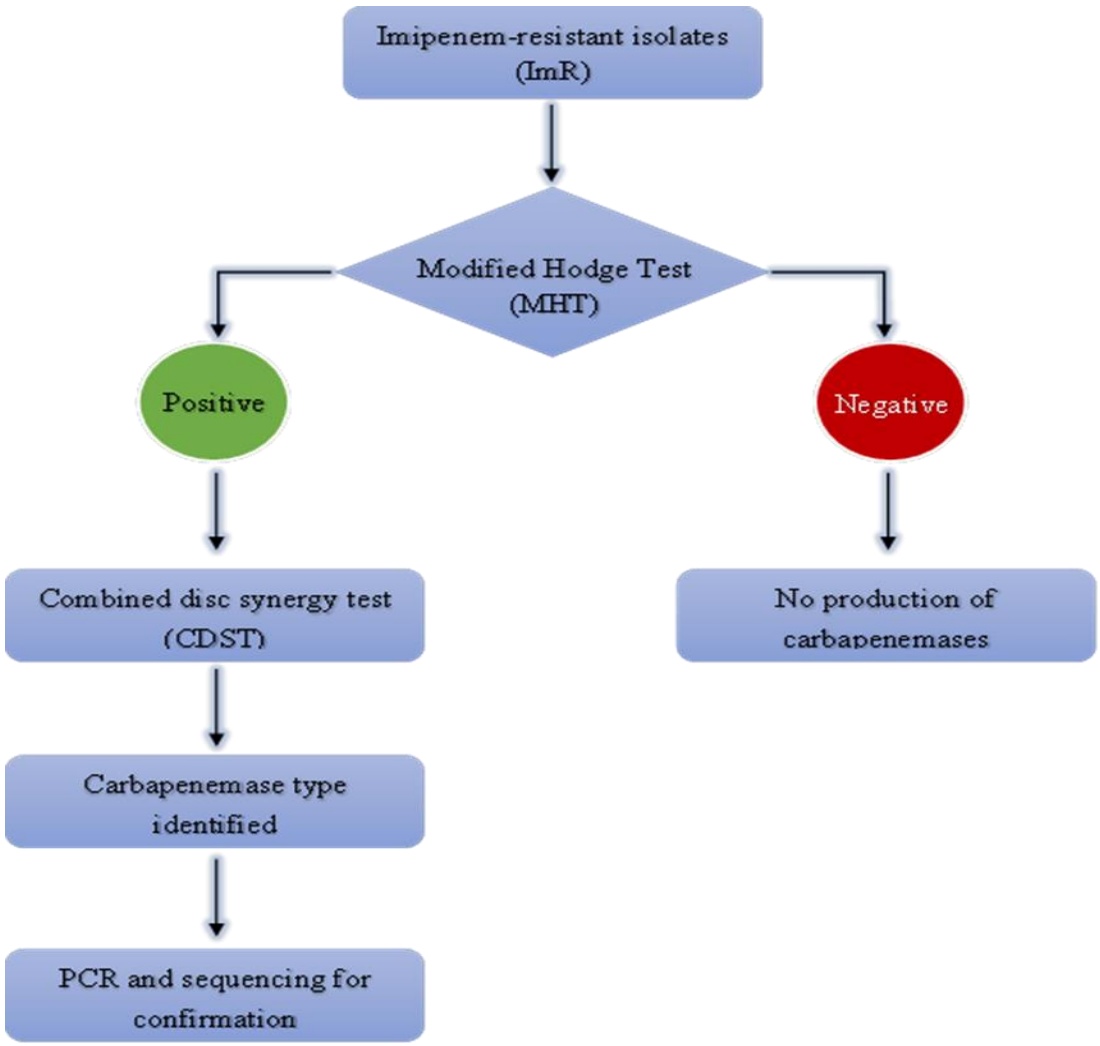


Figure 2.3: Flow chart for the detection of carbapenemase production used in the current study

2.2.3.2 Combined disc synergy test (CDST) 'inhibition tests'

As the MHT does not differentiate the type of β-lactamase (carbapenemase) produced by the bacteria, a combination of the carbapenem disc with different inhibitors was used in the inhibition assays. These inhibition tests are based upon the

inhibition of different classes of carbapenemase by specific inhibitors (Figure 2.4). For example, AmpC and class A carbapenemases are inhibited by boronic acid, and class B metallo-B lactamases are inhibited by zinc chelators e.g. EDTA or dipiclonic acid. None inhibit class D, while cloxacillin inhibits AmpC but not class A (Nordmann *et al*., 2012).

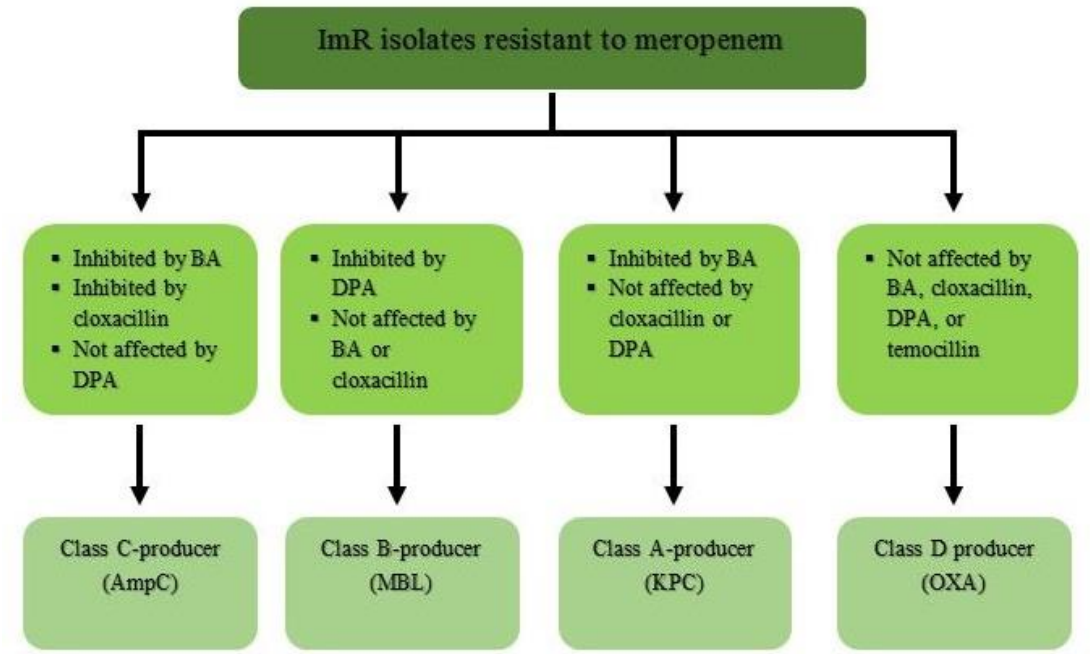


Figure 2.4: Diagram showing the possible results for CDST (ROSCO's Diagnostic, Denmark)

(BA = boronic acid; DPA = dipicolinic acid; MBL= metallo-β-lactamase; KPC = *Klebsiella pneumonia*e carbapenemase; OXA = Oxacillinase).

All positive MHT bacterial isolates were tested to determine the type of β-lactamase using the imipenem/meropenem-β-lactamase inhibitors combined disc synergy test 'CDST' (ROSCO's Diagnostic, Denmark).

There are three possible reasons why an organism shows reduced susceptibility to carbapenems:

1. The organism over-produces AmpC and then will be inhibited by cloxacillin and boronic acid.

2. The organism is producing a Class B (metallo-β-lactamase - MBL) enzyme and this enzyme is inhibited by dipicolinic acid (DPA) or EDTA.

3. The organism is producing a Class A e.g. (KPC) enzyme which is inhibited by boronic acid only.

Alternatively, the organism may be producing a Class D enzyme (oxacillinase - OXA-48 or similar). These enzymes are not inhibited by any substances used in the combination discs and are also resistant to Temocillin.

The differentiation between bacteria is shown in Table 2.7. Some bacteria may produce more than one type of enzyme.

Table 2.7: Susceptibility outcomes of antibiotic resistant bacteria in inhibitions tests

	β-lactamase activity			
Inhibition test	Class A - positive (KPC)	Class B - positive (MBL)	Class C - positive (AmpC)	Class D - positive (OXA)
Meropenem 10 µg (MRP10) disc	Resistant	Resistant	Resistant	Resistant
Meropenem 10µg + boronic acid (MRPBO)	Susceptible (≥ 5mm increase in the inhibition zone)	Resistant (≤ 3 mm increase in the inhibition zone)	Susceptible (≥ 5mm increase in the inhibition zone)	Resistant (≤ 3 mm increase in the inhibition zone)
Meropenem 10µg + cloxacillin (MRPCX)	Resistant (≤ 3 mm increase in the inhibition zone)	Resistant (≤ 3 mm increase in the inhibition zone)	Susceptible (≥ 5mm increase in the inhibition zone)	Resistant (≤ 3 mm increase in the inhibition zone)
Meropenem 10µg + dipicolinic acid (MRPDP)	Resistant (≤ 3 mm increase in the inhibition zone)	Susceptible (≥ 5mm increase in the inhibition zone)	Resistant (≤ 3 mm increase in the inhibition zone)	Resistant (≤ 3 mm increase in the inhibition zone)
Temocillin 30µg (TEMOC)	Variable	Variable	≥ 12mm	Resistant (no zone of inhibition)

In general, a difference of ≥ 5mm after combination indicated the presence of related enzyme activity (CLSI, 2012b).

2.2.4 Study of multiple resistance profiling

Carbapenemase-producing bacteria are often associated with resistance to many antimicrobials and are often multi-drug resistant isolates (MDRs) (Nordmann *et al*., 2012).

To test for multi-drug resistance, isolates were inoculated upon iso-sensitest agar according to BSAC guidelines and tested using the disc diffusion method. Two sets of plates were inoculated and tested for susceptibility. Isolates were incubated for 24 hours at 25 ^{o}C and 37 ^{o}C for the environmental and clinical isolates, respectively. The susceptibility of the isolates to these antibiotics was determined by the absence of an inhibition zone (Abaidoo *et al*., 2002; D'Costa *et al*., 2007).

The density of the inoculum suspension of the tested bacterial strain was standardized to contain a fixed number of bacteria per unit volume of fluid. Three to four separate colonies of the tested microorganism were added to peptone water broth and the optical density (OD) measured by spectrophotometer (Cecil Instruments Ltd., Cambridge, UK) at 500 nm. A specific volume, depending on the OD and the bacterial strain, was then transferred from the broth culture to 5 ml of sterile distilled water (DW), as shown in Table 2.8.

Table 2.8: Volume of liquids to be transferred from broth culture to a tube containing 5 ml of sterile distilled water (BSAC, 2013)

OD at 500 nm	Volume of broth culture to be transferred to DW (µL)
0.01- 0.05	250
0.05- 0.1	125
0.1- 0.3	40
0.3- 0.6	20
0.6- 1.0	10

This standard bacterial suspension was used in the inoculation of Iso-Sensitest agar plates. Iso-Sensitest agar plates were inoculated from this standard suspension using a sterile cotton swab, first by making a central cross over the plate then by

spreading in three different planes to achieve an even, semi-confluent bacterial growth.

2.2.5 Molecular characterisation of antimicrobial resistant bacteria

2.2.5.1 Extraction of crude cell lysate containing DNA

DNA extraction was performed as described by Senda *et al*. (1996), by taking two to three fresh colonies from a plate and mixing in 50 µl of sterile distilled water. The cells were mixed using a small vortex machine for 3-5 seconds and then heated using a PCR thermal cycler (Bio-Rad C1000™ Thermal Cycler) at 94 ^{o}C for two minutes followed by centrifugation at 13000 rpm for two minutes using a microcentrifuge (Mini Spin Plus, Eppendorf). Crude lysate containing DNA was stored at -20 ^{o}C for use over a long period. DNA extraction was assessed prior to use in the PCR, using the NanoDrop 1000 spectrophotometer and gel electrophoresis.

2.2.5.2 PCR amplification of DNA

PCR mimics the DNA replication process in nature, with some modifications carried out *in vitro*. It consists of three steps: denaturation, which allows the separation of the two DNA strands of interest (carried out at a high temperature, usually at 94 ^{o}C for 2-8 minutes initially and then for 1-2 minutes for subsequent cycles); the annealing of a specific synthetic short oligonucleotide primer complementary to the target sequence (carried out usually for 1-2 minutes at 55 ^{o}C, depending upon the length of the primer, GC contents, and concentrations of the primer used); and lastly the extension from this annealed primer using the enzyme Taq DNA polymerase, which is obtained from *Thermus aquaticus,* an organism which naturally lives in a high temperature environment of about 110 ^{o}C and can withstand repeated heating for many cycles (Cariello *et al.,* 1991). The extension step is usually carried out at 72 ^{o}C (which is near to the optimum temperature for Taq DNA polymerase) for 1 minute or longer. These steps are repeated multiple times (25-40 cycle) with an exponential duplication of the DNA produced in each cycle (Lo and Chan, 2006; Beaz-Hidalgo *et al*., 2008).

PCR was performed to detect the genes responsible for the antimicrobial resistance for imipenem within the isolated microorganisms. PCR was also used for the amplification of the 16S rRNA gene to be used in the identification of the

isolated microorganism. The sets of primers used therein (Table 2.5) were selected according to those used in previous studies.

The volume added to each 1 µl of DNA sample from the master mix contained two sets of primer: 0.3 µM of the forward (F) and 0.3 µM of the reverse (R) sequences (Eurofins MWG Operon, Germany); 2.5 units of Taq DNA polymerase enzyme and 1x reaction buffer; 1.5 mM of magnesium chloride ($MgCl_2$) (Bioline Ltd, UK); 0.2 mM of dNTPs (Roche, Germany), and made up to a total volume of 25 µl with sterile distilled water (sdH_2O).

PCR conditions were applied depending on the primers described in previous studies (see Table 2.5), using different annealing temperatures for the different selected primers, as follows: preheating (initial) at 94 °C for five minutes, followed by 30 cycles of denaturation at 94 °C for one minute, annealing for one minute (see Table 2.5) and elongation at 72 °C for two minutes, with a final extension at 72 °C for 10 minutes performed using a Bio-Rad C1000^{TM} thermal cycler. In the presence of some problems with the amplification of the target gene (e.g. weak bands or non-specific secondary bands after the PCR reaction) which hindered the assessment of the PCR product and further analysis, a gradient PCR technique was carried out, which allowed the empirical determination of an optimal annealing temperature (Özdemir, 2009). This was achieved by choosing a temperature range (e.g. 50, 52, 56, 58, and 60 °C), and setting this across different columns of wells (at different annealing temperatures) in the thermocycler. For example, column 1 for 50 °C, column 2 for 52 °C, and column 3 for 56 °C (i.e. gradually raising the annealing temperature in order to amplify the target gene). With each sample, the PCR mix had the same concentrations for all the reactants. Thus, each reaction was performed at a different annealing temperature, with all other values being the same. PCR is normally started at 5 °C below the calculated (referenced) temperature of the primer melting point (T_m). Theoretically, the annealing temperature (T_A) = (T_m) – 5 °C. The melting point (T_m) can also be calculated using the following equation: T_m (°C) = 4 (no. of G + C) + 2 (no. of A + T) (Suggs *et al.*, 1981).

2.2.5.3 Detection of amplified products and extracted DNA using agarose gel electrophoresis

The principle of this technique is to separate DNA (and RNA) fragments in a mixed population according to their size in order to assess the length of DNA and RNA fragments which are negatively charged (Kryndushkin *et al.*, 2003). This can be achieved by applying an electric current across which DNA and RNA will migrate from the negative charge (cathode) through an agarose matrix to the positive charge (anode). Shorter fragments migrate faster, so they move further than longer ones, as shorter fragments move more easily through the pores of the gel (Sambrook and Russell, 2001).

Tris-Borate-EDTA x10 (TBE) buffer (Fermentas Life Science) was diluted using distilled water to x0.5. The appropriate concentration of agarose gel in x0.5 TBE buffer (depending upon the expected amplicon size of DNA e.g. to give a percentage of agarose (w/vol) of between 0.8-2%) was prepared in a glass flask, and then heated and dissolved in a microwave oven (Sanyo EM-SL40S) on a low power setting (300W). After that, the agarose was allowed to cool until hand hot with continued shaking to avoid solidification of the agarose. At that point, a 1X Syber-SAFE DNA stain (Invitrogen) was added to the agarose and mixed before being poured into the gel tray and then allowed to solidify inside the gel tray. The agarose gel containing the dye was then placed inside the electrophoresis tank, which was filled with x0.5 TBE buffer to the limit point.

The PCR sample (5 µl) to be electrophoresed was mixed with the loading dye (1 µl) (Fermentas Life Science) and pipetted into wells within the gel. A DNA size standard composed from different sizes of DNA fragments (Fermentas Life Science, Appendix A1) was also loaded into the gel. DNA was electrophoresed at 80-100V until the DNA fragments were separated according to their size. The resulting DNA fragments were visualized using a UV transilluminator at 302 nm (Bio-Rad, Molecular Imager®, ChemiDoc™ XRS+ with Image Lab™ Software).

2.2.5.4 DNA sequencing and alignment

PCR products were sequenced by LGC Genomics GmbH (Germany), using Sanger sequencing. The sequence data was proofread, assembled, and aligned using BioEdit Sequence Alignment Editor version 7.0.0

(**http://www.bioedit.software.informer.com/).** A BLAST (Basic Local Alignment Search Tool) (**http://blast.ncbi.nlm.nih.gov/Blast.cgi**) was used to study the homology and Clustal W2 software from EMBL/EBI (The European Molecular Biology Laboratory/EuropeanBioinformatics Institute) (**http://www.ebi.ac.uk/Tools/msa/clustalw2/**) was used to construct the multiple alignments.

2.2.5.5 Isolation of bacterial genomic DNA

Bacterial genomic DNA was extracted from bacteria using a PowerMicrobial® Maxi DNA Isolation Kit (MoBio Laboratories Inc., California). The quantity of genomic DNA was measured using a NanoDrop ND-1000 spectrophotometer, while its quality was assessed via agarose gel electrophoresis. For isolates that showed low quantity and/or quality, the DNA was re-extracted with some modifications suggested by the manufacturers.

2.2.5.6 Bacterial genome sequencing

Bacterial genomes were sequenced by the NGS Laboratory (Eurofins Genomics, Germany) using de novo sequencing by Illumina MiSeq with chemistry v3 (Genomic shotgun protocol, with paired-end sequencing).

2.2.5.7 Bacterial genome data analysis

After assembled contigs were obtained from NGS Laboratory (Eurofins Genomics, Germany), genome sequences were annotated and further studied using RAST (Rapid Annotation using Subsystem Technology) (**http://rast.nmpdr.org/**). Additional analysis was performed using analysis tools from the CGE (Centre for Genomic Epidemiology) (**http://www.genomicepidemiology.org/**). Related ARGs were also investigated using BLAST (Altschul *et al*., 1997) against the GenBank database.

2.2.6 Statistical analysis

Statistical analyses (significance tests), including the t-test and one-way ANOVA test, were done using Vassarstats (www.vassarstats.net). The t-test is used to compare whether there are significant differences between two independent variables

(populations). The one-way analysis of variance (ANOVA) is performed when comparing multiple variables to detect any significant differences between the means of more than two independent (unrelated) groups. Differences between variables are considered statistically significant when the *P* value is $\leq$ 0.05 (Matthew and Farewell, 2007; Bowers, 2008).

Chapter 3: Phenotypic Characterisation of Antibiotic Resistant Bacteria from Natural Environments and Clinical Settings

3.1 Introduction

The spread of antimicrobial resistance in clinical settings leads to increases in both morbidity and mortality in humans (Paul *et al*., 2010) and has been the cause of considerable social and economic damage. In Europe in 2007, for example, 400,000 cases of multi-drug resistant (MDR) bacterial infections led to 25,000 deaths and over 2.5 million extra hospital-stay days at a cost of more than 1.5 million euros per year (ECDC, 2009; Bush *et al*., 2011). It is now established that there is a close relationship between antimicrobial resistance in clinical settings and in the non-clinical environment, with natural environments including soils and water playing an important role in the spread of resistant bacteria by acting as a reservoir for and source of these resistant isolates (Wellington *et al*., 2013). The soil environment is particularly important as many of the antibiotics used in clinical settings were originally extracted from naturally-occurring antimicrobial-producing microorganisms isolated from soil (Dantas and Sommer, 2014). Additionally, prescribed antimicrobial medicines are excreted by patients and enter the environment via the sewage system and can select for increased resistance in environmental bacteria. Together with naturally-occurring antibiotic-resistant bacteria, these organisms and resistant genes can then be transferred to humans by direct contact and/or via the food chain (Wellington *et al*., 2013).

Bacteria are continuously developing resistance to antimicrobials, partly as a consequence of the use of many different antimicrobials in agriculture and animal feeding; a situation which is leading to the selection and greater prevalence of antimicrobial resistant strains (Phillips *et al*., 2004; Kummerer, 2005). Another activity that increases the spread of antibiotic resistant bacteria is the use of animal manure to fertilise agricultural land (Giger *et al*., 2003). Animal manure frequently carries antibiotic-resistant genes (ARGs) existing in gut bacteria and also contains the residues of veterinary antimicrobials that are used in feedstocks as a prophylactic treatment (Witte, 2000). ARGs can be transmitted to other bacteria via horizontal gene transfer and consequently to bacteria in humans via the food chain. Residual

antimicrobials can persist in the soil for many years (Gaze *et al*., 2008). These selection pressures in the environment, together with the presence of antimicrobials which are naturally secreted by certain organisms (Jensen and Demain, 1995), has led to the appearance of numerous bacterial species which are multi-drug resistant (MDR). Of recent concern is the emergence of resistance to carbapenem antibiotics, these are important broad-spectrum antibiotics, generally only used as a last resort treatment for clinically-important antibiotic resistant bacteria, including *Klebsiella pneumoniae*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa* (Shah 2008; Hawkey and Livermore, 2012). The mechanisms implicated in carbapenem resistance are thought to be either decreased membrane permeability to carbapenems or to the production of carbapenemase enzymes that hydrolyse the antibiotic (Nordmann *et al*., 2012).

Carbapenemases, which are classified into four classes (A, B, C, and D, see section 1.7.1.7), are found worldwide. As carbapenems (including imipenem, ertapenem, meropenem, and doripenem) belong to the group of β-lactam antimicrobials, so the development of resistance to carbapenemase action is associated with resistance to other β-lactams, including penicillins and cephalosporins (Nordmann *et al*., 2011). Research into imipenem resistance in both environmental and clinical settings has shown variable levels of resistance. Rossolini and co-workers (2001) found widespread imipenem-resistant bacterial isolates in soil obtained from different environmental sites, including hills and farm treated with manure in Italy. They identified carbapenem resistant isolates of *Stenotrophomonas maltophilia*, *Chryseobacterium* spp., *Aeromonas hydrophila* and *Janthinobacterium lividum*. They also reported the detection of a novel class of metallo-β-lactamase in a *J. lividum* isolate (Rossolini *et al*., 2001). Girlich and colleagues (2010) discovered the imipenem-resistant *Oxa-23*-producing *Acinetobacter baumannii* in both aquatic and soil environments and also in hospital settings. In comparing the environmental and clinical isolates they found evidence for genetic exchange, as they detected the *Oxa-23* gene of the environmental isolate in the clinical isolates, and they have not excluded the possibility that resistance could have been transferred from the environment to the clinical bacteria (Girlich *et al*., 2010).

In clinical imipenem resistant isolates, Nordmann and colleagues (2011) reported the worldwide spread of carbapenemase classes A, B, and D at varying

frequencies in different geographical locations. Class A *Klebsiella pneumoniae* carbapenemase (KPC) was found to be more prevalent in the USA and Greece, while class B metallo-β-lactamases (MBLs) were more prevalent both in Europe and especially in Asia (VIM and IMP), whilst Oxa-48 β-lactamases were more abundant in Mediterranean Europe. They also reported the need to try to minimise the spread of these resistant isolates in the community. However, there are a number of factors contributing to the spread which are highly difficult to control, e.g. the increase in worldwide travel, the overuse and misuse of antimicrobials, and the lack of personal hygiene (Nordmann *et al*., 2011).

It is often the case that most imipenem-resistant bacteria are also resistant to other antimicrobials. For example, some of these bacteria have been found to produce gentamicin-modifying enzymes leading to resistance to gentamicin, and others show co-production of AmpC with ESBLs, leading to resistance to aztreonam (Livermore, 2009). This multi-resistance can be confirmed by testing ImR bacteria against a range of antimicrobials related to different classes and with different mechanisms of action (EUCAST, 2012).

Vancomycin is a member of the glycopeptide group of antimicrobials (see Section 1.3.1) and is used in the treatment of a wide variety of Gram-positive bacteria, including *Staphylococcus* and *Enterococcus*, and again as an antibiotic of last resort for treating multi-resistant bacteria (Uttley *et al*., 1988). However, resistance to vancomycin has been acquired by clinical isolates of *Enterococcus* in both Europe and in the USA, as long ago as 1988 (Leclercq *et al*., 1988). One of the causes of the appearance and spread of vancomycin resistance in clinical settings was the use of antimicrobials in agriculture. For example, the vancomycin-analogue avoparcin used in pig feed was the source of vancomycin-resistant enterococci (VRE) in humans (Witte, 1997), and it was consequently banned throughout Europe in 1997 (Heuer *et al*., 2002). Vancomycin-resistant enterococci (VRE) have been detected in the stools of farm animals, in the stools of healthy asymptomatic humans (Bates, 1997), in sewage water, animal products and aquatic environments (Harwood *et al*., 2001; Iversen *et al*., 2002). Horizontal gene transfer mediating movement of vancomycin resistance between various bacteria is common by conjugation in manure (Schwartz *et al*., 2003). More dangerous is the passage of vancomycin-resistant genes like *vanA* from Enterococci to Staphylococci in the environment,

leading to the emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA) (Weigel *et al*., 2003). Emergence of such VRSA leads to the loss of last-resort treatment of MRSA with vancomycin.

The first aim of this chapter was to quantify the relative abundance of culturable imipenem-resistant bacteria (as the first priority) and vancomycin-resistant bacteria (as the second priority) in natural environments, specifically, river water and agricultural soils. However, if imipenem resistance was detected in both environments, the vancomycin-resistant isolates were not tested for further characterisation. This is because imipenem resistance is a more important finding and there are fewer available studies on imipenem resistance than on vancomycin resistance. The second aim was to phenotypically characterise and compare imipenem resistant isolates from both natural environments and clinical settings in terms of their β-lactamase type and multi-antibiotic-resistant profiles.

3.2 Results

3.2.1 Isolation and enumeration of antibiotic resistant bacteria in river water

The abundance of bacteria resistant to imipenem and vancomycin in river water was investigated using culture-based approaches. Water samples were taken from the Beverley Beck section of the River Hull [53.839511N, -0.410579E] on 14th September 2011.

Water samples from four different sites (S1, S2, S3 and S4) were taken from pontoons at seven meter intervals, at a depth of 15 cm below the surface of the water. The samples were serially diluted and 100 µl aliquots were plated onto Plate Count Agar (PCA) and MacConkey agar (MCA) and onto PCA and MCA each supplemented with either imipenem (1µg ml^{-1}) or vancomycin (8µg ml^{-1}) (Nordmann *et al*., 2009; CLSI, 2012a). Viable counts and numbers of antibiotic resistant culturable bacteria were determined after 24 (MCA) and after 72 hours (PCA) (Appendix A2.1).

Numbers of culturable bacteria (total viable counts) were higher on PCA (mean ± SD of 670 ± 16.3 CFU ml^{-1}) than on MCA (mean ± SD of 330 ± 37.4 CFU

ml^{-1}). Numbers of both vancomycin-resistant (VmR) and imipenem-resistant (ImR) bacteria were higher on PCA (mean ± SD of 362.5 ± SD 155.9 and 75 ± 28.9 CFU ml^{-1}, respectively) than on MCA (mean ± SD of 145 ± 60.3 and 5 ± 5.8 CFU ml^{-1}, respectively) (Figure 3.1). The relative abundance of VmR and ImR bacteria was determined as a percentage of the total viable bacterial counts. On PCA, 54.1% of isolates were resistant to Vm, while 11.2% of bacteria were resistant to Im. On MCA, 43.9% of isolates were resistant to Vm, while 1.5% were resistant to Im.

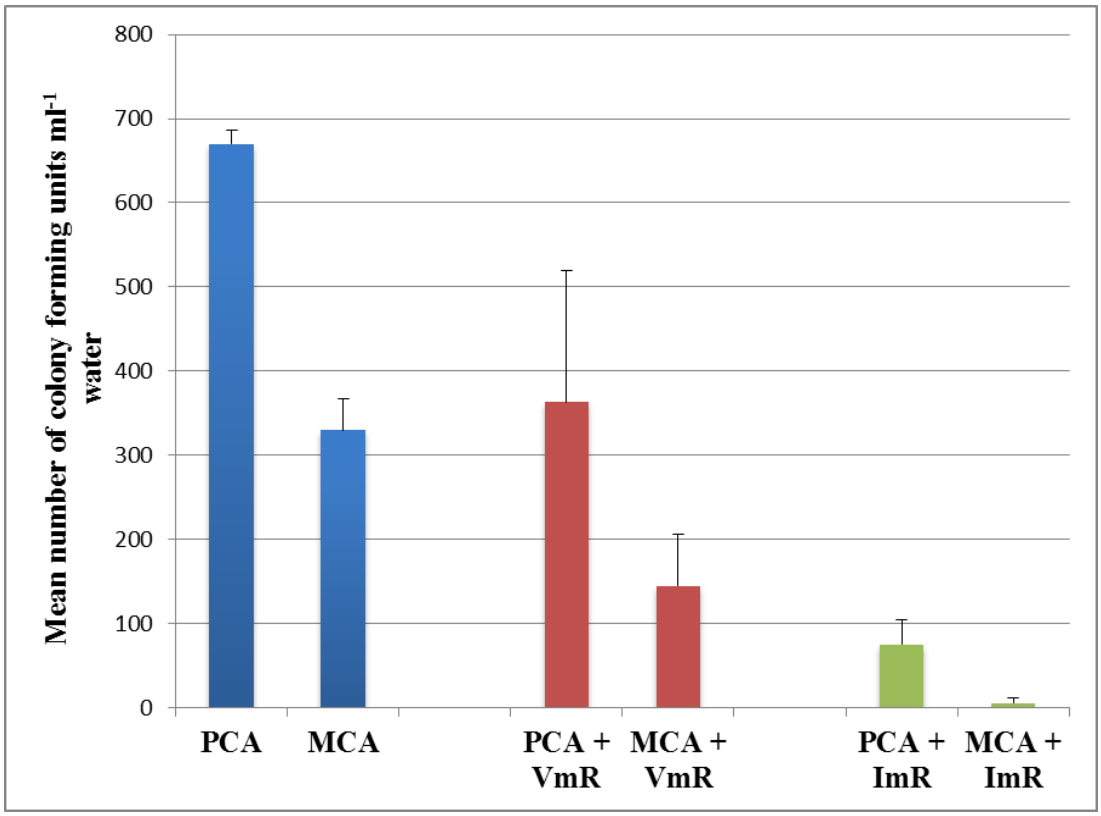


Figure 3.1: Total viable and antibiotic resistant bacteria counts (CFU ml^{-1} water) isolated from river water from Beverley Beck, East Yorkshire, U.K.

Bacteria were isolated on either MCA or PCA and on MCA or PCA supplemented with either vancomycin (Vm) or imipenem (Im). Mean values ± SD are shown (n=4).

3.2.2 Isolation and enumeration of antibiotic resistant bacteria in agricultural soils

The abundance of bacteria resistant to the antibiotics imipenem and vancomycin in agricultural soils was investigated by culture-based approaches. Total numbers of culturable bacteria (total viable counts) and of ImR and VmR bacteria were determined in soil samples taken from three fields at Riseholme (R) Farm and three fields at Lodge (L) Farm, on 26th November 2012 (Appendix A2.2). These farms differed in their agricultural practice, with manure used as fertiliser at Riseholme and conventional inorganic fertilisers used at Lodge Farm (see Table 2.6). The sample fields at the three farms were planted with different crops as follows. Riseholme: Field RA (winter wheat), Field RB (sugar beet), and Field RC (spring beans). Lodge Farm: Field LA (winter wheat), Field LB (sugar beet) and Field LC (spring beans). Soil samples were taken in triplicate from each field and the total viable counts (on PCA) and numbers of antibiotic resistant bacteria (PCA + Im or PCA + Vm) were determined following serial dilution.

3.2.2.1 Variation in total viable counts

The total viable bacterial counts (TVC) in the six fields ranged from $4.27 \times 10^6 \pm 5.82 \times 10^6$ CFU g^{-1} soil in Field RA to $1.53 \times 10^6 \pm 3.82 \times 10^5$ CFU g^{-1} soil in Field RC, with the highest counts in Field RA (Figure 3.2). No significant variation was identified using pairwise t-tests, in the total viable counts between bacterial numbers in any two individual fields ($P \geq 0.18$).

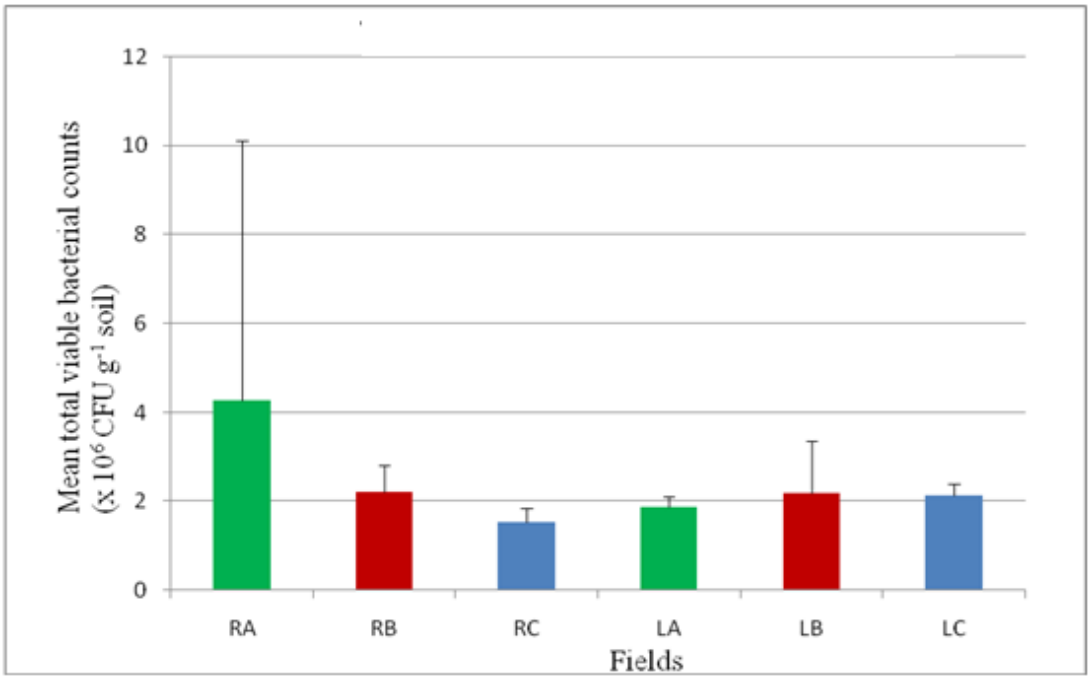


Figure 3.2: Mean total viable bacteria counts (x 10^6 CFU g^{-1} soil) isolated from six agricultural fields, Lincolnshire, U.K.

Mean values ± SD are shown (n=3). RA= Riseholme winter wheat , RB= Riseholme sugar beet, RC= Riseholme spring beans, LA= Lodge winter wheat, LB= Lodge sugar beet, and LC= Lodge spring beans fields.

Overall, the mean numbers of viable bacteria were higher in Riseholme Farm than in Lodge Farm soils (Figure 3.3), driven primarily by the higher viable counts found in field RA at Riseholme (Figure 3.2). However, overall, no significant variation in bacterial numbers was found overall between the two farms using pairwise t-tests (p=0.414).

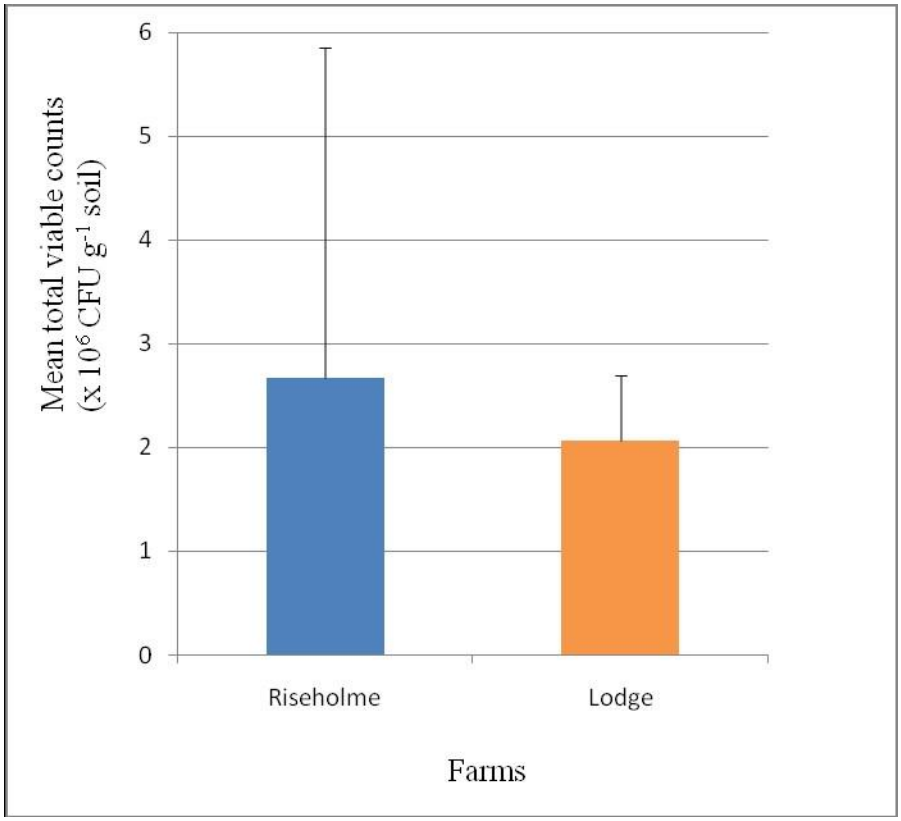


Figure 3.3: Mean total viable bacteria counts (x 10^6 CFU g^{-1} soil) in Riseholme and Lodge Farms, Lincolnshire, U.K.

Mean values ± SD are shown (n=9).

Comparing the total viable bacterial counts in soils between the three crops (Figure 3.4), bacterial counts were slightly higher in soil from the winter wheat field than in the sugar beet field. The field planted with spring bean crops showed the lowest number of isolated bacteria and no significant variation (p=0.645) was detected in bacterial counts between crops using the ANOVA test.

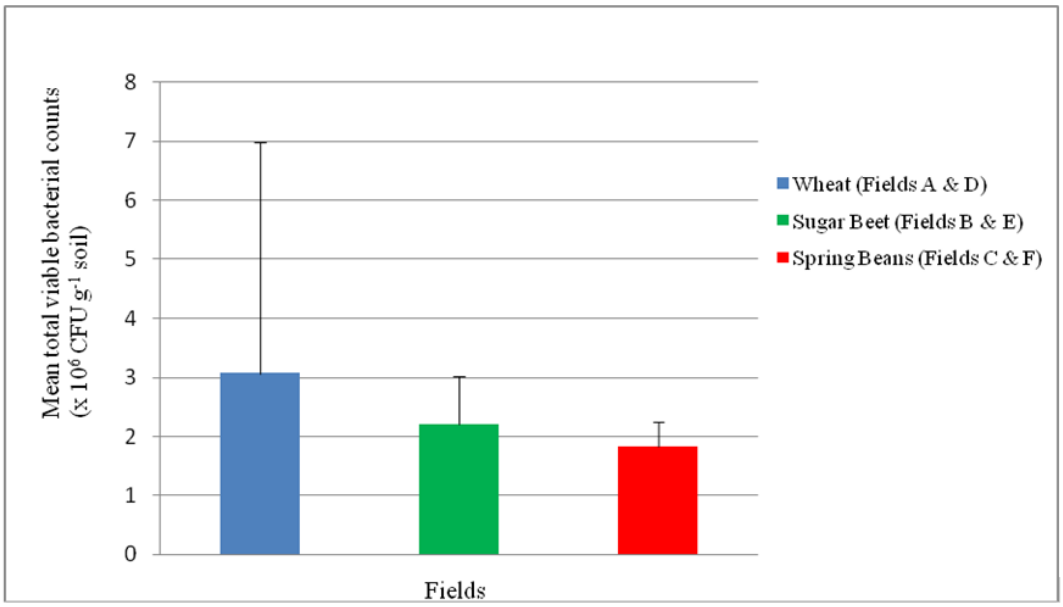


Figure 3.4: Mean total viable bacteria counts (x 10^6 CFU g^{-1} soil) isolated from soils planted with three crops in two farms in Lincolnshire, U.K.

Mean values ± SD are shown (n=6).

3.2.2.2 Variation in numbers of culturable antibiotic resistant bacteria

In all six fields, the number of VmR isolates was higher than the number of ImR isolates (Figure 3.5). For ImR isolates, the highest numbers (0.5 X 10^6 $\pm$ 0.6 x10^6 CFU g^{-1} soil) were detected in Field RA, while the lowest numbers were detected in Field LB (0.4 X 10^4 $\pm$ 0.1 x10^4 CFU g^{-1} soil). Significant variation between some individual fields was identified using the t-test (Table 3.1). The number of VmR isolates was highest in Field RA (1.0 X 10^6 $\pm$ 0.2 x10^6 CFU g^{-1} soil), which had significantly higher numbers of ImR bacteria than all of the other fields except Field LA (Table 3.1). Lowest numbers of ImR bacteria were found in Field LB (0.2 X 10^6 $\pm$ 0.1 x10^6 CFU g^{-1} soil) which had significantly lower numbers of ImR bacteria than in Fields RA, RC and LA (Table 3.1).

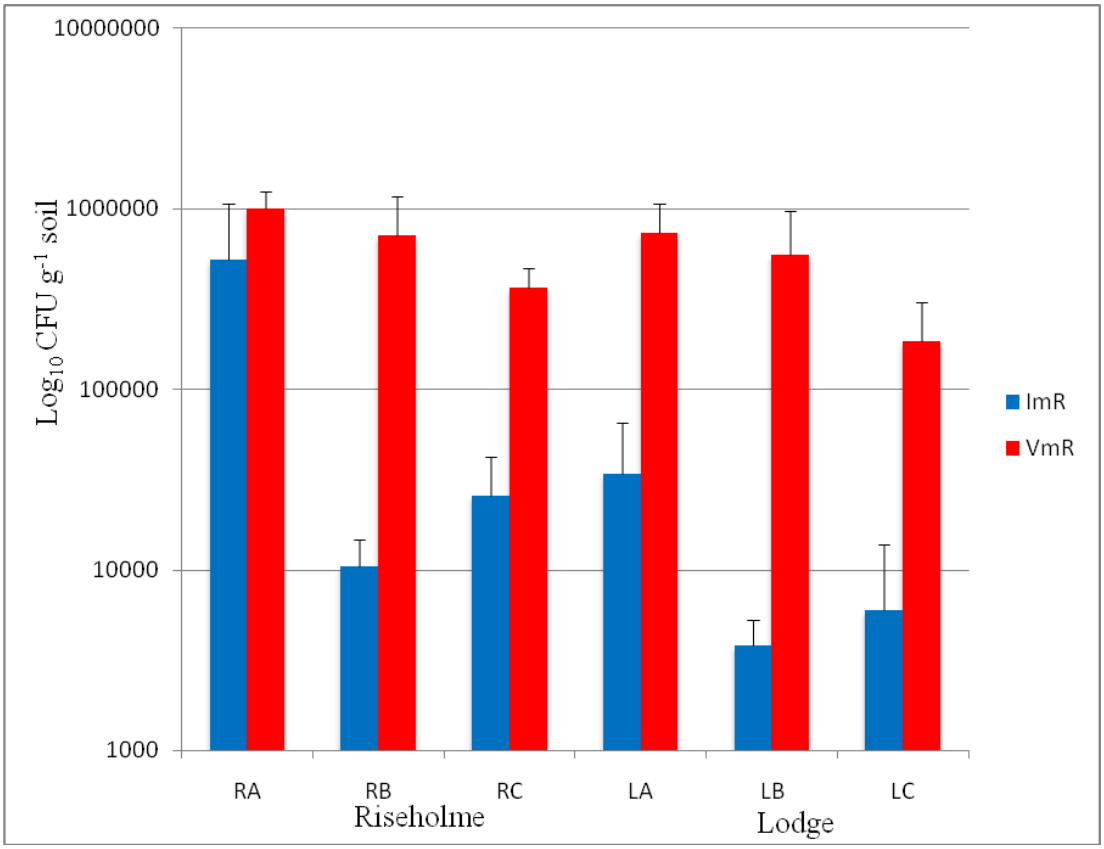


Figure 3.5: Antibiotic resistant bacterial counts (Log_{10} CFU g^{-1} soil) isolated from six agricultural fields in two farms in Lincolnshire, U.K.

Mean values ± SD are shown (n=3).
(ImR= Imipenem resistant, VmR= Vancomycin resistant, RA= Riseholme winter wheat, RB= Riseholme sugar beet, RC= Riseholme spring beans, LA= Lodge winter wheat, LB= Lodge sugar beet, and LC= Lodge spring beans fields).

Table 3.1: Pairwise comparison using the t-test showing variation between numbers of ImR bacteria between individual fields.

Pairwise Comparison (Field vs. Field)	*P* value
RA vs. RB	**0.000497**
RA vs. RC	**0.023121**
RA vs. LA	0.059880
RA vs. LB	**0.000145**
RA vs. LC	**0.000435**
RB vs. RC	**0.020563**
RB vs. LA	**0.007739**
RB vs. LB	0.226244
RB vs. LC	0.105374
RC vs. LA	0.271003
RC vs. LB	**0.006093**
RC vs. LC	0.151286
LA vs. LB	**0.002272**
LA vs. LC	0.062150
LB vs. LC	**0.033234**

Significant *P* values ≤ 0.05 are marked in bold.

Comparing the two farms (Figure 3.6) overall, the mean numbers of ImR bacterial counts were significantly higher in Riseholme than in Lodge Farm, (p= <0.0001) using the t-test. Conversely, no significant variation was seen in VmR bacterial numbers between the two farms (p= 0.452) using the t-test.

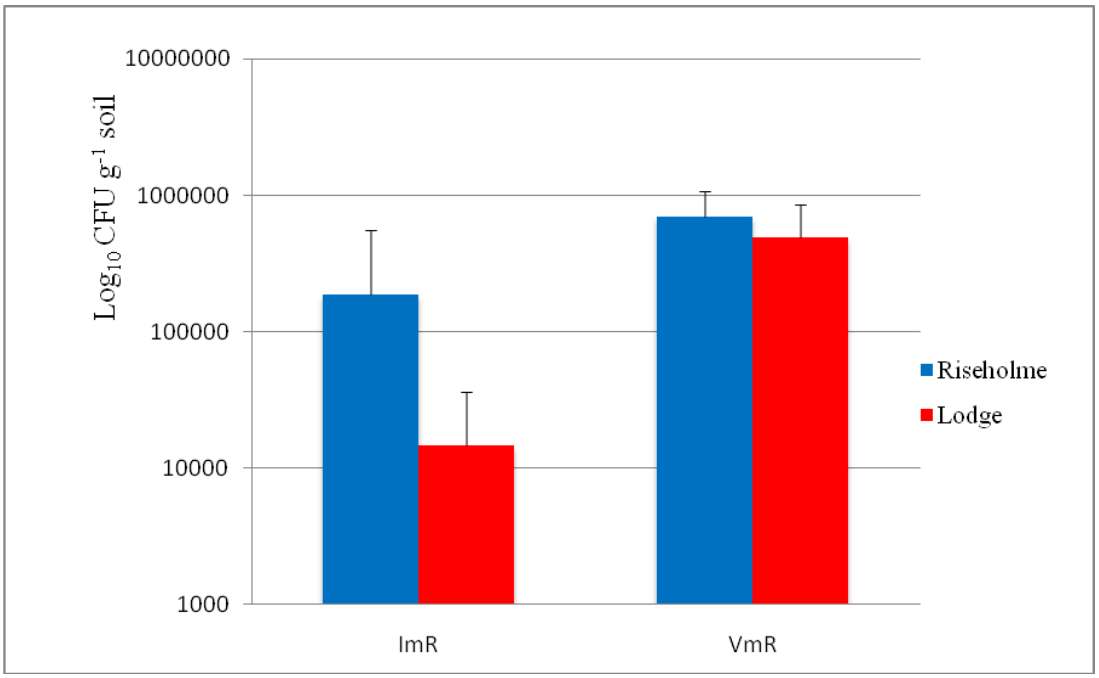


Figure 3.6: Mean antibiotic resistant bacteria count (Log_{10} CFU g^{-1} soil) isolated from two farms in Lincolnshire, U.K.

Mean values ± SD are shown (n=9).

Comparing the antibiotic-resistant bacterial counts obtained from soils from fields planted with the three crops (Figure 3.7), the highest numbers of both ImR and VmR bacteria were detected in the winter wheat field, while the lowest numbers were detected in the sugar beet and spring beans fields, respectively. The number of VmR isolates was higher than the number of ImR isolates in soil samples from all fields. No significant variation was detected in bacterial counts between crops for VmR, whereas significant variation in the numbers of ImR between individual crops was shown using the t-test (Table 3.2).

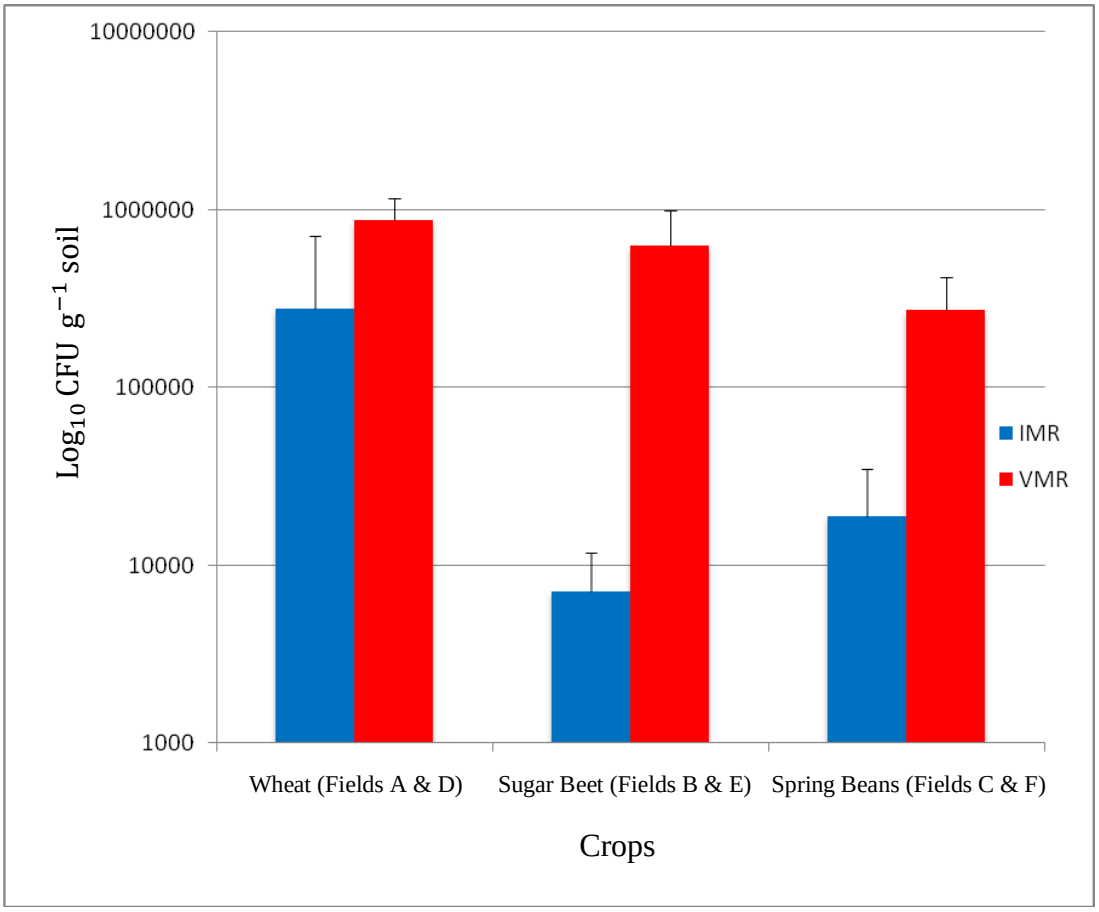


Figure 3.7: Mean antibiotic resistant bacterial counts (Log_{10} CFU g^{-1} soil) isolated from fields planted with three crops in Lincolnshire

Mean values ± SD are shown (n=6).

Table 3.2: Pairwise comparison using the t-test showing variation between numbers of ImR bacteria in fields planted with different crops.

Pairwise Comparison Crop vs. Crop	***P* value**
Winter wheat vs. Sugar beet	**0.0001**
Winter wheat vs. Spring beans	**0.000661**
Sugar beet vs. Spring beans	**0.009324**

Significant *P* value ≤ 0.05 are shown in bold

3.2.3 Antibiotic resistant bacteria from hospitals

109 bacteria isolated from different clinical samples were provided as ImR by three different hospitals (in York, Sheffield and Hull). In the current study, these isolates were screened for resistance to imipenem by determining MIC, resulting in

42 being categorised as ImR (MIC ≥ 4 mg L^{-1}) (CLSI, 2012a). 61.3% of the clinical isolates obtained from Hull were confirmed as ImR, while 35.5% and 38.7% of the isolates obtained from York and Sheffield respectively showed confirmation of ImR.

3.2.4 Phenotypic characterisation of imipenem resistant bacteria from natural environments and clinical settings

Cultured ImR isolates were chosen for further study. A total of 168 isolates (30 from water, 96 from soil and 42 from hospitals) were characterised by phenotypic methods (this chapter) and molecular methods (Chapter 4). The identification of isolates by 16S rRNA gene sequencing is reported in Chapter 4.

3.2.4.1 Determination of minimal inhibitory concentration for ImR bacteria

In order to quantify the resistance levels of isolated strains, the MIC of these isolates was determined within a range of 0.004-32 mg L^{-1} imipenem, by the quantitative microdilution method using microtiter plates following the EUCAST 2012 guidelines. Resistance to imipenem among these isolates ranged from between 4 and ≥32 mg L^{-1} (Table 3.3). MIC results for individual water, soil and clinical isolates are shown in Appendix A2 (Table A2.4, A2.5 and A2.6, respectively).

After identifying the MICs of 30 ImR water bacterial isolates, the majority (63.4%) of these isolates (3 *Caulobacter* spp., 1 *Chitinophaga* spp., 1 *Kinneretia* spp.,4 *Pedobacter* spp., 2 *Proteus* spp., 1 *Pelomonas* spp., 2 *Epilithonimonas lactis*, 1 *Chryseobacterium* spp., 2 *Sphingomonas* spp.,1 *Acidovorax* spp. and 1 *Brevundimonas lenta*) were found to have a MIC of 4 mg L^{-1}. Higher MICs of between 8 and >32 mg L^{-1} were less frequent, occurring in 12 isolates of either *Caulobacter* spp. or *Stenotrophomonas* spp. Seven isolates (*Caulobacter* spp.) showed a MIC of 16 mg L^{-1} (23.4%) of water isolates, while a MIC of 32 mg L^{-1} or greater was detected in one *Caulobacter* spp. and three *Stenotrophomonas* spp. strains (13.3%).

Table 3.3: Minimal inhibitory concentration of imipenem for ImR bacteria isolated from water

Bacterial species	No. of isolates	Number of isolates with MIC (mg L^{-1}) of				
		4	8	16	32	>32
Caulobacter segnis	1	1				
Caulobacter vibrioides	1	1				
Caulobacter spp.	9	1		7	1	
Stenotrophomonas spp.	3				2	1
Chitinophaga spp.	1	1				
Kinneretia spp.	1	1				
Pedobacter alluvionis	1	1				
Pedobacter spp.	2	2				
Pedobacter koreensis	1	1				
Proteus spp.	2	2				
Pelomonas spp.	1	1				
Epilithonimonas lactis	2	2				
Chryseobacterium spp.	1	1				
Sphingomonas spp.	2	2				
Acidovorax spp.	1	1				
Brevundimonas lenta	1	1				
Total numbers	30	19	0	7	3	1

The MICs of 96 ImR bacterial strains isolated from farm soil were then determined (Table 3.4). 42.7% of isolates (41) (6 *Xanthomonas oryzae* pv. *Oryzae*, 2 *Xanthomonas retroflexus*, 2 *Microbacterium* spp., 8 *Acetobacter pasteurianus*, 4 *Pedobacter* spp, 3 *Flavobacterium*, 3 *Chryseobacterium* spp., 1 *Sporocytophaga* spp., 1 *Epilithonimonas* lactis, 2 *Acidovorax facilis*, 8 *Pseudomonas* spp. and 1 *Duganella zoogloeoides*) had a MIC of 4 mg L^{-1}, while a MIC 8 mg L^{-1} was less frequent, found in only two (*Janthinobacterium lividum*) isolates (2.1%). MICs of 16 mg L^{-1} were detected in 15 isolates (9 *Stenotrophomonas maltophilia*, 1 *Stenotrophomonas* spp. and 5 *Janthinobacterium lividum*) isolates. MICs of 32 mg L^{-1} were detected in 29 (15 *Stenotrophomonas rhizophila*, 8 *Stenotrophomonas maltophilia*, 2 *Stenotrophomonas* spp. and 4 *Janthinobacterium lividum*), while MICs of >32 mg L^{-1} were detected in 9 (6 *Stenotrophomonas rhizophila*, 2 *Stenotrophomonas maltophilia* and 1 *Mucilaginibacter mallensis*) isolates.

Table 3.4: Minimal inhibitory concentration of imipenem for ImR bacteria isolated from soil

Bacterial species	No. of isolates	Number of isolates with MIC (mg L^{-1}) of				
		4	8	16	32	>32
Stenotrophomonas rhizophila	21				15	6
Stenotrophomonas maltophilia	19			9	8	2
Stenotrophomonas spp.	3			1	2	
Janthinobacterium lividum	11		2	5	4	
Mucilaginibacter mallensis	1					1
Xanthomonas oryzae pv. oryzae	6	6				
Xanthomonas retroflexus	2	2				
Microbacterium foliorum	1	1				
Microbacterium oxydans	1	1				
Acetobacter pasteurianus	8	8				
Pedobacter agri	1	1				
Pedobacter suwonensis	1	1				
Pedobacter terrae	1	1				
Pedobacter wanjuense	1	1				
Flavobacterium saccharophilum	1	1				
Flavobacterium johnsoniae	1	1				
Flavobacterium pectinovorum	1	1				
Chryseobacterium spp.	1	1				
Chryseobacterium soldanellicola	1	1				
Chryseobacterium hominis	1	1				
Sporocytophaga spp.	1	1				
Epilithonimonas lactis	1	1				
Acidovorax facilis	2	2				
Pseudomonas poae	1	1				
Pseudomonas veronii	1	1				
Pseudomonas geniculata	6	6				
Duganella zoogloeoides	1	1				
Total numbers	96	41	2	15	29	9

For the 42 ImR clinical bacterial isolates (Table 3.5), 68% of isolates (11 *Stenotrophomonas maltophilia*, 1 *Pseudomonas geniculate*, 4 *Escherichia* spp., 11 *Klebsiella pneumoniae* and 3 *Enterobacter* spp.) had a MIC of >32 mg L^{-1}. 3 (7.2%)

Stenotrophomonas maltophilia isolates showed MIC at 16 mg L^{-1} and only one *Stenotrophomonas* species showed MIC at 8 mg L^{-1}, while two (4.7%) *Stenotrophomonas maltophilia* and three *Pseudomonas aeruginosa* had MICs of 4 mg L^{-1}.

Table 3.5: Minimal inhibitory concentration of imipenem for ImR bacteria isolated from clinical samples

Bacterial species	No. of isolates	Number of isolates with MIC (mg L^{-1}) of				
		4	8	16	32	>32
Acinetobacter spp.	1			1		
Stenotrophomonas maltophilia	16	2		3		11
Stenotrophomonas spp.	1		1			
Pseudomonas aeruginosa	3	3				
Pseudomonas geniculata	1					1
Escherichia coli	2					2
Escherichia spp.	2					2
Klebsiella pneumoniae	11					11
Enterobacter aerogenes	4				2	2
Enterobacter cloacae	1					1
Total numbers	42	5	1	4	2	30

3.2.4.2 Characterisation of carbapenemase and β-lactamase activity of ImR bacteria

Modified Hodge tests were performed to test for the production of carbapenemases by environmental and clinical isolates (Figure 3.8). 63 out of the 126 ImR environmental isolates (for water 9/30 and for soil 54/96) and 42 out of the 109 clinical isolates tested positive for carbapenemase production.

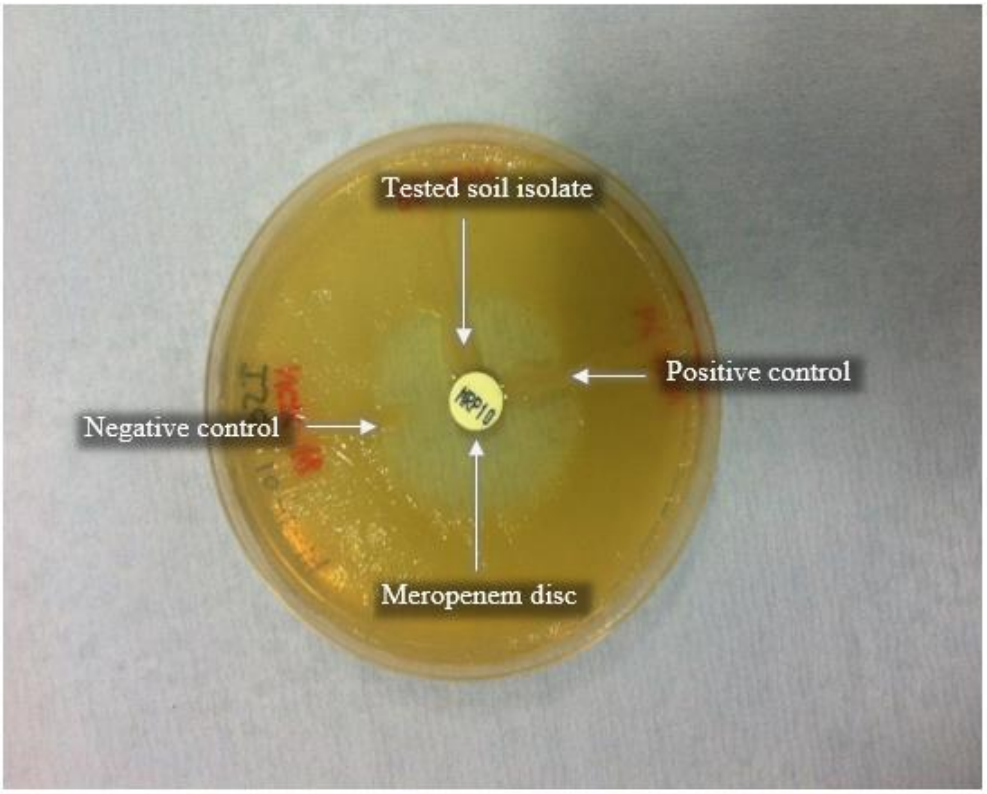


Figure 3.8: Example of a Modified Hodge test plate for a soil isolate

MHT using meropenem 10 µg (MRP10) discs on a Muller-Hinton (Iso-Sensitest) agar plate. Tested soil isolate: *Stenotrophomonas maltophilia*; positive control: *Klebsiella pneumoniae* NCTC 13439-producing metallo-β-lactamase, negative control: *Escherishia coli* NCTC 10418.

3.2.4.2.1 Combined disc synergy test (CDST)

Four classes of carbapenemase are known (A, B, C and D), with class C being very rare (Nordmann *et al*., 2012). The CDST was performed in order to determine the class of carbapenemase carried by the MHT positive isolates, possibly indicating the production of β-lactamase 'carbapenamase'. CDST results for individual water, soil and clinical isolates are shown in Appendix A2 (Table A2.4, A2.5 and A2.6, respectively. Examples of CDST plates are shown in Figure A2.1, A2.2 and A2.3). Most of these environmental isolates produced class B (MBL) in 8/9 (88.9%) of water isolates and 45/54 (83.4%) of soil samples. Class A (KPC) carbapenemases were produced by 9/54 of soil isolates (16.7%) but were not observed in the water isolates. Neither the soil nor the water isolates produced class D (OXA) by the CDST. The AmpC enzyme was detected in 3/9 of the water isolates (33.3%) and in 16/54 of the soil isolates (29.6%). It should be noted that 24 (38.1%) isolates produced more than one type of enzyme. These include *S. maltophilia* (12), *S.*

rhizophila (5), *Stenotrophomonas* spp. (2), *Caulobacter* spp. (1), *J. lividum* (4) and *M. mallensis* (1). Also, β-lactamase activity from an unknown source was detected in 1/9 of the water isolates (11.1%) and in 5/54 of soil isolates (9.3%), i.e. a positive MHT result, but the class of β-lactamase was not identified using CDST (Table 3.6).

Table 3.6: Combined disc synergy tests (β-lactamase activity tests) for MHT-positive strains isolated from water and soil

Isolate	Source	No.	Class A (KPC) No. (%)	Class B (MBL) No. (%)	Class C (AMPC) No. (%)	Class D (OXA) No. (%)	Unknown No. (%)
Stenotrophomonas maltophilia	Water	-	-	-	-	-	-
	Soil	19	-	16 (84.2%)	12 (66.7%)	-	3 (16.7%)
Stenotrophomonas rhizophila	Water	-	-	-	-	-	-
	Soil	21	5 (23.8%)	21 (100%)	-	-	-
Stenotrophomonas spp.	Water	3	-	3 (100%)	2 (66.7%)	-	-
	Soil	3	3 (100%)	-	-	-	-
Caulobacter spp.	Water	6	-	5 (83.4%)	1 (16.7%)	-	1 (14.3%)
	Soil	-	-	-	-	-	-
Janthinobacterium lividum	Water	-	-	-	-	-	-
	Soil	10	1 (10%)	7 (70%)	4 (40%)	-	2 (20%)
Mucilaginibacter mallensis	Water	-	-	-	-	-	-
	Soil	1	1 (100%)	1 (100%)	-	-	-
Total	Water	9	-	8 (88.9%)	3 (33.3%)	-	1 (11.1%)
	Soil	54	10 (18.5%)	45 (83.4%)	16 (29.6%)	-	5 (9.3%)

(No.= number of isolates)

For the 42 clinical isolates that gave positive MHT, CDST analysis (Table 3.7) showed that 17 isolates (40.5%) produced class A (KPC), while 16 isolates (38.1 %) produced class B (MBL) carbapenemases. class D (OXA) enzymes were not produced by any of the isolates. The AmpC enzyme was detected in 7 isolates of *S.*

maltophilia (16.7%). One of these isolates (2.4%) produced more than one type of enzyme (MBL and AmpC). β-lactamase activity from an unknown source was also detected in three (7.2%) isolates (Table 3.7).

Table 3.7: Combined disc synergy tests (β-lactamase activity test) for MHT-positive strains isolated from clinical settings

Isolate	No.	Class A (KPC) No. (%)	Class B (MBL) No. (%)	Class C (AMPC) No. (%)	Class D (OXA) No. (%)	Unknown No. (%)
Acinetobacter spp.	1	-	1 (100%)	-	-	-
Stenotrophomonas maltophilia	16	-	10 (62.5%)	7 (43.7%)	-	-
Stenotrophomonas spp	1	-	-	-	-	1 (100%)
Pseudomonas aeruginosa	3	-	2 (66.7%)	-	-	1 (33.4%)
Pseudomonas geniculata	1	-	-	-	-	1 (100%)
Escherichia coli	2	-	2 (100%)	-	-	-
Escherichia spp	2	2 (100%)	-	-	-	-
Klebsiella pneumoniae	11	10 (90.9%)	1 (9.1%)	-	-	-
Enterobacter aerogenes	4	4 (100%)	-	-	-	-
Enterobacter cloacae	1	1 (100%)	-	-	-	-
Total No (%)	42	17 (40.5%)	16 (38.1 %)	7 (16.7%)	0 (0%)	3 (7.2%)

(No. = number of isolates)

3.2.4.3 Multiple drug resistance (MDR) profiling of ImR bacteria isolated from natural environments and clinical settings

As the bacteria resistant to imipenem are often resistant to other available antimicrobials (Nordmann *et al*., 2011), the ImR isolates in this study were tested against a range of antimicrobials of different classes. MDR profiles for individual water, soil, clinical isolates are shown in Appendix A2 (Table A2.4, A2.5 and A2.6, respectively. An example of MDR test is shown in Figure A2.4).

From the MDR profile of water isolates (Table 3.8), although all isolates were resistant to imipenem, some isolates were susceptible to meropenem e.g. *Pedobacter* spp. *and Epilithonimonas lactis*. Many isolates were susceptible to tetracycline, and minocycline. All isolates were resistant to gentamicin and most of the isolates were resistant to ceftazidime and aztreonam. The highest percentage of MDR level were found for the *Stenotrophomonas* spp. isolates (77.8%) followed by *Caulobacter* spp. (76.9%), and the lowest % MDR was found for the *Sphingomonas* spp. isolates (54.1%).

Table 3.8: Multiple resistance profiling for MDR isolated bacteria from water

		Pedobacter spp. (4)	*Sphingomonas* spp. (2)	*Stenotrophomonas* spp. (3)	*Caulobacter* spp. (9)	*Epilithonimonas lactis* (2)
TIM	N	0	0	3	9	2
	%	(0%)	(0%)	(100%)	(100%)	(100%)
CN	N	4	2	3	9	2
	%	(100%)	(100%)	(100%)	(100%)	(100%)
CAZ	N	2	2	3	9	2
	%	(50%)	(100%)	(100%)	(100%)	(100%)
ATM	N	3	2	3	9	1
	%	(75%)	(100%)	(100%)	(100%)	(50%)
IPM	N	4	2	3	9	2
	%	(100%)	(100%)	(100%)	(100%)	(100%)
MEM	N	3	0	3	9	1
	%	(75%)	(0%)	(100%)	(100%)	(50%)
CIP	N	4	2	3	5	2
	%	(100%)	(100%)	(100%)	(55.6%)	(100%)
LEV	N	1	0	0	3	0
	%	(25%)	(0%)	(0%)	(33.3%)	(0%)
W	N	4	2	3	9	2
	%	(100%)	(100%)	(100%)	(100%)	(100%)
SXT	N	2	1	1	3	1
	%	(50%)	(50%)	(33.3%)	(33.3%)	(50%)
TE	N	0	0	3	9	0
	%	0%	0%	100%	(100%)	(0%)
MH	N	0	0	0	0	1
	%	(0%)	(0%)	(0%)	(0%)	(50%)
Total MDR	%	56.3%	54.1%	77.8%	76.9%	66.7%

(TIM= Ticarcillin-clavulanate, CN= Gentamicin, CAZ= Ceftazidime, ATM= Aztreonam, IPM= Imipenem, MEM= Meropenem, CIP= Ciprofloxacin, LEV= Levofloxacin, W= Trimethoprim, SXT= Co-trimoxazole, TE= Tetracycline, MH= Minocycline, N= number of resistant isolates). Data in the table are not for all water isolates but only for those species isolated which had at least two or more isolates for that species. Detailed results in Appendix A2.

For soil isolates (Table 3.9), the most resistant species was *Xanthomonas oryzae* pv. *oryzae* showing 72.3% resistance to all examined antimicrobials collectively, followed by *Stenotrophomonas maltophilia* (69.3%) while the species to show lowest levels of MDR to tested antimicrobials was *Acetobacter pasteurianus* (53.1%). Most isolates showed resistance to imipenem, gentamicin and trimethoprim. In spite of all isolates being resistant to imipenem some of the isolates were susceptible to meropenem. Most of the isolates were susceptible to both tetracycline and minocycline. Also it is noted that although all isolates were resistant to imipenem some of them showed susceptibility to ceftazidime.

Table 3.9: Multiple resistance profiling for MDR isolated bacteria from soil

		S. maltophilia (19)	*S. rhizophilia* (21)	*Pseudomonas geniculata* (6)	*J. lividum* (11)	*Acetobacter pasteurianus* (8)	*Xanthomonas oryzae* pv. *oryzae* (6)
TIM	N	5	9	4	10	3	6
	%	(26.3%)	(42.9%)	(66.7%)	90.9%	37.5%	(100%)
CN	N	17	20	6	11	8	6
	%	(89.5%)	(95.2%)	(100%)	(100%)	(100%)	(100%)
CAZ	N	17	20	6	11	3	6
	%	(89.5%)	(95.2%)	(100%)	(100%)	(37.5%)	(100%)
ATM	N	17	20	6	2	8	6
	%	(89.5%)	(95.2%)	(100%)	(18.2%)	(100%)	(100%)
IPM	N	19	21	6	11	8	6
	%	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
MEM	N	17	8	3	5	2	6
	%	(89.5%)	(38.1%)	(50%)	(45.5%)	(25%)	(100%)
CIP	N	19	17	6	2	8	6
	%	100%	(81.0%)	(100%)	(18.2%)	(100%)	(100%)
LEV	N	4	6	0	8	0	0
	%	(21.1%)	(28.6%)	(0%)	(72.7%)	(0%)	0%
W	N	19	21	6	11	8	6
	%	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
SXT	N	5	8	3	3	3	4
	%	(26.3%)	(38.1%)	(50%)	27.3%)	(37.5%)	(66.7%)
TE	N	19	15	0	3	0	0
	%	100%	71.4%	(0%)	(27.3%)	(0%)	0%
MH	N	0	7	0	0	0	0
	%	0%	33.3%	0%	0%	0%	0%
Total MDR	%	69.3%	68.3%	63.9%	58.3%	53.1%	72.3%

(S.= *Stenotrophomona*, *J.*= *Janthinobacterium*, TIM= Ticarcillin-clavulanate, CN= Gentamicin, CAZ= Ceftazidime, ATM= Aztreonam, IPM= Imipenem, MEM= Meropenem, CIP= Ciprofloxacin, LEV= Levofloxacin, W= Trimethoprim, SXT= Co-trimoxazole, TE= Tetracycline, MH= Minocycline). Data in the table are not for all soil isolates but only for those species isolated which had at least two or more isolates for that species. Detailed results in Appendix A2.

For the clinical isolates (Table 3.10), most of the Enterobacterial isolates, including *Escherichia coli*, *Escherichia* spp., *Enterobacter aerogenes, Enterobacter cloacae and Klebsiella pneumonia*, showed the highest levels of MDR. The highest levels of MDR was found for *Enterobacter aerogenes* (77.1%) followed by *Klebsiella pneumoniae* (74.2%). The lowest levels of MDR for the 12 tested antimicrobials were detected in *Stenotrotrophomonas maltophilia* species (47.9%) followed by *Pseudomonas aeruginosa* (55.5%). The Enterobacteria from clinical isolates showed a higher MDR pattern than the non-fermentative group of Gram-negative bacilli.

Table 3.10: Multiple resistance profiling for MDR isolated bacteria from clinical settings

		Stenotrotrophomonas maltophilia (16)	*Pseudomonas aeruginosa* (3)	*Escherichia coli* (2)	*Escherichia* spp. (2)	*Klebsiella pneumoniae* (11)	*Enterobacter aerogenes* (4)
TIM	N	4	1	1	2	7	2
	%	(25%)	(33.3%)	(50%)	(100%)	(63.6%)	(50%)
CN	N	4	2	1	1	6	2
	%	(25%)	(66.7%)	(50%)	(50%)	(54.6%)	(100%)
CAZ	N	5	1	1	1	7	3
	%	(31.3%)	(33.3%)	(50%)	(50%)	(63.6%)	(75%)
ATM	N	5	1	1	1	7	2
	%	(31.3%)	(33.3%)	(50%)	(50%)	(63.6%)	(100%)
IPM	N	16	3	2	2	11	4
	%	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
MEM	N	16	3	2	2	11	4
	%	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
CIP	N	6	1	1	1	9	3
	%	(37.5%)	(33.3%)	(50%)	(50%)	(81.8%)	(75%)
LEV	N	5	1	1	1	8	3
	%	(31.3%)	(33.3%)	(50%)	(50%)	(72.7%)	(75%)
W	N	16	3	2	2	11	4
	%	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
SXT	N	5	1	1	1	7	2
	%	(31.3%)	(33.3%)	(50%)	(50%)	(63.6%)	(50%)
TE	N	6	2	1	1	8	2
	%	(37.5%)	(66.7%)	50%	(50%)	(72.7%)	(50%)
MH	N	4	1	1	1	6	2
	%	(25%)	(33.3%)	(50%)	(50%)	(54.6%)	(50%)
Total MDR	%	47.9%	55.5%	62.5%	66.7%	74.2%	77.1%

TIM= Ticarcillin-clavulanate, CN= Gentamicin, CAZ= Ceftazidime, ATM= Aztreonam, IPM= Imipenem, MEM= Meropenem, CIP= Ciprofloxacin, LEV= Levofloxacin, W= Trimethoprim, SXT= Co-trimoxazole, TE= Tetracycline, MH= Minocycline). NB: N: number of resistant isolates.

3.3 Discussion

River water is affected by the physical, chemical and biological activity caused by the human population living nearby. As a consequence of this activity, rivers are vulnerable to contamination by different pollutants (Wellington *et al*., 2013). The

soil environment is also likely to be affected by similar activity and can also be contaminated, for example, by animal manures, by antibiotics used in agriculture and animal feeding and also by naturally-occurring antibiotic-secreting bacteria (Dantas and Sommer, 2014). All these factors may lead to the generation, selection and spread of antimicrobial-resistant bacteria, which presents a significant threat to our society today.

Bacterial resistance to antimicrobial agents covers almost all classes of antimicrobials available on the market, and is leading the world towards a return to the conditions of the pre-antimicrobial era (D'Costa *et al*., 2006). Trying to understand the sources and mechanisms of the spread of such resistance may help to confine or at least delay this development. The rise of the natural environment, including soil and water, as a reservoir of bacterial resistance, and the spread of such resistance to clinical isolates of bacteria can occur in various ways, including via the food chain (Wellington *et al*., 2013). Thus, in the current study, investigation of bacterial isolates from different environmental samples was carried out to estimate their resistance to imipenem and vancomycin. These two drugs are normally reserved as a last resort in the treatments of infections caused by otherwise resistant bacteria. At the time of writing there are few available studies comparing resistance to these antimicrobials, in particular, comparing imipenem resistance in environmental samples.

In the first phase of this research, water samples were taken from Beverley Beck in East Yorkshire. This short canal receives biologically-treated effluent from the nearby sewage works. Antibiotic resistant (AR) bacteria in water can originate from hospital waste, aquaculture e.g. fish farms, and municipal effluents. There are also naturally produced antibiotics from environmental bacteria such as *Actinomycetes*, leading to the evolution of AR by other bacteria. The presence of such AR bacteria in a natural water environment such as a river represents a source of threat to human health, as AR bacteria can infect people in direct contact with the water. For example, the consumption of water during bathing or the consumption of fish containing AR bacteria can lead to infection. Also, HGT (horizontal gene transfer) can play a role in transmitting these AR genes to other bacterial isolates (Lu *et al*., 2010).

In the river water samples used in the current study, resistance to vancomycin was higher than resistance to imipenem, on both the MCA and PCA plates. The phenotypic resistances to vancomycin were found to be 43.9% on MCA and 54.1% on PCA; those for imipenem were found to be 1.5% on MCA and 11.2% on PCA. This is most likely to be attributable to the predominance of Gram-negative bacteria in the current study, which lack the target for this antibiotic. A study by Kuhn *et al*. (2005), carried out in a number of European countries including Sweden, Spain and the United Kingdom, observed VmR. However, this was attributed to the prevalence of VRE in raw and treated sewage water, which constituted 8.3% of all detected isolates. Similar to our findings for imipenem resistance in samples taken from the River Hull, another study carried out in the USA by Ash *et al.* (2002) detected that 2.19% of the water isolates from rivers from different states were also resistant to imipenem. These isolates belonged to the genera *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Pseudomonas*, and *Serratia*.

In this current study, the growth of vancomycin-resistant isolates on PCA (54.1%) was higher than that on MCA (45.9%), and the growth of imipenem-resistant isolates on PCA (11.2%) was higher than that observed on MCA media (1.5%) due to the permissive effects of PCA, allowing growth of many types of bacteria from water, as this is the standard medium for this purpose (Reasoner and Gerldeich, 1985), while MCA media permits growth of enteric Gram-negative isolates. This supports the preference of PCA in studying and isolating organisms from water and other environmental specimens (Wehr and Frank, 2004; Eaton *et al*., 2005).

Results showing the presence of vancomycin-resistant bacteria in river water differ according to locality. For example, Rathnayake *et al.* (2011) similarly did not detect any vancomycin resistance in bacterial strains isolated from the Coomera River in South East Queensland, Australia. However, they reported resistance to other antimicrobials, e.g. gentamicin and ciprofloxacin, in the same isolates. Also, Blaak and colleagues (2011) did not observed VmR in their study using samples from three rivers: the Meuse (at Eijsden), the Rhine (at Lobith), and the New Meuse (at Brienenoord), in the Netherlands. In contrast, Novais *et al*. (2005) reported that 33% (6/18) of their isolates from Portuguese rivers were were VmR. In another study

carried out in Germany, Schwartz *et al*. (2003) detected the presence of vancomycin-resistant heterotrophic bacteria in 20% of drinking water.

Imipenem resistance has been found to vary between studies according to the type of media used and the species of bacteria under study. For example, in a study done in Portugal by Vaz-Moreira *et al.* (2011), the highest percentage of imipenem resistant isolates from drinking water samples (17.7%) was found on Tergitol-7-agar (TTC) and the lowest percentage of isolates (2.9%) was found on R2A media, which is recommended by some researchers to be used for water samples (Eaton *et al*., 2005; Vaz-Moreira *et al*., 2011). The imipenem resistant isolates were confined to 25.9% of *Sphingomonas* species isolates while *Sphingobium*, *Novosphingobium*, *Sphingopyxis*, and *Blastomonas* isolates were not resistant to imipenem (Vaz-Moreira *et al*., 2011).

In the current study, the proportion of imipenem resistance was lower than that of vancomycin resistance in the water environment (Figure 3.1). In a similar study by Ash and colleagues (2002) conducted in waters from different sites in USA rivers, they detected that the mean percentage of imipenem resistant bacteria (1.6%) in these rivers was lowest when compared to percentages of bacteria resistant to other antimicrobials including cephalothin (72.3%), cefotaxime (10%), ceftazidime (1.9%) and amoxicillin + clavalanic acid (67%), which were included in their study.

In the second phase of this study, soil samples were collected from the agricultural farms of Riseholme Farm, where cattle manure was used in fertilisation, and Lodge Farm, where inorganic fertilisers (ammonium nitrate and triple super phosphate) were used. Higher viable bacterial counts were found in soils from fields, where animal manure was used to fertilise the soil (Riseholme), than in fields where inorganic fertilisers were used (Lodge) (Figure 3.3). Fields from the two farms not only showed a difference in total viable bacterial counts but also in both vancomycin and imipenem resistance counts, which were collectively higher at Riseholme than at Lodge Farm (Figure 3.6).

The effect of manure on antibiotic resistance in soil bacteria has previously been reported by some researchers. For example, a recent study from Switzerland (Udikovic-Kolic *et al*., 2014) showed that there were greater numbers of resistant bacteria and β-lactamase-producing bacteria in all ten samples of soils mixed with

organic manure (obtained from dairy cows which had not received antibiotics) than in soil that had been treated with inorganic fertilisers (containing nitrogen, phosphorus, and potassium (NPK)). Also, this manure caused an increase in the proliferation of certain species of resistant soil bacteria, including *Pseudomonas* spp., *Janthinobacterium* spp. and *Psychrobacter pulmonis* (Udikovic-Kolic *et al.*, 2014).

Most studies of animal manure have been based on pig manure; however, cattle-based manure (which was applied in fields analysed in the current study) has been implicated not only in the transmission of known antimicrobial-resistant genes but also in the emergence of novel genes encoding resistance to antibiotics, including β-lactams (Wichman *et al*., 2014). Thus the effects of the animal manure used on Riseholme Farm may be a factor in the increased proportions of antimicrobial resistance in the soil there.

Similar to the findings in the water environment, the soil samples in the current study showed higher percentages of vancomycin-resistant isolates compared to imipenem-resistant isolates. The soil environment is an important reservoir of antimicrobial-resistant genes, and due to the close contact between human beings and soil via the food chain, these genes can be easily transmitted to humans (Gaze *et al*., 2008). One hypothesis is that the genes encoding for vancomycin resistance in human beings, including that for aminoglycoside-modifying kinases, originated from antibiotic-secreting organisms living in soil (Marshall *et al*., 1998; D'Costa *et al*., 2006). Some studies have reported that the occurrence of vancomycin-resistant soil bacteria is due to the presence of antibiotics in soil (Hong *et al*., 2002) and that this can also occur in the absence of vancomycin use (Guardabassi and Dalsgaard 2004). However, in this current study it is expected that the high proportions of vancomycin resistant culturable bacteria are likely to be attributable to the predominance of Gram-negative bacteria, which lack the target for this antibiotic.

Another study of imipenem resistance was conducted as part of a screening study of resistant bacteria in soil samples in Lisbon, Portugal. It was found that resistance to imipenem was species-dependent, as all species of isolated *Acinetobacter* were susceptible to both imipenem and meropenem, while the majority of *Stenotrophomonas maltophilia* isolates were resistant to imipenem, but only some of these isolates were susceptible to meropenem (De Carvalho., 2011).

Among the clinical isolates examined for imipenem resistance in the current study, the highest proportion of ImR isolates was detected among isolates obtained from Hull hospital (61.3% of isolates), while imipenem resistance was observed in 38.7% and 35.5% of isolates obtained from Sheffield and York hospitals, respectively. This difference in the detection of resistant clinical isolates was found to be related to the MDR activity of the isolates. For example, McCormick *et al.* (2003) indicated that multi-drug resistant bacteria were more widespread than singly antimicrobial resistant isolates.

The problem of the variable imipenem resistance pattern is actually a warning sign, especially in clinical settings, where imipenem has been reserved for the treatment of multidrug-resistant organisms (Woodford *et al*., 2014).

The MIC values for imipenem vary between studies depending on the different species of organisms under study. However, similar to the findings in the current study, most studies have shown that clinical isolates have more antibiotic resistances and higher MICs than environmental isolates. For example, *Pseudomonas aeruginosa* isolated from a patient in India was found to be more resistant to antibiotics than for corresponding environmental isolates (Muthu *et al*., 2006). In another study of susceptibility of *Aeromonas* spp. from clinical, animal, and environmental origins, the isolates of human origin, although susceptible to imipenem (Benassi *et al*., 2001) and meropenem (Aravena-Roman *et al*., 2012), showed higher MICs to other antimicrobials than the isolates from animal and environmental sources. Also, clonally-related *Acinetobacter baumannii* isolated from an intensive care unit in Turkey showed variable resistance patterns and MICs to imipenem (4-32 mgL^{-1}) for reasons which are not yet understood (Bogaerts *et al*., 2006; Ozen *et al*., 2009).

In a similar study in Ireland, 13 isolates out of 23 (56.5%) of KPC-2 producing *Klebsiella pneumoniae* had MICs of $32\geq$ mgL^{-1}. These strains were isolated from outbreak cases of hospital-acquired infection in two hospitals. All isolates were also resistant to meropenem (Morris *et al*., 2012), indicating the higher resistance pattern of such clinical isolates. Typically, the MICs of enterobacteria to imipenem have been expected to be variable, giving difficult results in its laboratory detection in terms of changing the susceptibility limits and criteria to carbapenems defined by CLSI or EUCAST (Nucleo *et al*., 2013). However, the presence of these higher

MICs for imipenem in clinical isolates, despite facilitating the detection of the resistant isolates (based upon these higher MICs), might help in the spread of resistant isolates due to their resistance to different antimicrobials (Nucleo *et al*., 2013).

The most common β-lactamases detected in the current study among environmental isolates were class B (MBLs) (84.2%) (Table 3.6). The β-lactamase enzymes present in the environmental isolates can be characterised by the acquired resistance mechanisms contained within these isolates (Avison *et al*., 2001). The importance of such enzymes in the environmental isolates lies in the possibility of their transfer to clinical isolates, resulting in the greater spread of resistant β-lactamase encoding genes. Similarly, Rossolini *et al.* (2001) also reported widespread distribution of MBLs in environmental bacterial isolates.

Class A (KPCs) (40.5%) (Table 3.7) were the most common β-lactamases observed in the clinical isolates in this study. This finding raises concerns about the spread of these mechanisms of antimicrobial resistance in clinical settings. Europe is considered as a green zone regarding the spread of carbapenem-resistant bacteria due to the low reporting percentages of such resistant isolates (EDCP, 2013 (website); Woodford *et al*., 2014); however, reports of carbapenem resistance are rising. Although the origin of carbapenemases in clinical isolates is not well-defined, all genes encoding these enzymes were acquired from environmental bacteria (Tofteland *et al*., 2013; Woodford *et al*., 2014); in particular, two types of enzymes, MBLs and KPCs, represent two members of the "big five" (KPC, OXA-48, IMP, NDM, and VIM) carbapenemases (Woodford, 2012 (website)), which are of great clinical significance in the spread of cabapenem resistance.

While identifying β-lactamase activity in the current study (Table 3.6 and Table 3.7) it was noted that some isolates (28.5%) produced more than one class of β-lactamase. This finding was also observed in a study done on *Klebsiella pneumoniae* isolates in rehabilitation hospitals in Italy. It was found that some isolates produced both the MBL and CTX-M types of β-lactamases (Nucleo *et al*., 2013). The production of different types and classes of β-lactamase by a single clinical bacterial isolate makes it more resistant to antimicrobial classes (pan-drug), producing offspring of resistant bacteria with much more detrimental effects upon morbidity and mortality of human beings (Nucleo *et al.,* 2013).

As some of the isolates in the current study produced more than one type of β-lactamase, it was worthwhile to study the multiple resistance profiles of the isolates to other antimicrobial classes. The results of MDR profiles (Table 3.8 and Table 3.9) showed that most of the isolates were resistant to more than one class of antimicrobials. The first observation is the presence of more multi-drug resistance patterns to many classes of antimicrobials among enterobacteria (*Escherichia, Klebsiella* and *Enterobacter* species) than among non-enterobacteria (*Pseudomonas, Acinetobacter*, and *Stenotrophomonas* species) in the clinical isolates. In fact, the observation of greater multiple resistance patterns to many classes of antimicrobials among enterobacteria compared to non-enterobacteria in the clinical isolates is a very important finding of the current study. This is because non-enterobacteria are considered as the most resistant microorganisms and include species of *Acinetobacter*, *Pseudomonas* and *Stenotrophomonas*, which have more than one type of resistance to many classes of antimicrobial (Chawla *et al*., 2013, Fu and Zheng, 2013). Being more resistant, having more than one type of β-lactamase as previously discussed, and being more prevalent in clinical infections, the enterobacteria in the current study should raise awareness regarding the spread of resistance genes in clinical settings.

The second observation is the detection of high frequencies of multi-drug resistant patterns among the environmental isolates which may be attributable to interensic resistance. It has been claimed that environmental bacteria are the most important source of AR (O'Toole, 2014).

Similar to these findings, another study recorded that in testing a number of environmental strains against various antimicrobials, all soil *Streptomyces* spp. strains showed resistance to seven or eight antimicrobials, i.e. all strains were multi-drug resistant, and five strains were resistant to vancomycin and to some newly introduced antimicrobials e.g. daptomycin (D'Costa *et al*., 2006).

The spread of resistant clinical isolates (nosocomial) is attributed to increased colonisation of these resistant isolates and their spread via the hands of healthcare providers when infection control is lacking (Corbellini *et al*., 2014). Multiple drug resistant *Klebsiella pneumoniae* was isolated from various clinical samples in Italy where it was found to be resistant to β-lactams (100% of isolates), amikacin (97.5%), and fosfomycin (77.5%). These points have led to the need for improved infection

control procedures to control the spread of such resistant bacteria (Corbellini *et al*., 2014). In another study investigating causative agents of urinary tract infections (UTIs) in different patients with UTIs in Chicago, USA, it was found that enterobacteria caused 87.6% of infections, while non-enterobacteria were incriminated in only 12.4% of the total cases of UTIs. Out of these enterobacteria, 19% of them were MDR enterobacteria (Khawcharoenporn *et al.,* 2014). This again demonstrates the role of MDR bacteria amongst the enterobacteria causing clinical infections.

3.4 Conclusion

A number of conclusions can be drawn from this study. Firstly, PCA is superior to MCA in isolating antibiotic-resistant bacteria from water and soil samples but MCA can be used selectively to isolate enteric bacteria (Enterobacteria). The presence of culturable antimicrobial-resistant bacteria in the current study indicates the importance of river water and farm soil environments as a reservoir and potential site for the transport of antibiotic-resistant bacteria. The manured soil contained more resistant organisms than the non-manured soil, which indicates the potential effect of manure in increasing antibiotic-resistance bacteria and their genes. The problem of the variable imipenem resistance pattern is actually a warning sign, especially in clinical settings, where imipenem has been reserved for the treatment of multidrug-resistant organisms. In addition, the importance of β-lactamases detected in the environmental isolates lies in the possibility of their transfer to clinical isolates, resulting in the greater spread of resistant β-lactamase encoding genes. Non-enteric bacteria are considered to be more resistant than Enterobacteria. However, the observation of greater multiple resistance patterns to many classes of antimicrobials among Enterobacteria compared to non- enteric bacteria in the clinical isolates is a very important finding of the current study.

Chapter 4: Molecular Characterisation of Imipenem-Resistant Bacteria from Natural Environments and Clinical Settings

4.1 Introduction

Carbapenems are members of a group of β-lactam antimicrobials that are usually reserved for the treatment of multiple antibiotic-resistant bacteria such as *Acintobacter* and *Pseudomonas* species. However, some strains of these bacterial genera have now developed resistance to carbapenems. The mechanisms of resistance are variable and include the production of carbapenemase enzymes (the most important mechanism), loss of porins and the high expression and production of efflux pumps (Poirel and Nordmann, 2006).

The development of carbapenemase enzymes and their spread among clinical bacterial isolates has detrimental consequences in the treatment of infections. Carbapenemase enzymes confer resistance not only to all β-lactams but also to other groups of antimicrobials, especially in the case of New Delhi metallo-β-lactamase (NDM) in *Enterobacteriaceae* (Livermore, 2009). This pattern of resistance has led to the emergence of pan-drug resistant *Enterobacteria* and *Acinetobacter* species which are untreatable and associated with high mortality rates (Saleem *et al*., 2010; Laxminarayan *et al*., 2013). Also, resistance to commonly used antimicrobials, including cefixime and ciprofloxacin, has been found in 50-60% of isolates of community-acquired Gram-negative *Escherichia coli* in urinary tract infections (Khan *et al*., 2010). Enterobacteriaceae resistance to carbapenems in the USA have increased from 0% in 2001 to 1.4% in 2011 (CDC, 2013). Bloodstream infections in India with carbapenemase-producing *Klebsiella pneumoniae* (KPC) increased from 2.4% in 2002 to 52% in 2009 (Datta *et al*., 2012).

The natural environment acts as a reservoir of carbapenem-resistant bacteria and also plays a part in their spread to clinical environments. Carbapenem-resistant *Pseudomonas fluorescens* and *Serratia fonticola*, for example, have been detected in water samples taken from the Seine river in Paris. These bacteria were found to produce BIC, SFC-1, and KPC-2 carbapenemases (Girlich *et al*, 2010b). Several studies have also detected carbapenemase genes in soil samples, using molecular

techniques in the identification and characterization of the bacteria. The most widely used technique for the detection of resistant genes is PCR, which requires prior sequence data to design primers to target the gene of interest. However, AR genes encoding resistance to particular antibiotics are typically diverse, making it difficult in some cases to design primers that target homologous genes from different species (Riesenfeld *et al*., 2004).

nmcA was the first carbapenemase gene detected in enterobacteria in 1993 (Naas and Nordmann, 1994), and this was later followed by the detection of many other genes, leading to their classification into four groups (A, B, C, and D) by Ambler (Queenan and Bush, 2007). Carbapenemases are distinguished by their different locations on the bacterial chromosome or plasmid, their different types of actions, and the different substances which inhibit their activities (Nordmann, 2011). See also Section 1.4 in Chapter 1 for details of antimicrobial resistance. For example, class C carbapenemases are chromosomally-determined and have uncharacterised clinical actions, while class A carbapenemases are inhibited by clavulanic acid and are more widespread in bacteria. These are either plasmid-mediated (KPC, GES, IMP-2) or chromosomally-mediated (NmcA, Sme, IMI-1, SFC-1), of which KPC has been found to be the most common (Queenan and Bush, 2007).

Class B carbapenemases are metallo-β-lactamases (MBLs) which are inhibited by EDTA and include the New Delhi metallo-β-lactamases (NDM), which have been recently detected, and the Verona integron–encoded metallo-β-lactamase (VIM). The first class B carbapenemase to be detected was IMP-1, which was found in Japan in 1991 (Queenan and Bush, 2007). Class B carbapenemases are widely prevalent except in America (Nordmann *et al*., 2011).

Class D carbapenemases are not inhibited either by clavulanate or EDTA; they are of the oxacillin-hydrolysing enzymes (OXA) type and include many different genes. The first detection of OXA-48 was in a *Klebsiella pneumoniae* isolate identified in Turkey in 2003. (Nordmann *et al*., 2011); other types include OXA-23, OXA -40, and OXA -58, which show weak carbapenemase activity in *Acinetobacter baumannii* (Girlich *et al*., 2010a).

There are two carbapenemase enzymes produced by *Stenotrophomonas maltophilia*, termed L1 and L2. L1 is related to class B enzymes, while L2 is related

to class A, and both types are either-chromosomally or plasmid-encoded (Saino *et al*., 1982; Saino *et al*., 1984, Avison *et al*., 2001).

Sequencing of the 16S rRNA gene can be used in the identification of different bacterial species (Girlich *et al*., 2010a and 2010b) and also to identify culturable and non-culturable soil bacteria (Riesenfeld *et al*., 2004). Species of *Stenotrophomonas maltophilia* can be identified and also divided into strains using sequencing of 16S rRNA genes (Furushita *et al*., 2005).

Genotypic methods are the most accurate, rapid and reliable. One of the most common genotypic methods used nowadays is based upon the sequencing of 16S rRNA obtained from PCR, followed by sequencing techniques for environmental bacterial isolates (Block and Ouellette, 2012) and also for clinical isolates (Jenkins *et al*., 2012).

The aims of this study were firstly to identify isolates of imipenem-resistant (ImR) bacteria from water and soil environments and from clinical settings, and secondly to use a suite of PCR primers targeting β-lactamases to detect and allow characterisation of imipenem (carbapenem) resistance genes in these isolates.

4.2 Results

4.2.1 PCR amplification and sequencing of DNA for 16S rRNA gene from environmental and clinical isolates

DNA was extracted and PCR amplifications were performed using primers 63F (Marchesi *et al*., 1998) and 1389R (Osborn *et al*., 2000) targeting the 16S rRNA gene. 16S rRNA genes were amplified from the 30 water, 96 soil, and 42 clinical ImR isolates (Figure 4.1). Sequence analysis of these products showed that these PCR products were specific and related to the 16S rRNA gene by BLASTN comparison to the GenBank non-redundant database. These resistant isolates from water, soil and clinical samples were classified into 17, 27 and 10 different species, respectively as detailed below.

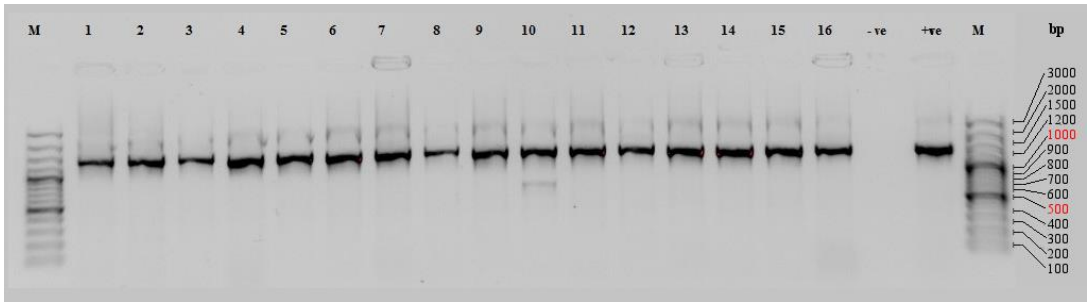


Figure 4.1 Agarose gel electrophoresis of PCR-amplified 16S rRNA genes in different ImR bacterial isolates

Lanes 1-16 show positive products (1300 bp) of the 16S rRNA gene for isolates (BBS1:15, BBS2:7, BBS4:5, BBS3, BBS1:7, BBS4:6, BBS4:2, BBS2:2, BBS4:7, BBS1, BBS4:4, BBS3:3, BBS2:12, BBS4:1, BBS4:15 and BBS1:14). M: molecular marker (Fermentas size as indicated); -ve: negative control (sterile water); +ve: positive control; DNA sizes are as indicated. Similar results were obtained from all other isolates.

4.2.2 16S rRNA gene sequence identification of ImR bacteria isolated from river water

The sequencing of the 16S rRNA gene in ImR strains isolated from river water identified 30 ImR bacteria (Table 4.1). This revealed the presence of 17 different species of bacterial isolates representing 12 different genera of bacteria. The most abundant genus in the current study was *Caulobacter*, where 11 isolates (36.7%) were identified relating to three different species of the genus. The second most common genera was *Pedobacter* (13.3%) for which three species were identified. Other genera and species which were less frequently detected were three isolates (10%) of *Stenotrophomonas* spp. and two isolates (6.7%) of each of *Proteus* spp., *Epilithonimonas lactis* and *Sphingomonas* spp. strains. Other isolates (one of each, 3.3%) were also isolated from different sites of the river as indicated in Table 4.1.

Table 4.1: 16S rRNA gene identification of ImR bacteria isolated from river water

Genus and species identification	Phylum (and class)	Number of isolates	Identity %	Strain name	Accession number of closest related sequence
Caulobacter segnis	Proteobacteria (Alphaproteobacteria)	1	99%	BBS1:15	AB680820.1
Caulobacter vibrioides		1	99%	BBS2:7	JQ361159.1
Caulobacter spp.		9	99%	BBS4:5 BBS2:5 BBS2:6 BBS1:21 BBS1:5 BBS4:18 BBS4:11 BBS3:20 BBS2:8	AJ227760.1 AJ227770.1 AB470462.1 AJ227773.1 AJ227760.1 AJ227770.1 AJ227766.1 AJ227760.1 DQ337547.1
Stenotrophomonas spp.	Proteobacteria (Gammaproteobacteria)	3	99%	BBS3 BBS4:12 BBS1:13	FJ668834.1 FJ668834.1 FJ668834.1
Chitinophaga spp.	Bacteroidetes (Sphingobacteriia)	1	97%	BBS1:7	JF710262.1
Kinneretia spp.	Proteobacteria (Betaproteobacteria)	1	99%	BBS4:6	JF958155.1
Pedobacter alluvionis	Bacteroidetes (Sphingobacteriia)	1	99%	BBS4:2	NR_044382.1
Pedobacter spp.		2	98%	BBS2:2 BBS2:4	HM204919.1 HM204919.1
Pedobacter koreensis		1	99%	BBS4:7	AB681397.1
Proteus spp.	Proteobacteria (Gammaproteobacteria)	2	99%	BBS1 BBS1:20	JN106439.1 JN106439.1
Pelomonas spp.	Proteobacteria (Betaproteobacteria)	1	98%	BBS4:4	AB542416.1
Epilithonimonas lactis	Bacteroidetes (Flavobacteriia)	2	99%	BBS3:3 BBS3:1	EF204460.2 EF204460.2
Chryseobacterium spp.		1	99%	BBS2:12	GU451166.1
Sphingomonas spp.	Proteobacteria (Alphaproteobacteria)	2	99%	BBS4:1 BBS4:2	AB076396.1 JQ665450.1
Acidovorax spp.		1	99%	BBS4:15	AJ864847.1
Brevundimonas lenta		1	99%	BBS1:14	GU188941.1

4.2.3 Identification of the 16S rRNA gene in ImR bacteria isolated from agricultural soil

Sequencing of the 16S rRNA gene in the ImR strains isolated from agricultural soil identified 96 ImR bacteria (Table 4.2). The most abundant genus identified was *Stenotrophomonas* (43 isolates, 44.8%) and the most common species of this genus was *Stenotrophomonas rhizophila* (21 isolates, 21.8%), followed by 19 isolates (19.8%) of *Stenotrophomonas maltophilia*. Other bacterial strains were also isolated, including *Janthinobacterium lividum* (11 isolates, 11.5%), *Xanthomonas* spp., *Acetobacter pasteurianus* and *Pseudomonas* spp. (8 isolates each, 8.3%), *Pedobacter* spp. (4 isolates, 4.2%), and *Flavobacterium* spp. (3 isolates, 3.2%). Other isolates (one of each, 1.1%) were also isolated from different sites of the farms as indicated in Table 4.2.

Table 4.2: 16S rRNA gene identification of ImR bacteria isolated from agricultural soil

Genus and species identification	Phylum (and class)	Number of isolates	Identity %	Strain name	Accession number of closest related sequence
Stenotrophomonas rhizophila	Proteobacteria (Gammaproteobacteria)	21	99%	RHWA1:7	JX908718.1
Stenotrophomonas maltophilia		19	100%	RHWA1:8	DQ862553.1
Stenotrophomonas spp.		3	99%	LFWD3:6 LFWD3:7 LFBF1:4	JX899633.1 KC618445.1 KF202769.1
Janthinobacterium lividum	Proteobacteria (Betaproteobacteria)	11	99%	RHWA2:1	EF111116.1
Mucilaginibacter mallensis	Bacteroidetes (Sphingobacteriia)	1	98%	LFSE1:9	FN400859.1
Xanthomonas oryzae pv. oryzae	Proteobacteria (Gammaproteobacteria)	6	99%	RHWA1:12	HM989021.1
Xanthomonas retroflexus		2	99%	RHWA2:10 LFBF1:20	JQ890537.1 JQ890537.1
Microbacterium foliorum	Actinobacteria (Actinobacteria)	1	99%	RHWA1:19	KC139419.1
Microbacterium oxydans		1	99%	RHWA3:6	JX869578.1
Acetobacter pasteurianus	Proteobacteria (Alphaproteobacteria)	8	99%	RHWA3:7	KC122706.1

Genus and species identification	Phylum (and class)	Number of isolates	Identity %	Strain name	Accession number of closest related sequence
Pedobacter agri	Bacteroidetes (Sphingobacteriia)	1	98%	RHSB1:1	JQ342863.1
Pedobacter suwonensis		1	98%	LFSE1:7	DQ297951.1
Pedobacter terrae		1	99%	LFSE2:1	GU385862.1
Pedobacter wanjuense		1	98%	LFSE3:8	AM279217.1
Flavobacterium saccharophilum	Bacteroidetes (Flavobacteriia)	1	99%	RHSB3:8	HM278518.1
Flavobacterium johnsoniae		1	98%	RHBC1:11	EU984151.1
Flavobacterium pectinovorum		1	99%	RHBC3:7	AM230490.1
Chryseobacterium spp.		1	99%	LFWD1:5	AY599655.1
Chryseobacterium soldanellicola		1	99%	LFSE1:13	EU834270.1
Chryseobacterium hominis	Bacteroidetes (Flavobacteriia)	1	96%	LFBF1:16	AM423081.1
Sporocytophaga spp.	Bacteroidetes (Cytophagia)	1	99%	RHBC3:13	AM179866.1
Epilithonimonas lactis	Bacteroidetes (Flavobacteriia)	1	98%	LFWD3:10	EF204460.2
Acidovorax facilis	Proteobacteria (Betaproteobacteria)	2	99%	LFSE2:3 LFSE2:4	JQ342846.1 JQ236816.1
Pseudomonas poae	Proteobacteria (Gammaproteobacteria)	1	99%	RHBC2:2	JQ782898.1
Pseudomonas veronii		1	99%	LFBF1:1	JQ317806.1
Pseudomonas geniculata		6	99%	HQ857772.1	HQ857772.1
Duganella zoogloeoides	Proteobacteria (Betaproteobacteria)	1	98%	LFBF1:8	JQ689172.1

Details of higher-incidence isolates (more than three) are given in Appendix A3.1

A comparison of the water and soil isolates (Table 4.3) shows that some strains were common to both environments. These bacterial isolates include *Stenotrophomonas* spp., *Pedobacter spp., Epilithonimonas lacti, Chryseobacterium*

spp. and *Acidovorax*. The other isolates were present either in water or soil but not in both.

Table 4.3: Comparison of numbers of isolates belonging to genera and species found in both water and soil

Genus/Species name	Numbers isolated from water	Numbers isolated from soil
Stenotrophomonas spp.	3	3
Pedobacter alluvionis	1	
Pedobacter spp.	2	
Pedobacter koreensis	1	
Pedobacter agri		1
Pedobacter suwonensis		1
Pedobacter terrae		1
Pedobacter wanjuense		1
Epilithonimonas lactis	2	1
Chryseobacterium spp.	1	1
Chryseobacterium soldanellicola		1
Chryseobacterium hominis		1
Acidovorax spp.	1	
Acidovorax facilis		2

4.2.4 Identification of the 16S rRNA gene in ImR bacteria isolated from clinical settings

Sequencing of the 16S rRNA gene from ImR strains isolated from three hospitals in the UK (in the cities of Sheffield, York, and Kingston-upon-Hull) identified 42 ImR bacteria (Table 4.4). These isolates represented 7 genera and 11 species. The most common genus isolated was *Stenotrophomonas* (17/42, 40.5%) with *Stenotrophomonas maltophilia* (16/42, 38.1%) being the most common species isolated. These were mainly from the Sheffield and York hospitals. This organism was also isolated in blood specimens (9/16, 56.3%) from the York hospital and in specimens from lower respiratory tract infections (pneumonias) (7/16, 43.8%) from the Sheffield hospital. The second most common species was *Klebsiella pneumoniae* (11/42, 26.2%) and this was the main species found among the Hull isolates. Sputum was the most common specimen containing the isolates of *Klebsiella pneumoniae* (6/12, 50%), which were mainly from the Hull hospital. Also, this organism was isolated from the urine (3/11, 27.3%) present in urinary tract infections from the

same hospital, and from faeces (1/12, 8.3%) from the York hospital. *Pseudomonas aeruginosa* isolates were obtained mainly from the Sheffield hospital and were isolated from wound infections (2/42, 4.8%) and from pneumonia (1/42, 2.4%). Four isolates of *Escherichia* (9.5%) were isolated from urine (UTI) and blood (septicaemia) in the York and Hull hospitals. Also, four isolates of *Enterobacter aerogenes* (9.5%) were isolated from pneumonia and septicaemia patients in the Hull hospital. Other isolates including *Acinetobacter* spp, *Pseudomonas geniculate* and *Enterobacter cloacae* (1/42, 2.4%) were isolated from sputum and urine from the Sheffield and Hull hospitals.

Table 4.4: 16S rRNA gene identification for ImR bacteria isolated from clinical settings

Genus and species identification	Phylum (and class)	Number of isolates	Identity %	Strain name	Accession number of closest related sequence
Acinetobacter spp.	Proteobacteria (Gammaproteobacteria)	1 (Sheffield-sputum)	100	SAT-1	KF815693.1
Stenotrophomonas maltophilia	Proteobacteria (Gammaproteobacteria)	6 (Sheffield-sputum)	99	SST-2 SST-3 SST-4 SST-6 SST-7 SST-8	KF542911.1 JN084034.1 HM755655.1 KF732977.1 HQ647282.1 KC503914.1
		1 (Sheffield-pneumonia)	99	SST-9	KF150498.1
		9 (York-blood)	99	YSM-1 YSM-2 YSM-3 YSM-4 YSM-5 YSM-6 YSM-7 YSM-8 YSM-9	KC136828.1 JX505430.1 KF542911.1 KC252696.1 KF150491.1 KF732971.1 KF254518.1 KF923813.1 KF923813.1
Stenotrophomonas spp.	Proteobacteria (Gammaproteobacteria)	1 (Sheffield-sputum)	99	SST-5	KF923813.1
Pseudomonas aeruginosa	Proteobacteria (Gammaproteobacteria)	2 (Sheffield-wound)	100	SPS-3 SPS-1	CP006983.1 CP006985.1
		1 (Sheffield-sputum)	99	SPS-2	CP006984.1
Pseudomonas geniculata	Proteobacteria (Gammaproteobacteria)	1 (York-blood)	99	YPSG-1	JN382217.1

Genus and species identification	Phylum (and class)	Number of isolates	Identity %	Strain name	Accession number of closest related sequence
Escherichia coli	Proteobacteria (Gammaproteobacteria)	1 (York-urine)	99	HEC-2	KF870457.1
		1 (Hull-blood)	99	HEC-4	JQ206366.1
Escherichia spp.	Proteobacteria (Gammaproteobacteria)	2 (Hull-urine)	99	HEC-3	JQ907528.1
Klebsiella pneumoniae	Proteobacteria (Gammaproteobacteria)	1 (York-stool)	99	YKP-1	KF974479.1
		3 (Hull-urine)	99	HKP-2 HKP-3 HKP-4	KC153122.1 JF489150.1 CP003999.1
		6 (Hull-sputum)	100	HKP-5 HKP-6 HKP-7 HKP-8 HKP-9 HKP-10	KC153122.1 HF543828.1 JF489150.1 HM371197.1 GQ259887.2 KC243319.1
		1 (Hull-blood)	99	HKP-11	JF690978.1
Enterobacter aerogenes	Proteobacteria (Gammaproteobacteria)	2 (Hull-sputum)	99	HENT-1 HENT-2	KF731618.1 KC990787.1
		2 (Hull-blood)	99	HENT-3 HENT-4	KF726081.1 AB844449.1
Enterobacter cloacae	Proteobacteria (Gammaproteobacteria)	1 (Hull-urine)	100	HENT-5	KC768786.1

4.2.5 Taxonomic relationship

Only the imipenem-resistant isolates were characterised and identified in this study, i.e. not all of the viable bacteria were investigated further. The most common class of bacteria was Gammaproteobacteria (Proteobacteria phylum). 115 Gammaproteobacteria were present out of the total of 168 isolates taken from both environmental and clinical sources (68.5%). All 42 clinical isolates were related to this class (100%), while the highest number of soil isolates also related to this class and phylum of bacteria: 69/96 (61.5%). The most common class of the water isolates was Alphaproteobacteria (Proteobacteria phylum): 16/30 (53.3%). The least

frequently found classes of imipenem-resistant bacteria were Cytophagia (Bacteroidetes): 1/168 (0.6%), followed by Actinobacteria (Actinobacteria): 2/168 (1.2%) (Table 4.5).

Table 4.5: The relationship of different imipenem-resistant bacteria to different classes and phyla of bacteria in both environmental and clinical isolates

Phylum (and class)	Genus	Water		Soil		Clinical		Phyla Total
		Numbers	Numbers (% of water)	Numbers	Numbers (% of soil)	Numbers	Numbers (% of clinical)	Numbers (% overall)
Proteobacteria (Alphaproteobacteria)	*Caulobacter*	11	15 (50%)	-	8 (8.3%)	-	-	23 (13.7%)
	Sphingomonas	2		-		-		
	Acidovorax	1		-		-		
	Brevundimonas	1		-		-		
	Acetobacter	-		8		-		
Proteobacteria (Betaproteobacteria)	*Kinneretia*	1	2 (6.7%)	-	14 (14.6%)	-	-	16 (9.5%)
	Pelomonas	1		-		-		
	Janthinobacterium	-		11		-		
	Acidovorax	-		2		-		
	Duganella	-		1		-		
Proteobacteria (Gammaproteobacteria)	*Stenotrophomonas*	3	5 (16.7%)	43	59 (61.5%)	17	42 (100%)	106 (63.1%)
	Proteus	2		-		-		
	Xanthomonas	-		8		-		
	Pseudomonas	-		8		4		
	Acinetobacter	-		-		1		
	Escherichia	-		-		4		
	Klebsiella	-		-		11		
	Enterobacter	-		-		5		
Bacteroidetes (Sphingobacteriia)	*Chitinophaga*	1	5 (16.7%)	-	5 (5.2%)	-	-	10 (6%)
	Pedobacter	4		4		-		
	Mucilaginibacter	-		1		-		
Bacteroidetes (Flavobacteriia)	*Epilithonimonas*	2	3 (10%)	1	7 (7.3%)	-	-	10 (6%)
	Chryseobacterium	1		3		-		
	Flavobacterium	-		3		-		
Bacteroidetes (Cytophagia)	*Sporocytophaga*	-	-	1	1 (1.0%)	-	-	1 (0.6%)
Actinobacteria (Actinobacteria)	*Microbacterium*	-	-	2	2 (2.1%)	-	-	2 (1.2%)
Total		30		96		42		168

4.2.6 PCR amplification and sequencing of imipenem-resistant genes (ImR) from environmental and clinical isolates

DNA was extracted from the environmental and clinical imipenem-resistant isolates and these were screened for the possible carriage of related imipenem-resistant genes using PCR primers targeting β-lactamase genes. PCR products were generated from some of the ImR environmental isolates (Figure 4.2) and from clinical isolates (Figure 4.3) using the primers targeting β-lactamase genes. Full details of PCR data are shown on an isolate by isolate basis in Appendix A3 (Tables A3.2, A3.3 and A3.4). Sequence analysis of these products from clinical isolates showed that these PCR products were specific and related to β-lactamase genes (Table 4.6). However in the environmental isolates, PCR products were non-specific and unrelated to β-lactamase genes (Table 4.6).

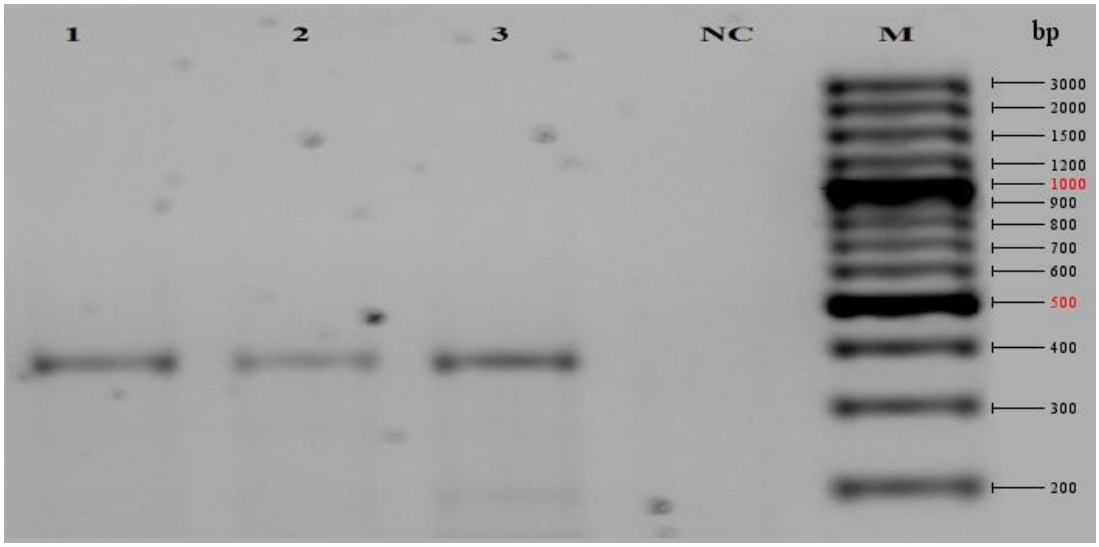


Figure 4.2: Agarose gel electrophoresis of PCR amplification products of suspected β-lactamase genes (VIM) from different environmental isolates

Agarose gel electrophoresis of PCR amplification for the suspected *vim* gene (~390 bp) from RHWA3:9 (*Stenotrophomonas maltophilia*) (1), RHWA3:10 (*Stenotrophomonas rhizophila*) (2) and LFWD3:7 (*Stenotrophomonas*) (3). NC: negative control (sterile dH2O). M: molecular marker; and DNA sizes are as indicated. Similar results were also obtained for other ImR environmental isolates.

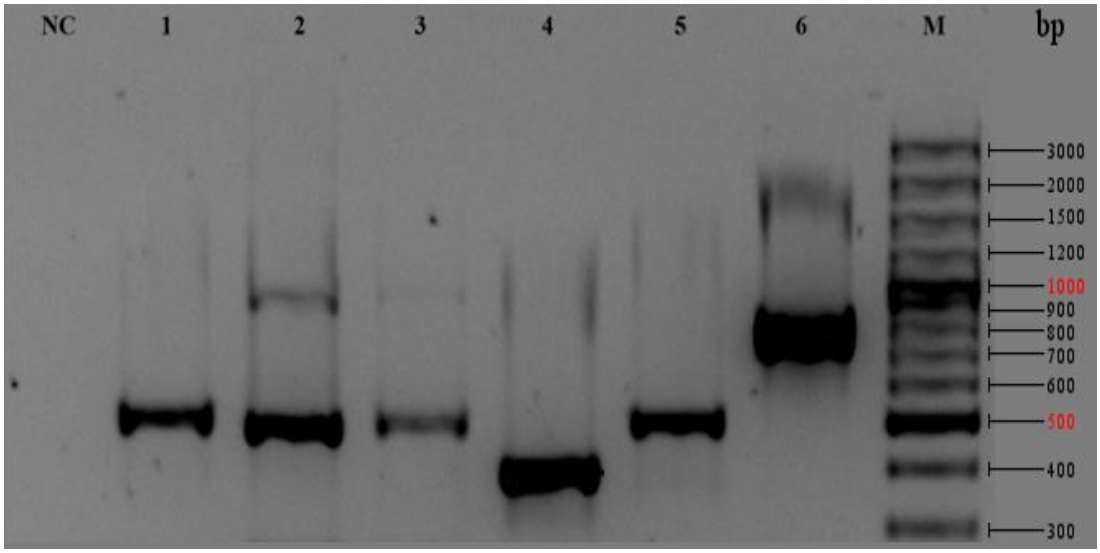


Figure 4.3: Agarose gel electrophoresis of PCR amplification products of different β-lactamase genes from ImR clinical isolates

PCR amplification of different β-lactamase genes from different ImR clinical isolates using different primers. NC: negative control (sterile dH2O). Lane 1: PCR amplification of $bla_{Oxa\text{-}23}$ (501 bp) from *Acinetobacter* spp. (SAT-1) Lanes 2 and 3: PCR amplification of *blaNDM-1*(621 bp) from *Pseudomonas aeruginosa* (SPS-1) and *Escherichia coli* (YEC-1), respectively. Lanes 4, 5 and 6: PCR amplification of $bla_{VIM\text{-}1}$ (390 bp), $bla_{IND\text{-}1}$ (580 bp) from *Klebsiella pneumoniae* (YKP-1) and $bla_{KPC\text{-}1}$ (798 bp) from *Klebsiella pneumoniae* (HKP-3). Similar results were also obtained for other ImR clinical isolates.

Table 4.6: Identification of PCR products of different β-lactamase encoding genes from environmental and clinical ImR bacterial isolates

Organism with PCR product	Source	Phenotypic detected β-lactamase	Suspected amplified ImR gene	Number of isolates	Strain name	Closest related sequence	Identity %	Accession
Stenotrophomonas maltophilia	Soil	Class B (MBL)	*Bla*$_{VIM-1}$	7	RHWA1:8, RHWA1:9, RHWA1:14, RHWA1:16, RHWA1:19, RHWA1:23, RHWA1:25	Drug resistant determinants	96	AM743169.1
				9	RHWA1:11, RHWA1:12, RHWA1:13, RHWA1:17, RHWA1:18, RHWA1:20, RHWA1:21, RHWA1:22, RHWA1:24	*tonB*-dependent receptor	97	HE798556.1
Stenotrophomonas rhizophila	Soil	Class B (MBL)	*Bla*$_{VIM-1}$	6	RHWA3:10, LFWD3:3, LFWD3:13, LFBF1:7, LFBF1:11, LFBF2:3	Cell division FtsK/SpoIIIE	95	CP001111.1
Stenotrophomonas spp.	Soil	Class B (MBL)	*Bla*$_{VIM-1}$	1	LFWD3:7	Cell division FtsK/SpoIIIE	96	CP001111.1
Stenotrophomonas spp.	Soil	Class B (MBL)	*Bla*$_{VIM-1}$	2	LFWD3:7, LFBF1:4	Hypothetical protein	97	CP001111.1
			Bla $_{L1}$	1	LFWD3:6	Ribonuclease R	94	CP002986.1
				1	LFBF1:4	Hypothetical protein	97	CP002986.1
	Water			1	BBS3:20	Hypothetical protein	95	CP001111.1
			Bla $_{IMP-1}$	1	BBS3	Hypothetical protein	92	CP009257.1
			Bla$_{NDM-1}$	1	BBS3	Hypothetical protein	96	CP009257.1

Organism with PCR product	Source	Phenotypic detected β-lactamase	Suspected amplified ImR gene	Number of isolates	Strain name	Closest related sequence	Identity %	Accession
Caulobacter spp.	Water	Class B (MBL)	*Bla* $_{CAU-1}$	3	BBS4:5, BBS4:9, BBS4:13	Hypothetical protein	95	CP000927.1
			Bla$_{Mbl1b}$	1	BBS4:6	Hypothetical protein	97	CP002008.1
Chryseobacterium spp.	Water	Class B (MBL)	*Bla* $_{B-1}$	1	BBS2:12	Hypothetical protein	94	AP014624.1
Epilithonimonas lactis	Water	Class B (MBL)	*Bla* $_{IMP-1}$	1	BBS3:1	Hypothetical protein	97	CP009257.1
Pedobacter koreensis				1	BBS4:7	Hypothetical protein	97	CP009257.1
Proteus spp.				1	BBS1:20	Hypothetical protein	96	CP009257.1
Janthinobacterium lividum	Soil	Class B (MBL)	*Bla* $_{IMP-1}$	3	RHWA2:1, RHSB2:16, LFWD3:15	Hypothetical protein	98	736784494
			Bla$_{IND-1}$	2	RHWA3:13, LFBF2:8	Hypothetical protein	95	736784737
			Bla $_{B-1}$	2	RHSB2:17, LFWD3:5	Hypothetical protein	94	736784346
Pseudomonas geniculata	Soil	Class B (MBL)	*Bla*$_{IND-1}$	1	RHWA1:18	Hypothetical protein	97	408543476
Pseudomonas geniculata	Blood	Class B (MBL)	*Bla*$_{NDM-1}$	1	YPSG-1	metallo-β-lactamase NDM-1 (*blaNDM-1*) gene	98	KF951481.1
Pseudomonas aeruginosa	Wound	Class B (MBL)	*Bla*$_{NDM-1}$	2	SPS-1, SPS-2	metallo-β-lactamase NDM-1 (*blaNDM-1*) gene	98	KF951481.1
Acinetobacter spp.	Sputum	None	*Bla*$_{OXA-23}$	1	SAT-1	*Oxa-23* gene	99	KF740470.1
Escherichia coli	Urine	Class B (MBL)	*Bla*$_{NDM-1}$	2	YEC-1, HEC-4	metallo-β-lactamase NDM-1 (*blaNDM-1*) gene	99	JX469383.1

Organism with PCR product	Source	Phenotypic detected β-lactamase	Suspected amplified ImR gene	Number of isolates	Strain name	Closest related sequence	Identity %	Accession
Escherichia spp.	Blood	Class A (KPC)	*Bla*$_{KPC-1}$	2	HEC-2, HEC-3	Plasmid pKP1780-*Kpc*	100	KF874497.2
Klebsiella pneumoniae	Urine	Class B (MBL)	*Bla*$_{VIM-1}$	1	YKP-1	Plasmid pKP-M4863 class I integron In4863 (*Vim* gene)	99	KF894700.1
			Bla$_{NDM-1}$	1	HKP-11	metallo-β-lactamase NDM-1 (*blaNDM-1*) gene	99	KF951468.1
	Sputum		*Bla*$_{IND-1}$	1	YKP-1	Positive for IND antibiotic resistance gene	98	CP006659.1
		Class A (KPC)	*Bla*$_{KPC-1}$	9	HKP-2, HKP-3, HKP-4, HKP-5, HKP-6, HKP-7, HKP-8, HKP-9, HKP-10	Plasmid pKP1780-*Kpc*	100	KF874497.2
Enterobacter aerogenes	Sputum and blood	Class A (KPC)	*Bla*$_{KPC-1}$	4	HENT-1, HENT-2, HENT-3, HENT-4	Plasmid pKP1780-*Kpc*	100	KF874497.2
Enterobacter cloacae	Urine	Class A (KPC)	*Bla*$_{KPC-1}$	1	HENT-5	Plasmid pKP1780-*Kpc*	100	KF874497.2

Thus, although amplification of suspected ImR genes from environmental isolates showed low similarity to some of the genes within GenBank, sequencing and alignment showed that they were not the genes responsible for imipenem resistance.

Water isolates (Table 4.6) yielded the lowest number of PCR products. These products indicated *Bla*$_{\text{IMP-1}}$ and *Bla*$_{\text{NDM-1}}$ in *Stenotrophomonas* spp (BBS3). *Caulobacter* spp. showed PCR products for *Bla*$_{\text{CAU-1}}$ in three strains (BBS4:5, BBS4:9, BBS4:13) and for *Bla*$_{\text{Mbl1b}}$ in one (BBS4:6). PCR products for *Bla*$_{\text{B-1}}$ were generated from one isolate of *Chryseobacterium* spp. (BBS2:12). *Bla*$_{\text{IMP-1}}$ products were also amplified from *Epilithonimonas lactis* (BBS3:1), *Pedobacter koreensis* (BBS4:7) and *Proteus* spp. (BBS1:20). All these PCR products were related to hypothetical proteins (Table 4.6).

For soil isolates (Table 4.6), PCR products were generated for *Bla*$_{\text{VIM}}$ from 16 *Stenotrophomonas maltophilia* isolates. Seven of these isolates (RHWA1:8, RHWA1:9, RHWA1:14, RHWA1:16, RHWA1:19, RHWA1:23 and RHWA1:25) yielded PCR products related to unidentified drug resistant determinants, while nine of them (RHWA1:11, RHWA1:12, RHWA1:13, RHWA1:17, RHWA1:18, RHWA1:20, RHWA1:21, RHWA1:22 and RHWA1:24) were related to *tonB*-dependent receptors. Six *Stenotrophomonas rhizophila* isolates (RHWA3:10, LFWD3:3, LFWD3:13, LFBF1:7, LFBF1:11and LFBF2:3), and one isolate of *Stenotrophomonas* spp. (LFWD3:7) yielded amplification products with the *Bla*$_{\text{VIM-1}}$ primers. However, these products were related to cell division FtsK/SpoIIIE genes. Another two isolates of *Stenotrophomonas* spp. (LFWD3:7 and LFBF1:4) which also yielded products with *Bla*$_{\text{VIM-1}}$ primers were related to hypothetical genes. Also, PCR products were generated with *Bla*$_{\text{L1}}$ primers from three isolates of *Stenotrophomonas* spp. (LFWD3:6, LFBF1:4 and BBS3:20). However, when these products were sequenced, they were found to be related to ribonuclease R. *Janthinobacterium lividum* yielded amplification products with *Bla*$_{\text{IMP-1}}$, *Bla*$_{\text{IND-1}}$ and *Bla*$_{\text{B-1}}$ primers in three (RHWA2:1, RHSB2:16 and LFWD3:15), two (RHWA3:13 and LFBF2:8) and two (RHSB2:17 and LFWD3:5) isolates, respectively. One isolate of *Pseudomonas geniculate* (RHWA1:18) showed the suspected detection of *Bla*$_{\text{IND-1.}}$ All of these products showed relationships to hypothetical genes.

Although the PCR products were amplified from the environmental isolates using primers targeting β-lactamase encoding genes, alignments of these sequences revealed low similarity to these β-lactamase genes (e.g. Figure 4.4).

Figure 4.4: CLUSTAL 2.1 sequence alignment between suspected amplified *Bla*$_{\text{VIM-1}}$ from *Stenotrophomonas* spp. (LFWD3:7) isolated from soil and the target gene

This figure shows that although the suspected amplified *Bla*$_{\text{VIM-1}}$ genes show ~ 25% similarity (hypothetical gene) to the target gene, they did not prove to be the genes responsible for imipenem resistance. Consensus-VIM-1: *Stenotrophomonas* spp. (LFWD3:7), ORIGIN: hypothetical gene (*Stenotrophomonas maltophilia* R551-3; Accession Number CP001111.1).

On the other hand, the amplification of genes from the clinical isolates was specific and related to imipenem resistance (Table 4.6). The most common gene detected in the current study was *Bla*$_{KPC-1}$ in 16 isolates in two *Escherichia* spp. (HEC-2 and HEC-3), nine *Klebsiella pneumoniae* (HKP-2, HKP-3, HKP-4, HKP-5, HKP-6, HKP-7, HKP-8, HKP-9 and HKP-10), four *Enterobacter aerogenes* (HENT-1, HENT-2, HENT-3 and HENT-4) and one *Enterobacter cloacae* (HENT-5) isolated from sputum, blood and urine from Hull. Detection of *Bla*$_{IND-1}$ was observed in two *Escherichia coli* (YEC-1and HEC-4), one *Pseudomonas geniculate* (YPSG-1), two *Pseudomonas aeruginosa* (SPS-1 and SPS-2), and one *Klebsiella pneumonia*e (HKP-11) isolates from urine, blood, wound, and urine, respectively, obtained from all three hospitals. Other genes, including *Bla*$_{VIM-1}$ and *Bla*$_{IND-1}$ were detected in two isolates of *Klebsiella pneumoniae* (YKP-1 and YKP-1 respectively), obtained from Hull, while *Bla*$_{OXA-23}$ was only observed in one *Acinetobacter* spp. (SAT-1) strain isolated from sputum from Sheffield.

After performing gene sequencing for PCR products of the different β-lactamase genes amplified from ImR clinical isolates, the results showed that the sequences were identical to those of β-lactamase gene sequences in the GenBank database (Table 4.6).

Some isolates showed weak bands following PCR for the suspected gene of around the expected size. Gradient PCR enabled bright bands of the targeted amplified genes to be obtained (Figure 4.5). These isolates include amplification of *Bla*$_{OXA-23}$ in *Acinetobacter* spp. (SAT-1) and *Bla*$_{NDM-1}$ in *Escherichia coli* (YEC-1) and *Pseudomonas geniculate* (YPSG-1). It was observed that the best annealing temperatures for amplifying these target genes were 58 °C and 60 °C.

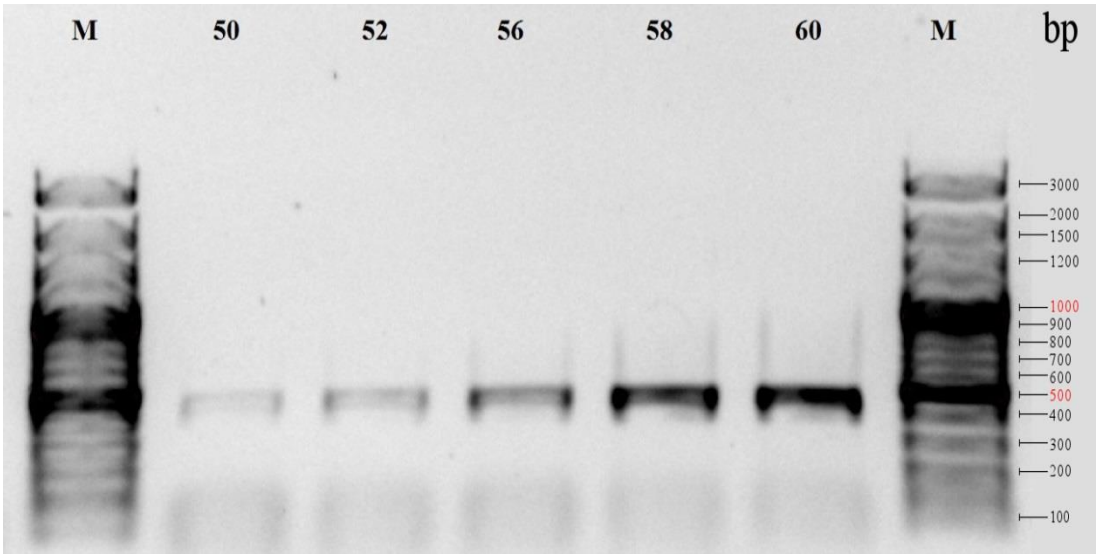


Figure 4.5: Agarose gel electrophoresis of gradient PCR amplification products of *Oxa-23 genes* in *Acinetobacter* spp. (strain SAT-1)

Agarose gel electrophoresis of a gradient PCR amplified *Oxa-23* gene (~501 bp) from *Acinetobacter* spp. (strain SAT-1), M: molecular marker and DNA sizes are as indicated. Similar results were also obtained for other isolates. Various annealing temperatures were used here (50, 52, 56, 58, and 60°C), as indicated.

4.3 Discussion

After the isolation of imipenem resistant isolates from both the natural environment and clinical settings, all of these isolates were identified by 16S rRNA gene sequencing. The molecular technique was chosen over the more traditional methods of phenotypic and biochemical identification because of its availability, convenience and cost-effectiveness. Other advantages include the presence of the 16S rRNA gene in all bacterial isolates, the relatively short length of the gene (1550 bp), its stability and its lower mutation rate. It has also been found to be useful in the identification of certain 'difficult-to-identify' bacteria (Block and Ouellette, 2012; Chen et al., 2014).

Studies of species composition isolated from river water differ according to the locality of the study and the study technique and objective. For example, in a similar study carried out on tap water and other specimens in Portugal, the most common isolates were 28 isolates of *Sphingobium*, 27 isolates of *Sphingomonas*, 12 of *Novosphingobium*, 7 of *Sphingopyxis*, and 12 of *Blastomonas* by using the 16S rRNA sequencing after isolation of the bacteria by culture based method on R2A media (Vaz-Moreira *et al*., 2011).

In a study carried out in the USA on 30 phenotypically imipenem-resistant bacteria isolated from rivers, the majority of isolates were *Enterobacteriaceae* species (22 isolates) while 6 of the isolates were *Stenotrophomonas maltophilia* and 1 was *Aeromonas hydrophila*, as confirmed by 16S rRNA gene analysis (Aubron *et al*., 2005).

In Asia, the urban surface water in Malaysia showed that 89.5% of the isolates to be enterobacteria-producing ESBLs and also resistant to imipenem (Tissera and Lee, 2013). In a river in India, there were also very high numbers of coliforms, which were identified by 16S rRNA and which exceeded the WHO recommended figures for drinking water quality (Mishra *et al*., 2013).

This indicates the importance of the water environment, as it contains numerous bacteria, some of which are antibiotic resistant (AR). Other AR bacteria in water can originate from hospital waste, aquaculture e.g. fish farms, and municipal effluents (Liu *et al*., 2014).

In Beverley Beck, the river water receives effluent from the sewage works after being biologically treated. Most of the isolated strains in the current study have, at some point, been implicated in the pathogenesis of various human infections. For example *Caulobacter*, which is an aquatic species and was the most common bacteria isolated from river water in the present study, has been isolated from a case of peritonitis in a chronic renal failure in Denmark in 2007. The identification of the organism was confirmed by sequencing the 16S rRNA gene (Justesen *et al*., 2007). *Caulobacter* spp. have also been isolated from a case of post neurosurgery meningitis in a 14-year old boy in Manitoba, Canada. The organism was isolated and identification was confirmed using 16S rRNA sequencing (Bridger *et al*., 2012).

Chryseobacterium, which was isolated in low numbers from a water environment, is a low-virulence microorganism; however, some species have been isolated and associated with clinical infections. For example *Chryseobacterium* spp. have been isolated from catheter-associated infections and peritonitis (Wang *et al*., 2011), and also from a case of septicaemia in an infant (Teke *et al*., 2014).

Pedobacter is an environmental organism which has been isolated from clinical specimens, although rarely. For example, it was detected in semen from HIV-infected patients in increasing numbers especially after the start of the anti-retroviral therapy (ART) (Liu *et al*., 2014).

Due to the great diversity of soil microorganisms there are large variations in composition and in species which have been identified by different studies (McCaig *et al*., 2001). Also, the species detected by the different studies have depended upon the selectivity of the media used in the study (McCaig *et al*., 2001). For example, in one study, *Pseudomonas* isolation agar (PIA) media detected 12 species, as it is selective for *Pseudomonas*, while the general lab tryptone soya agar (TSA) detected 21 highly different species of soil microorganisms to those detected on PIA. The 16S rRNA method of isolate identification was also found to be the most accurate compared to traditional culture methods (McCaig *et al*., 2001). There are other factors responsible for the diversity of isolates. These include the chemical and physical characteristics of soil, geographical locations and changes in climate (McCaig *et al*., 1999). Due to the culture-based selection and the media used in the current study, only the imipenem-resistant isolates were identified and further characterised, as they were the species of interest in this study.

The predominance of both *Stenotrophomonas rhizophila* and *Stenotrophomonas maltophilia* in the soil environments in the current study can be explained by the association of these species with the plants in the studied farms. There is a beneficial relationship between *Stenotrophomonas* and plants as they have a role in the nitrogen and sulphur cycles (Banerjee and Yesmin, 2002; Park *et al*., 2005). Also, soil is considered as an environmental reservoir of these bacteria. The fimbriae and pili present in *Stenotrophomonas* species help in the establishment of roots in some plants like wheat, but also allow colonisation of surfaces of medical equipment leading to human infections (Wolf *et al*., 2002; Ryan *et al*., 2009).

In the current study, *Janthinobacterium lividum* was the second most common species to be isolated from soil samples. This Gram-negative bacterium is most common in soil and aquatic environments, but is rarely isolated from clinical infections in human beings. It was found to be an opportunistic pathogen, occasionally causing fatal septicaemia (Pantanella *et al*., 2006). This microorganism is also implicated in the degradation of animal and plant tissue. In animals it has been found to cause spoilage of stored rabbit meat (Giaccone *et al*., 2008), spoilage of poultry meat (Cox, 1975), and pasteurized milk (Eneroth *et al*., 2000). In plants it has been suspected of causing rotting in mushrooms crops (Pudełko, 2013) and of spoiling stored vegetables (Koburger and May, 1982).

Based on the clinical isolates provided by the three hospitals from York, Sheffield and Hull, the most common microorganism identified amongst these isolates was *Stenotrophomonas maltophilia* (38.1%) from the York and Sheffield hospitals but this was not obtained from Hull. These species were mainly isolated from respiratory tract infections and from blood cultures. The most common species amongst the Hull isolates was *Klebsiella pneumoniae* (50%) and this was detected in a variety of infections including urinary and respiratory tract infections.

The predominance of *Stenotrophomonas maltophilia* amongst the York and Sheffield clinical isolates in the current study reveals how these normal soil-inhabiting microorganisms can cause many types of infections, including pneumonias and septicaemia. Another study indicated that *S. maltophilia* is not a highly virulent organism and its pathogenicity is related to being one of the most important nosocomial infectious agents nowadays with mortality rates ranging from 14 to 69% in cases with bacteraemia (Falagas *et al*., 2009). Brooke (2012) claims

that this organism has been isolated from many sources including soil, animals, plant roots, water and water treatment systems, lakes, sink holes, hemodialysis water and dialysate samples, tap water, biofilms on different surfaces, hand washing soaps and even in some antiseptics. Consequently, there is a wide range of infections associated with *S. maltophilia* including pneumonia, skin and soft tissue infections, cellulitis, endocarditis, endophthalmitis, UTIs, meningitis, and catheter associated infections (Brooke, 2012).

Thus, *S. maltophilia*, which is a MDR environmental microorganism, is recognised nowadays as an important species in hospital-acquired infections in many parts of the world. This may raise the importance of setting up control measures to prevent this type of infection in hospital settings (Brooke, 2012).

The second most common clinical imipenem-resistant microorganism was *Klebsiella pneumoniae*, especially among the Hull isolates. Infections from such carbapenem-resistant bacteria are associated with increased rates of morbidity and mortality due to the limited availability of antimicrobials for their control and treatment (Arnold *et al*., 2011).

Clinical infections from such *Enterobacteriaceae* are now increasingly encountered in Europe. For example, infections caused by carbapenemase-producing enterobacteria (CPE) have been reported in Belgium during 2010 and 2011 (17 and 13 CPE species, respectively) without any history of travelling abroad of infected patients. These CPE were isolated from urine, rectal swabs, wound discharges and lower respiratory tract infections (Huang *et al*., 2011). Also, infections with carbapenem-resistant *Klebsiella pneumoniae* have been reported in many areas in Italy and in some instances there have been outbreaks of infections in intensive care units. Infection control measures in such cases are compulsory and include hand hygiene and isolation procedures to limit the spread of infection (Gaibani *et al*., 2014). The clinical spread of CPE is considered a global crisis, as organisms have been isolated from many countries worldwide. In Europe, in addition to the above-mentioned countries, CPE isolates have been found in Greece, Switzerland, Ireland, the United Kingdom, France, Sweden, Norway, the Netherlands, France and Denmark (Tzouvelekis *et al*., 2012).

Regarding the detection of ImR genes amongst the environmental isolates in the current study, some of the PCR amplifications showed suspected ImR genes. However, sequence analysis for these genes showed that the resistant genes within these river water and farm soil isolates were not related to the ImR genes in antibiotic-resistant clinically important species.

Thus, the amplification results of suspected ImR genes among the environmental isolates showed similarities with some of the genes within GenBank. However, sequencing and alignment showed that these genes encode unknown protein functions (hypothetical protein) and so were not proven to be the genes responsible for imipenem resistance in this study. This may indicate that the primers were lacking in specificity and led to the amplification of other non-targeted genes. Also, it may indicate the presence of a potentially large and divergent gene pool for imipenem resistance within natural environments, and the wider importance of river water and agriculture soil as a reservoir for novel antibiotic resistance (D'Costa *et al*., 2006). These suspected β-lactamase encoding genes were L1, VIM, IND, IMP, Mbl1b, BlaB and CAU-1 in isolates of *S.maltophilia, S, rhizophila, Stenotrophomonas* spp.*, Epilithonimonas lactis, Pedobacter koreensis, Proteus* spp.*, Caulobacter* spp.*, Chryseobacterium* spp.*, J. lividum* and *P. geniculate.*

For example, although the suspected $Bla_{\text{VIM-1}}$ gene was amplified (similar size to the target gene) from 16 strains of *S. maltophilia* isolated from soil, sequence analysis of these products showed that these PCR products were related to the gene encoding *tonB* protein and for undefined drug resistance determinants in nine and seven isolates, respectively. The *tonB* genes encode the production of a bacterial membrane protein called the *tonB*-dependent receptor (TBDR) which is involved in the iron-siderophore active transport process in Gram-negative bacteria. The *tonB* gene, which is more common in *Xanthomonas* species, was found to be present in higher numbers in environmental isolates of *Stenotrophomonas maltophilia* than in others, as it is related to scavengers of complex carbohydrates in aquatic environments (Ryan *et al*., 2009). The over-expression of *tonB* genes was found to be related to resistance to many antimicrobial agents in *Pseudomonas* species, while *tonB*-deficient mutants were found to be unusually susceptible to many antimicrobial agents (Zhao *et al*., 1998), indicating the relation of such receptors to antimicrobial resistance in the environmental isolates detected in the current study. It was found

that TBDR is one of the proteins related to the pathogenicity, virulence and antimicrobial resistance in the clinical isolates of *Stenotrophomonas maltophilia* in fish models (Ferrer-Navarro *et al*., 2013).

Girlich *et al*. (2010b) have detected novel genes encoding carbapenem-hydrolyzing β-lactamase from the Seine River, Paris, and they report the limited detection of all genes encoding carbapenemase resistance in the environment.

In this study, some isolates screened for ImR genes yielded weak bands. Gradient PCR using different annealing temperatures was applied, which generated bright bands of the targeted amplified genes. This indicates the importance of optimizing PCR conditions to obtain reliable PCR products (Özdemir, 2009)

The clinical isolates in the present study showed PCR amplification of ImR genes and the sequence analysis showed that these genes were specific and related to ImR and β-lactamase genes within antibiotic-resistant clinically important species. These enzymes were KPC, VIM, NDM, IND and OXA-23 in isolates of *S. maltophilia*, *P. geniculate*, *P. aeruginosa*, *Acinetobacter* spp., *Escherichia coli*, *Escherichia* spp., *K. pneumoniae*, *E. aerogenes* and *E. cloacae*.

The most common gene detected in the current study was bla_{KPC} (class A) in 16 isolates of *Enterobacteriaceae* (9 *Klebsiella pneumoniae*, 4 *Enterobacter aerogenes*, 2 *Escherichia* spp. and 1 *Enterobacter cloacae*) isolated from sputum, blood and urine from the Hull hospital.

The problem of carbapenemase-producing *Enterobacteriaceae* (CRE) is one of the most challenging threats to public health in the current era (CDC, 2009). Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has spread steadily around the world since it was first identified in North Carolina in 2001 (Arnold *et al*. 2011). France experienced the first non-US outbreak of CRKP and this has since been followed by reports of *Enterobacteriaceae*-producing KPCs found in South America, the Middle East, the Far East and elsewhere in Europe (Saidel-Odes and Borer, 2013).

CRKP presents a particularly difficult challenge as it is resistant to most of the antimicrobials available today. It is responsible for high morbidity and mortality rates in hospitals and healthcare settings, especially where there is critical illness and exposure to invasive devices such as catheters (CDC, 2009). The gene encoding the

KPC enzyme is carried on mobile genetic elements such as plasmids, which increases the risk of spreading (Mathers *et al*. 2013).

The importance of performing PCR targeting such genes in the current study is that CRE can be difficult to detect phenotypically because there are strains which carry KPC and which yet show minimal inhibitory concentrations (MICs) that are high, but still within the susceptible range for carbapenems (CDC, 2009). Nordmann *et al*. (2011) have also pointed out that strains are sometimes not identified as potentially dangerous since standard carbapenem susceptibility tests are not always accurate. Thus the inadvertent transmission of CRKP has occurred during nosocomial outbreaks of infection. Measures to contain the spread of infection include effective sterilisation techniques and decontamination procedures (Saidel-Odes and Borer, 2013).

Detection of NDM-1 was observed in 6 isolates (2 *Escherichia coli*, 1 *Pseudomonas geniculate*, 2 *Pseudomonas aeruginosa* and 1 *Klebsiella pneumoniae*) from samples of urine, blood, and wound and urine, respectively, which were obtained from the three hospitals. The detection of this gene in these three localities in the UK may demonstrate the warning signs of the further spread of antibiotic-resistant strains, which need to be contained and controlled (ECDC, 2013).

The findings in the current study correlate partly with the survey results that were obtained in 2013 from 28 countries of the European Union for the detection of CPE and CRAB, where it was reported that the most frequent carbapenemases were KPC, VIM, IMP and the NDM-1 for enterobacteria. In *Acinetobacter* species, the most common mechanism was production of the *oxa-23*genes (ECDC, 2013), as similarly detected in the current study with one *Acinetobacter* spp. isolated from sputum in the Sheffield hospital.

The low-detection of the $Bla_{\text{Oxa-23}}$ gene, which encodes one of the most common enzymes of the class D carbapenemase-hydrolyzing β-lactamases and plays a major role in the resistance of *Acinetobacter baumannii* and other bacteria, may be attributable to the presence of another variant resistant gene, as class D enzymes are located on transmissible plasmids and include five types: oxa-23-like (oxa-23, oxa-27 and oxa-49), oxa-24-like (oxa-24, oxa-25, oxa-26, oxa-40 and oxa-72), oxa-51-like, oxa-58-like, and oxa-143 (de Figueiredo *et al*., 2011; Carvalho *et al*., 2011).

The finding of NDM-1 prevalence actually represents a very important aspect of this study. There are several reports warning of the global spread of such genes, with the consequent spread of antimicrobial-resistant bacteria to all continents except South America and Antarctica. Returning back to the first case in which NDM-1 was detected in 2008 in a patient of Indian origin, and revising the situation where more than forty countries were affected globally in 2013, then the problem of the spread of this type of gene can be understood (Johnson and Woodford, 2013). Global spread of such antimicrobial-resistant genes requires the cooperation of international authorities in controlling the spread by imposing the necessary regulations and procedures (Nordmann *et al*., 2011).

Several factors play a part in the spread of such resistant bacteria globally. Firstly, the MDR pattern of NDM-producing bacteria that are resistant to all β-lactams except monabactam, and for which the only available antimicrobials for treatment are colistin, fosfomycin and tigecycline. Secondly, the higher percentage of these bacteria in countries like India, where 70-90% of enterobacteria are ESBL-producers, and where tourism aids global spread. Thirdly, the presence of the NDM gene on a plasmid, where it is easily spread to other bacterial isolates (Majewski *et al*., 2012). These factors may indicate that the world is now approaching a state akin to the pre-antimicrobial era (Carlet *et al* 2012).

Some studies have also reported the predominance of NDM in the U.K. and have attributed the cause of that predominance to the historical link between the U.K. and India, where this gene type is widespread in India (Grundmann *et al*., 2010).

Verona integron encoded metallo-β-lactamase (VIM), related to class B enzymes, which was the second carbapenemase to be reported in Europe after IMP (Lauretti *et al*., 1999) was detected in *Klebsiella pneumoniae* obtained from Hull. VIM was found to be present in 37 countries in all five inhabited continents; and is mainly present in enterobacteria (Hawkey and Jones, 2009; Grundmann *et al*., 2010).

4.4 Conclusion

The most abundant genera identified were *Caulobacter* (36.7%), *Stenotrophomonas* (44.8%) and *Stenotrophomonas* (40.5%) from water, soil and

clinical environments, respectively. Infections from carbapenemase-producing bacteria are now increasing at an unprecedented rate in many parts of Europe. The resistance genes detected in the river water and farm soil isolates in the present study were not sufficiently closely related to ImR genes within antibiotic-resistant clinically important species to enable their detection by PCR. This indicates a potentially large and divergent gene pool for imipenem resistance within natural environments, and the wider importance of river water and agricultural soil as a reservoir for novel antibiotic resistance. The low detection of ImR genes in the environmental isolates may be attributable to the presence of other variants of resistance genes, or to the presence of novel genes encoding such a resistance pattern. It should be noted that the low frequency of detection of ImR genes in the environmental isolates was also often associated with corresponding low ImR MICs (4 mgL^{-1}) in these isolates that may indicate the possible absence of carbapenamases in some of these isolates. In contrast, the more frequent detection of ImR genes in clinical isolates was associated with the higher MICs (≥ 8$mgL^{-}1$) observed among these isolates. The application of rapid diagnostic measures for the detection of carbapenemase-producing bacteria is very important in surveillance programmes. Strict isolation procedures to contain infections and proper hygienic measures by healthcare providers are highly important in limiting the spread of such MDR organisms. It is recommended that many different arrays of primers are used when testing for ImR genes in environmental bacterial isolates due to the great diversity of such genes. Performing gradient PCR is useful when using molecular techniques in order to reach the optimal temperature needed to detect the gene of interest. The higher rates of isolation of *Stenotrophomonas* spp. from both water and soil and in the hospitals, may support its role as an opportunistic pathogen and merits further analysis including via genome sequencing (see Chapter 5).

Chapter 5: Genomic analysis of *Stenotrophomonas maltophilia* isolates from natural environments and clinical settings

5.1 Introduction

Bacterial resistance to the action of antimicrobials is one of the most important burdens and threats facing healthcare providers all over the world. The number of newly discovered antimicrobials is decreasing, while the number of antimicrobials to which resistance has been developed is increasing (Crossman *et al.,* 2009). The emergence of multi-drug resistant (MDR) bacteria which limit the choice of effective antimicrobials, together with infections caused by opportunistic pathogens, could drive the world back to a pre-antimicrobial era; this is now a real threat (Martinez *et al*., 2007).

The carriage of antibiotic resistances varies markedly between different bacterial isolates, with strain-to-strain variation typical. These resistances are frequently intrinsic in many of the opportunistic pathogenic bacteria arising from environmental sources, including water and soil. Another resistance mechanism, which is also the most important, is one that is acquired, either by mutation or the transfer of a resistant gene(s) by mobile genetic elements via horizontal gene transfer (HGT). This mechanism is responsible for the spread and dissemination of antimicrobial resistance to different bacterial isolates (Martinez *et al.,* 2007; Torres *et al.,* 2007). The intrinsic mechanism of resistance in opportunistic pathogens can be directed at the specific antimicrobial or group of antimicrobials or it may be part of the resistance mechanisms exerted by a microorganism towards a wider range of toxic compounds and heavy metals. In the latter condition, antimicrobial resistance occurs by chance (Crossman *et al.,* 2007).

Water and soil environments contain various concentrations of heavy metals such as mercury, chromium, cadmium and lead, which, when present in high concentrations, are toxic to animals, plants and human beings. However, a number of bacteria have become resistant to the toxic effects of these heavy metals and have developed enzymes and metabolic pathways to deal with them. Genes for heavy metal resistance are carried on bacterial chromosomes or plasmids and are present mainly in Gram-negative bacteria. These metal resistance genes are often linked (i.e.

carried on the same mobile genetic element as antibiotic resistance genes, thereby driving their co-selection; Baker-Austin *et al.*, 2006). For example, resistance to multiple antimicrobials has been found in *Stenotrophomonas maltophilia* after exposure to certain heavy metals, including selenium, cadmium, and tellurium (Pages *et al*., 2008).

Stenotrophomonas maltophilia, which is present in soil and water as a free-living microorganism, is opportunistically infectious to human beings. The organism has intrinsic resistance to some β-lactams, macrolides, and aminoglycosides (Sader and Jones, 2005). This intrinsic resistance is mediated by efflux pumps, low permeability to the entrance of antimicrobials, or the production of antimicrobial hydrolyzing enzymes by the microorganism. However, an intrinsically resistant microorganism can also acquire resistance to other antimicrobials, leading to the emergence of a pan-drug-resistant microorganism (Crossman *et al.*, 2007).

Although, the antimicrobial resistance pattern observed in *Stenotrophomonas maltophilia* is common, the genetic analysis by genome sequencing of various, mostly clinical, strains shows great diversity in most studies. Genomic diversity has been observed among different environmental isolates (Crossman *et al.*, 2007; Nunvar *et al.*, 2014).

Despite the low susceptibility or even the pan-resistance pattern of *S. maltophilia*, which makes it difficult to treat with regard to clinical infections, the organism has importance in some beneficial biotechnical applications, including: 1) the biodegradation of pollutants and large molecules such as phenolic compounds, e.g. *p*-nitrophenol and 4-chlorophenol, toluene, benzene and xenobiotics; 2) the biogeochemical cycling of heavy metals, such as cadmium, cobalt, lead, zinc, silver and mercury from soil heavily contaminated with these metals, as well as xenobiotics in the soil; 3) the production of molecules that have economic value, such as antibiotics and various enzymes (proteases, glucanases, chitinases, DNases, RNase, etc.); and 4) the biocontrol and improvement of plant growth by the prevention of plant pathogens through several mechanisms (Ryan *et al.*, 2009; Lira *et al*., 2012).

The genetic diversity of *S. maltophilia* is not limited to environmental strains. There is also great genetic diversity among strains isolated from clinical specimens.

Such genetic diversity can lead to the identification of novel strains that are different from those stored in GenBank. This identification is important because infection and antimicrobial resistance is associated with certain genotypic strains (Tanimoto, 2013).

In the current study, *Stenotrophomonas* was the only genus isolated from both the natural environment (water and soil) and hospital samples which showed MDR patterns phenotypically. However, when applying molecular methods to detect genes resistant to imipenem (Chapter 4), PCR assays failed to amplify imipenem resistance genes from environmental isolates, despite β-lactamase activity being demonstrated by functional assays (Chapter 3). This highlights the limitations of PCR-based approaches for detecting and allowing characterisation of divergent functional (including antibiotic resistance) genes. Hence in this chapter, shotgun genomic analysis of multiple antibiotic resistance *Stenotrophomonas* isolates was undertaken to enable the identification of putative antibiotic resistance genes, including those potentially encoding imipenem resistance. Specifically, the objectives of this chapter were to identify and describe the ARGs (including those encoding β-lactamases) present in multiple antibiotic resistant *Stenotrophomonas* spp. isolates taken from river water, agricultural farm soil and clinical settings, and to establish whether the phenotypes are consistent with the presence or absence of ARGs.

5.2 Results

5.2.1 Bacterial genome isolation

Genomic DNA was extracted from eight imipenem (and multiple antibiotic resistant) *Stentotrophomonas* spp. isolates, as described previously in Section 2.2.5.5. DNA was eluted in a final volume of 1000 µl and 10 µl was electrophoresed on a 0.7 % (w/vol) agarose gel. DNA concentration and quality (A_{260}/A_{280} ratio) were quantified by analysis of 1 µl using a NanoDrop® ND-1000 spectrophotometer. The DNA samples that were to be used for whole genome sequencing had to be of sufficient quality (Figure 5.1) and also of sufficient quantity (Table 5.1) (> 100 ng μl^{-1}). In some cases, re-extraction of DNA was necessary, e.g. for isolate RHBC2-15 (Figure 5.1, lane 3).

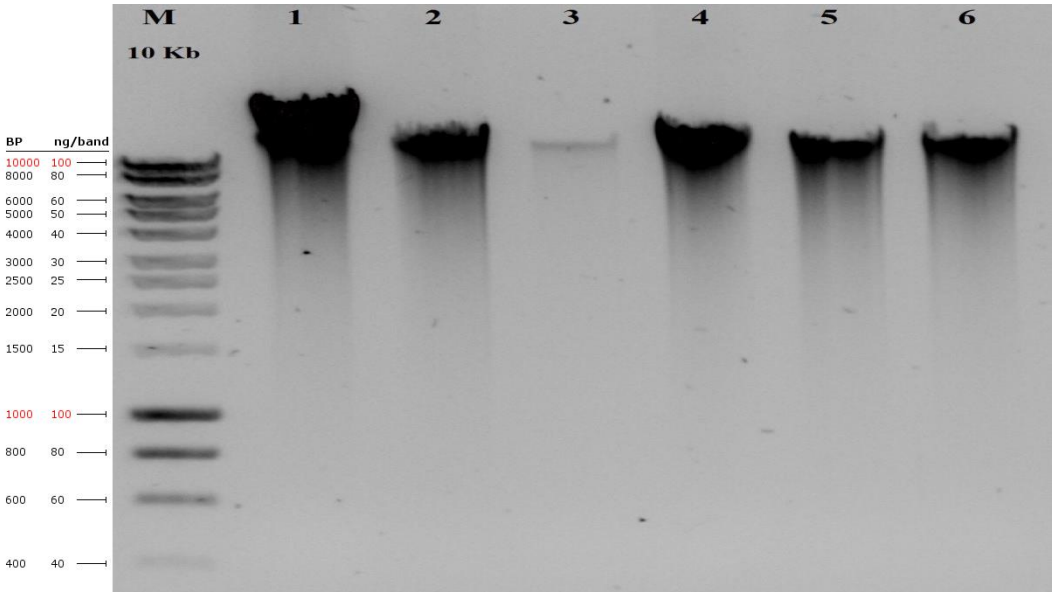


Figure 5.1: Agarose gel electrophoresis (0.7%) of genomic DNA extracts from *Stenotrophomonas* spp. isolates

Lane 1: BBS4:12 (water); Lane 2: BBS3 (water); Lane 3: RHBC2-15 (soil); Lane 4: LFBF1-10 (soil); Lane 5: BBS1:13 (water); and Lane 6: LFWD3:7 (soil). M: 10 Kb molecular marker (size as indicated).

Table 5.1: Quantification and purity (A_{260}/A_{280} ratio) of genomic DNA isolated from *Stenotrophomonas* spp. isolates

Strain name	Isolate source	A_{260}	A_{280}	260/280 ratio	DNA concentration $ngµl^{-1}$
BBS1:13	Beverley Beck	5.137	2.727	1.88	256.9
BBS4:12	Beverley Beck	2.167	1.171	1.85	108.4
BBS3	Beverley Beck	3.655	1.947	1.88	182.8
RHBC2:15	Riseholme beans field	4.338	2.279	1.90	216.9
LFWD3:7	Lodge Farm wheat field	4.575	2.548	1.80	228.8
LFBF1:10	Lodge Farm beans field	2.852	1.540	1.85	142.6
SST-8	Sheffield Hospital sputum	2.765	1.460	1.89	138.3
YSM-3	York Hospital blood	3.500	1.776	1.97	175.0

5.2.2 Bacterial genome sequencing

Genome sequences were generated by Eurofins Genomics, (https://www.eurofinsgenomics.eu/Germany) using *de novo* sequencing on an Illumina MiSeq instrument with v3 chemistry using a genomic DNA shotgun protocol, with paired-end sequencing. Eurofins supplied data following clipping to remove adapter sequences and reads with a Phred score of <20, and also clipped reads that were shorter than 150 bp. An overview of the raw data sequencing report showing overall yields and %Q30 data is shown in Table 5.2. Sequences were supplied in FASTQ format as raw data (i.e. unassembled) and secondly as contig assemblies (FASTA format), with assembly conducted using NEWBLER. Contigs with a minimum length of 1,000 bp (defined by Eurofins as 'large contigs') were used for all subsequent downstream bioinformatics analysis.

Table 5.2: Overview of DNA sequence yields and quality of *Stenotrophomonas* spp. isolates

Strain name	Isolate source	Yield (MBp)	%Q30	Mean Q
BBS1:13	Beverley Beck	1,196	65.57	27.28
BBS4:12	Beverley Beck	1,280	65.88	27.42
BBS3	Beverley Beck	1,141	65.70	27.34
RHBC2:15	Riseholme beans field	1,046	64.09	26.86
LFWD3:7	Lodge Farm wheat field	1,023	65.30	27.19
LFBF1:10	Lodge Farm beans field	3,502	66.08	27.47
SST-8	Sheffield Hospital sputum	1,242	72.32	28.98
YSM-3	York Hospital blood	1,137	70.75	28.98

Yields are number of bases generated in mega bases (MBp). % Q30 represents the percentage of bases with a quality (Q; Phred) score of at least 30 (A Q score of 30 = a base call accuracy of 1 error in 1000 bp or 99.9% accuracy; a Q score of 20 = a base call accuracy of 1 error in 100 bp or 99% accuracy). The mean Q score (Phred score) is the Q score for all sequence data generated for each genome.

5.2.3 Genome annotation and analysis

Annotation of the environmental and clinical isolates of *S. maltophilia* genome sequences was primarily undertaken using RAST (Rapid Annotation using Subsystem Technology) (http://rast.nmpdr.org/). An overview of the input data used for the eight genomes is shown in Table 5.3. For the six environmental isolates, they had a mean genome size (for large contigs only) of 4.685 ± 0.287 (StDev) Mbp. GC contents ranged from 66.2 to 67%. In contrast the two clinical isolates (SST-8 and YSM-3) had a mean genome size (for large contigs only) of 7.44 ± 0.051 (StDev) Mbp and GC contents of 53.7%.

Table 5.3: Overview of input data used for RAST analysis of *Stenotrophomonas* spp. isolates

Strain name	Total number of contigs	Number of base pairs/ genome size (all contigs)	Number of contigs (≥1000 bp)	Number of base pairs/ genome size (large contigs only)	Mean contig size (bp) for large contigs only	GC content (%)	Q40 plus bases (%)
BBS1:13	191	4,609,592	137	4,589,610	33,500	66.2	99.98
BBS4:12	145	4,601,151	119	4,593,243	38,598	66.3	99.99
BBS3	154	4,915,531	125	4,903767	39,230	66.3	99.99
RHBC2:15	108	4,216,923	90	4,209,114	46,767	67	99.99
LFWD3:7	221	4,814,993	190	4,804,344	25,286	66.2	99.98
LFBF1:10	126	5,033,008	66	5,012207	75,942	66.2	100
SST-8	317	7,430,977	235	7,404,253	31,507	53.7	99.98
YSM-3	203	7,489,512	162	7,477,131	46,155	53.7	99.99

The % of Q40 Plus bases is the proportion of individual bases with a Q score of at least 40 (a Q score of 40 = a base call accuracy of 1 error in 10,000 bases or 99.99 accuracy)

5.2.3.1 Identification of mixed cultures of clinical *Stenotrophomonas maltophilia* isolates

Initial observations of individual contig data using RAST, in comparison to other closely related sequences, immediately suggested that the two genomes from

the clinical isolates contained genome sequences from both *Stenotrophomonas* spp. and *Staphylococcus aureus* bacteria (data not shown). Additionally, their large genome sizes in combination with their GC contents that differed markedly from those of the environmental *Stenotrophomonas* spp. isolates suggested that the genome sequences for isolates SST-8 and YSM-3 were derived from mixed isolates (i.e. co-cultures). As these two isolates had been provided from clinical laboratories, it was deemed essential to confirm whether or not, the isolates being examined were, in fact, mixed cultures. Firstly, the genome sequences were analysed by Genome Peek (http://edwards.sdsu.edu/GenomePeek/) that identifies 16S rRNA, *recA*, *rpoB* and *groEL* genes in genome (or metagenome) data and enables taxonomic assignment of genomes. Both genome sequences were found to include both *Stenotrophomonas maltophilia* and *Staphylococcus aureus* sequences for each of these four taxonomic marker genes. This result was in direct disagreement with the original 16S rRNA gene sequencing data (Chapter 4, section 4.2.4) in which both isolates had been identified as *Stenotrophomonas maltophilia*, i.e. mixed 16S rRNA gene sequences were not generated by PCR. Consequently, the isolates were recovered from frozen culture stocks. Repeated sub-culturing using streak plates followed by Gram stain analysis (data not shown) of individual purified isolates demonstrated that each of these two clinical isolates were a mixture of Gram-negative rod-shaped bacteria and Gram-positive Staphylococci.

The phenotypic characterisation of the newly purified *Stenotrophomonas maltophilia* isolates were repeated to compare these profiles to those produced in Chapter 3, i.e. which were now established to have been conducted on mixed cultures. Phenotypic profiling of the *Staphylococcus aureus* isolates was not undertaken. The results of the phenotypic analyses, namely imipenem resistance MICs, β-lactamase classification and mulitiple antibiotic resistance profiles are shown in Table 5.4. Only one phenotypic difference was observed between the purified (single) and the mixed cultures of these two strains. In the purified SST-8 strain, the MIC of imipenem was 16 μg ml^{-1}, which was lower than in the mixed culture of SST-8 (32 μg ml^{-1}).

Table 5.4: Comparison of antibiotic resistance phenotype of mixed clinical isolates of *Stenotrophomonas maltophilia* SST-8 and YSM-3 strains before and after streak plate isolation to purity

		SST-8 (Mixed)	SST-8 (Single)	YSM-3 (Mixed)	YSM-3 (Single)
ImR MIC (μgml^{-1})		**32**	**16**	>32	>32
Combined Disc Synergy Tests (β-lactamase activity tests)	Class B (MBL)	+	+	+	+
	Class A (KPC)	-	-	-	-
	Class D (OXA)	-	-	-	-
	Class C (AMPC)	+	+	-	-
Multi-Antibiotic-Resistance profiling	TIM	S	S	S	S
	CN	S	S	R	R
	CAZ	S	S	S	S
	LEV	S	S	S	S
	ATM	S	S	S	S
	IPM	R	R	R	R
	CIP	S	S	R	R
	MEM	R	R	R	R
	W	R	R	R	R
	SXT	S	S	S	S
	TE	S	S	S	S
	MH	S	S	S	S

TIM= Ticarcillin-clavulanate, CN= Gentamicin, CAZ= Ceftazidime, ATM= Aztreonam, IPM= Imipenem, MEM= Meropenem, CIP= Ciprofloxacin, LEV= Levofloxacin, W= Trimethoprim, SXT= Co-trimoxazole, TE= Tetracycline, MH= Minocycline. +: positive, -: negative, R: resistant, S: susceptible.

5.2.3.2 Overview of *Stenotrophomonas* spp. isolates genome sequences

RAST (Rapid Annotation using Subsytems Technology) analysis with reference to the SEED Viewer was used to analyse the *Stenotrophomonas* spp. isolate genome sequences. The genome sequences of all six of the *Stenotrophomonas* spp. isolates from environmental sources, and also of the two clinical isolates (SST-8 and YSM-3) were found to be most closely related to genomes of *Stenotrophomonas*

maltophilia strains in SEED. The genomes of two of the isolates from Beverley Beck (BBS1:13 and BBS4:12) and clinical isolate SST8 were found to be most closely related to the genome of the *S. maltophilia* strain R551-3, isolated originally as an endophytic bacterium from a poplar tree (Taghavi *et al.,* 2009). The genomes of the water isolate BBS3, two of the soil isolate (RHBC2:15 and LFBF1:10) and the clinical isolate YSM-3 were most closely related to the genome of the *S. maltophilia* strain K279a isolated originally from a patient in a hospital in Bristol, U.K. suffering from septicaemia following a course of chemotherapy (Crossman *et al.,* 2008). The genome of the soil isolate LFWD3:7 was most closely related to that of the *S. maltophilia* SKA14 strain isolated from the Baltic Sea.

Description of the RAST analysis of the genomes of the two clinical isolates is not provided as their genome data comprised both *Stenotrophomonas maltophilia* and *Staphylococcus aureus* sequence data. Table 5.5 presents an overview of genomic features of the six environmental *S. maltophilia* isolates in comparison to those of their closest related genome sequences in the SEED Viewer. Subsystem coverage was typically higher for the six environmental isolate genomes than in the reference strain genomes, with coverage of between 45-48% for the environmental isolates and of 40-43% for the reference strains. The number of protein encoding genes (PEGs) identified in the environmental isolates ranged from 3,676 PEGs in the soil isolate RHBC2:15 up to 4,609 PEGs in the soil isolate LFBF1:10. The proportion of non-hypothetical genes (i.e. genes for which known functions have been established) in the six isolates ranged from 67.84% for strain LFBF1:10 up to 71.02% for strain RHBC2:15. These proportions were similar to those described for the genome sequences of the reference strains R551-3 and K279a. RAST predicted that between 66 (LFBF1:10) and 120 LFWD3:7) PEGs were possibly missing from the genomes of the environmental isolates. The predicted number of RNA genes in the soil and water isolates was lower than those predicted in the genomes of R551-3 and K279a, but similar to the numbers of RNA genes present in SKA14.

Table 5.5: Comparison of genome features between environmental isolates of *S. maltophilia* and their closest related sequences in the SEED Viewer

Strain name	Number of contigs	Genome size (bp)	Subsystems	Subsystem coverage (%)	Protein encoding genes	Number of non-hypothetical genes	Number of hypothetical genes	Possible missing genes	Number of RNAs
BBS1:13	191	4,609,592	439	45	4074	2840 (69.71%)	1234 (30.29%)	74	69
BBS4:12	145	4,601,151	441	45	4081	2843 (69.66%)	1238 (30.33%)	70	69
BBS3	154	4,915,531	459	45	4465	3080 (68.98%)	1385 (31.01%)	89	73
RHBC2:15	108	4,216,923	438	48	3676	2611 (71.02%)	1045 (28.42%)	77	68
LFWD3:7	221	4,814,993	448	45	4336	2954 (68.13%)	1382 (31.87%)	120	72
LFBF1:10	126	5,033,008	463	45	4609	3127 (67.84)	1482 (32.15%)	66	74
Genome sequences of related organisms in SEED-Viewer									
R551-3	1	4,573,969	389	40	4090	2876 (70.31%)	1214 (29.68%)	N/A	86
K279a	11	4,839,721	425	41	4399	3187 (72.45%)	1212 (27.55%)	N/A	91
SKA14	50	4,990,006	444	43	4469	2990 (66.57%)	1501 (33.42%)	83	74

Subsystems are defined as a set of functional roles that an annotator has decided should be thought of as related (http://www.theseed.org/wiki/Glossary). Subsystem coverage refers to the % of protein encoding genes that can be classified in the SEED Viewer tool. Possible Missing Genes and Numbers of RNAs were calculated by RAST.

The RAST overview of the environmental isolates of *Stenotrophomonas maltophilia* showed that the numbers of genes encoding antibiotic resistance (AR) ranged from 32–58 ARGs. The highest number of these genes were detected in water isolates (BBS1:13 and BBS4:12), whereas the lowest were observed in the isolate from the Riseholme manured farm soil (RHBC2:15). The numbers of ARGs in the other isolates were similar to one another (55, 56, and 56 in LFBF1:10, BBS3, and LFWD3:7 respectively). The numbers of ARGs in the environmental isolates were higher than the numbers observed in the closest related sequences in the R551-3 (31) and K279a (40) isolates. However, these were similar to the number of ARGs (58) observed in SKA14 (Table 5.6).

One of the water isolates (BBS3) showed the highest number of genes that encode resistance to heavy metals (MRGs) (55), whereas the Riseholme (RHBC2:15) manured isolate showed the lowest number (24). In the LFWD3:7 and LFBF1:10 isolates, 49 and 52 MRGs were observed, respectively. Also, the numbers of MRGs in the environmental isolates were higher than observed in the closest related sequences in R551-3 (21). However, this number was similar to the number of MRGs in SKA14 (49) and K279a (51) (Table 5.6).

Regarding mobile genetic element (bacteriophage-related) genes, the highest numbers were detected in the LFBF1:10 isolate (42), whereas the lowest numbers (4) were observed in RHBC2:15. RAST analysis did not identify any other MGE genes either in the environmental isolates or in the isolates RSS1-3, K279a or SKA14. Two water isolates showed moderate numbers of phage related genes (6 and 9) in BBS1:13 and BBS4:12, respectively. Twenty-three MGEs were observed in BBS3, which represented the highest number among water isolates. The closest related sequences in R551-3 and K279a showed lower numbers of MGEs than the amount detected in the environmental isolates (1 and 3 genes, respectively). In contrast, SKA14 had the same number of phage-related genes (23) to isolates BBS3 and LFWD3:7 (Table 5.6).

Table 5.6: Numbers of antibiotic resistance and heavy metal resistance-protein encoding genes and numbers of mobile genetic element (bacteriophage-related) genes identified by RAST analysis of the genomes of *Stenotrophomonas maltophilia* water and soil isolates, compared with genome sequences of related organisms

Strain	Resistance to antibiotics	Resistance to heavy metals	Mobile genetic element genes
BBS1:13	58	25	6 phage
BBS4:12	58	25	9 phage
BBS3	56	55	23 phage
RHBC2:15	32	24	4 phage
LFWD3:7	56	49	23 phage
LFBF1:10	55	52	42 phage
R551-3	31	21	1 phage
K279a	40	51	3 phage
SKA14	58	49	23 phage

5.2.3.3 Overview of antibiotic resistance genes in environmental and clinical isolates of *S. maltophilia*

Various antibiotic resistance genes were detected within the isolates of environmental *S. maltophilia* using RAST. For the two clinical isolates (SST-8 and YSM-3), only antibiotic resistant genes (ARGs) related to *Stenotrophomonas* were counted and included in the subsequent analysis, whereas those related to *Staphylococcus* were excluded.

Genes encoding resistance to antimicrobials can be divided into those mediating resistance to specific antimicrobial classes and those encoding efflux pump systems (Figure 5.2). Genes mediating resistance to specific groups of antimicrobials, such as β-lactamases, and resistance to fluoroquinolones were detected in moderate numbers in most of the isolates. However, genes encoding resistance to aminoglycosides were detected in low numbers in different isolates. The most abundant specific ARGs detected in all of the isolates were β-lactamases encoding genes. These ranged from 8 β-lactamases in the RHBC2:15 strain isolated from the manured Riseholme farm soil to 13 in the non-manured Lodge farm soil

isolate LFWD3:7. The highest numbers of genes encoding β-lactamases (15 genes) were detected in both of the clinical isolates; the YSM-3 strain isolated from a blood sample and the SST-8 strain isolated from sputum. The second most abundant specific class of ARGs were those mediating resistance to fluoroquinolones. Four genes encoding resistance to fluoroquinolones were detected in each of the six environmental isolates, whereas twice that number (8) was detected in each of the clinical isolates (SST-8 and YSM-3). Another observation was the detection of specific ARGs for those mediating resistance to aminoglycosides. There was no trend in the distribution of resistance genes to aminoglycoside. The genes mediating multidrug resistance efflux pumps were the most commonly found resistance genes for all of the isolates, ranging from 23 to 32 genes (Figure 5.2). The lowest number found was 23 in the RHBC2:15 soil isolate, and the highest number found was 32 in both the BBS-3 isolate from water and the YSM-3 clinical isolate (Figure 5.2).

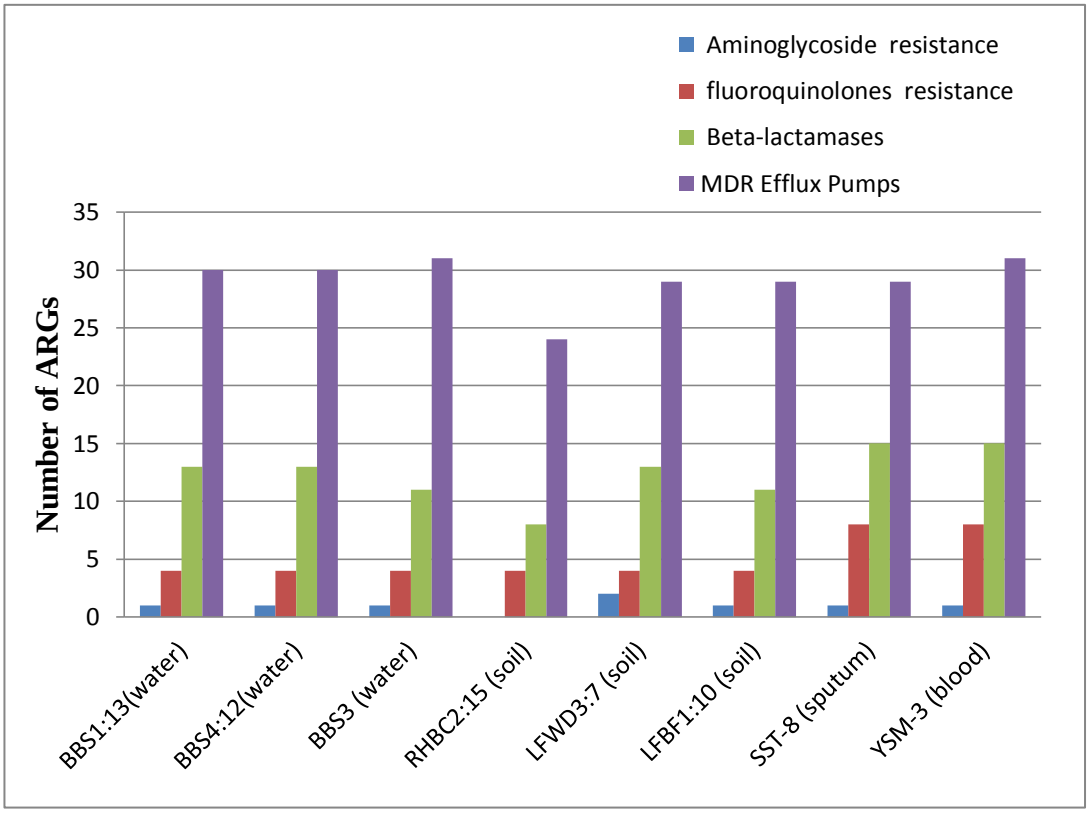


Figure 5.2: Overview of types of antibiotic resistance genes found in environmental and clinical isolates of *S. maltophilia* identified by RAST

5.2.3.3.1 Identification of β-lactamases

Genome analysis using the RAST software system demonstrated that the six environmental and two clinical isolates of *S. maltophilia* produced various classes of β-lactamases (Table 5.7). Although RAST detected a number of β-lactamases produced by each isolate, the exact enzyme class was not definitively given as an output of RAST. Therefore to provide more detailed identification of potential antibiotic resistance genes, a BLAST (Basic Local Alignment Search Tool) search was performed for each of the PEGs identified as β-lactamases by RAST. BLAST is a rapid search tool used in DNA and protein sequencing (Altschul *et al*., 1990; Donkor *et al*., 2014). Analysis of the translated gene sequences (i.e. into protein sequences) from the environmental and clinical isolates of *S. maltophilia* were analysed by BLASTP to identify specific β-lactamases (Table 5.7).

This analysis detected various classes of β-lactamases. Class A β-lactamase (L2) was detected in all eight isolates (BBS1:13, BBS4:12, BBS3, LFBF1:10, RHBC2:15, LFWD3:7 SST-8 and YSM-3). This detection is not consistent with the phenotypic identification in six isolates because this class was observed phenotypically in only two isolates, RHBC2:15 and LFWD3:7 (Table 5.7).

Class B β-lactamase (MBL) was observed in seven isolates (BBS1:13, BBS4:12, BBS3, LFBF1:10, RHBC2:15, SST-8 and YSM-3) and was not detected in one isolate (LFWD3:7). This was broadly consistent with the phenotypic detection in all isolates, with the exception of isolate RHBC2:15, for which class B β-lactamases were only identified from genome data (Table 5.7).

Detection of AmpC, which is related to class C β-lactamase, was observed in only three isolates (BBS1:13, BBS4:12 and LFWD3:7). This was consistent with the phenotypic activity in only one isolate (BBS1:13). However, although isolates BBS3, LFBF1:10 and SST-8 showed phenotypic production of this enzyme (Table 5.7), genes encoding class C β-lactamases were not identified in their genomes.

Class D β-lactamases were not detected either in the phenotypic or molecular characterisation for all of these isolates (Table 5.7).

The closest related sequences for these β-lactamases were those related to various strains of *Stenotrophomona*s previously studied by other researchers, which

were isolated from various sources. These strains include *S. maltophilia*, *S. maltophilia* 5BA-I-2 (soil), *Stenotrophomonas* sp. SKA14 (Baltic Sea), *S. maltophilia* K279a (blood), *S. maltophilia* R551-3 (rhizosphere), *Stenotrophomonas* sp. RIT309 (shrub willow 'salix') and *S. maltophilia* EPM1 (stool) (Table 5.7).

Moreover, the BLAST search identified other β-lactamases (related to *Stenotrophomonas* spp) within all eight isolates. However, these were not classified under known classes. These include 10, 10, 9, 6, 11, 9, 13 and 13 β-lactamases in isolates BBS1:13, BBS4:12, BBS3, RHBC2:15, LFWD3:7, LFBF1:10, SST-8 and YSM-3, respectively.

Table 5.7: Phenotypic and molecular characteristics of β-lactamases in *S. maltophilia* isolates

Isolate name	Source	Phenotypic β-lactamase activity *				Gene length (bp)	Molecular β-lactamase identification (BLAST)					
		Class A	Class B	Class C	Class D		β-lactamase identification	QC %	Identity %	Accession	Closest related sequence (isolation source)	Reference
BBS1:13	Beverley Beck Site 1	N	Y	Y	N	912	β-lactamase Class A (L2)	100	90	WP_025874687.1	*S. maltophilia* (NA)	NA
						1059	β-lactamase Class C (AmpC)	100	85	EVT73212.1	*S. maltophilia* 5BA-I-2 (soil)	Nunvar *et al*., 2014
						801	β-lactamase Class B (MBL)	100	98	EVT73642.1	*S. maltophilia* 5BA-I-2 (soil)	Nunvar *et al*., 2014
BBS4:12	Beverley Beck Site 4	N	Y	N	N	912	β-lactamase Class A (L2)	99	83	CAB63491.1	*S. maltophilia* (blood)	Avison *et al*., 2001
						1059	β-lactamase Class C (AmpC)	100	84	EED37816.1	*Stenotrophomonas* sp. SKA14 (Baltic Sea)	Hagstrom *et al*., 2008
						801	β-lactamase Class B (MBL)	100	92	WP_008264769.1	*Stenotrophomonas* sp. SKA14 (Baltic Sea)	Hagstrom *et al*., 2008
BBS3	Beverley Beck Site 3	N	Y	Y	N	912	β-lactamase Class A(L2)	100	99	CAB63490.1	*S. maltophilia* K279a (blood)	Avison *et al*., 2001
						801	β-lactamase Class B (MBL)	100	99	WP_024958474.1	*S. maltophilia* (NA)	NA
RHBC2:15	Riseholme beans 30130	Y	N	N	N	900	β-lactamase Class A(L2)	99	81	ACF52835.1	*S. maltophilia* R551-3 (rhizosphere)	Lucas *et al*., 2008
						801	β-lactamase Class B (MBL)	100	86	YP_001973501.1	*S. maltophilia* K279a (blood)	Crossman *et al*., 2008

Isolate name	Source	Phenotypic β-lactamase activity *				Gene length (bp)	Molecular β-lactamase identification (BLAST)					
		Class A	Class B	Class C	Class D		β-lactamase identification	QC %	Identity %	Accession	Closest related sequence (isolation source)	Reference
LFWD3:7	Lodge Farm wheat Field	Y	N	N	N	912	β-lactamase Class A(L2)	100	93	ACF52835.1	*S. maltophilia* R551-3(rhizosphere)	Lucas *et al*., 2008
						1116	β-lactamase Class C (AmpC)	96	89	EZP42591.1	*Stenotrophomonas* sp. RIT309 (shrub willow 'salix')	Gan *et al*., 2014
LFBF1:10	Lodge Farm beans 142	N	Y	Y	N	912	β-lactamase Class A (L2)	100	99	CAB63490.1	*S. maltophilia* K279a (blood)	Avison *et al*., 2001
						801	β-lactamase Class B (MBL)	100	96	WP_005418893.1	*S. maltophilia* (NA)	NA
SST-8	Sheffield sputum	N	Y	Y	N	912	β-lactamase Class A (L2)	100	99	AAG15385.1	*S. maltophilia* (NA)	NA
						801	β-lactamase Class B (MBL)	100	99	CAQ47213.1	*S. maltophilia* K279a (blood)	Crossman *et al*., 2008
YSM-3	York blood	N	Y	N	N	912	β-lactamase Class A(L2)	100	99	EMF59206.1	*S. maltophilia* EPM1 (stool)	Sassera *et al*., 2013
						801	β-lactamase Class B (MBL)	100	98	YP_001973501.1	*S. maltophilia* K279a (blood)	Crossman *et al*., 2008

NA: not available, both source of sample and reference (unpublished); bp: base pair; QC: query cover.

* Phenotypic data is as determined in Chapter 3 (Section 3.2.4.2.1) or for the clinical isolates for single strains in Table 5.4.

5.2.3.3.2 Identification of antibiotic resistance using ResFinder

The ResFinder tool from CGE (Centre for Genomic Epidemiology) (http://www.genomicepidemiology.org/) uses BLAST to identify acquired antibiotic-resistance genes (ARGs) in genome data (Zankari *et al.*, 2012). For the two clinical isolates (SST-8 and YSM-3), only ARGs related to *Stenotrophomonas* were counted and included in the subsequent analysis, whereas those related to *Staphylococcus* were excluded. This identified the various ARGs responsible for resistance to aminoglycosides, β-lactams, sulphonamide and tetracycline (Table 5.8). *sph* genes encoding resistance to aminoglycosides were observed in six of the environmental isolates (BBS1:13, BBS4:12, BBS3, LFBF1:10 and LFWD3:7) and in one clinical isolate (YSM-3). However, this gene was not detected in the RHBC2:15 and SST-8 isolates. Other genes encoding aminoglycoside resistance (*aph(3')-IIc* and *aac(6')-Iz*) were only detected in two clinical isolates (SST-8 and YSM-3, respectively). Class B metallo β-lactamase (*blaL1*) was the most commonly detected gene-encoding antibiotic resistance detected by this approach, being found in seven isolates (BBS1:13, BBS4:12, BBS3, LFBF1:10, LFWD3:7, SST-8 and YSM-3), whereas this enzyme was not observed in RHBC2:15. Only two isolates showed *sul1*, which is responsible for sulphonamide resistance. It should be noted here that ARGs were not detected in the RHBC2:15 isolate using ResFinder.

The closest related sequences for these ARGs were those related to various strains previously studied by other researchers, which were isolated from various sources. These strains include *S. maltophilia* strain KJ, *S. maltophilia* strain K1, uncultured bacterium plasmid PB5 (activated sludge), *Escherichia coli*, uncultured bacterium plasmid pIPO2T (wheat rhizosphere), *S. maltophilia* strain K279a (blood) and *S.maltophilia* (sputum) (Table 5.8).

Table 5.8: Antibiotic resistance genes identified in *S. maltophilia* isolates using ResFinder

Strain name	Source	ARGs	% Identity	Predicted phenotype resistance	Accession number	Closest related sequence (isolation source)	Reference
BBS1:13	Beverley Beck Site 1	*sph*	86.47	Aminoglycoside resistance	HQ424461	*S. maltophilia* strain KJ (*sph*) gene (NA)	Huang *et al*., 2010
		blaL1	87.01	β-lactam resistance	EF126059	*S. maltophilia* strain K1 (L1) β-lactamase gene (NA)	Yang *et al*., 2006
BBS4:12	Beverley Beck Site 4	*sph*	86.47	Aminoglycoside resistance	HQ424461	*S. maltophilia* strain KJ (*sph*) gene (NA)	Huang *et al*., 2010
		blaL1	87.01	β-lactam resistance	EF126059	*S. maltophilia* strain KJ (*sph*) gene (NA)	Huang *et al*., 2010
BBS3	Beverley Beck Site 3	*sph*	99.38	Aminoglycoside resistance	HQ424461	*S. maltophilia* strain KJ (*sph*) gene (NA)	Huang *et al*., 2010
		blaL1	89.43	β-lactam resistance	EF126059	*S. maltophilia* strain K1 (L1) β-lactamase gene (NA)	Yang *et al*., 2006
		sul1	100	Sulphonamide resistance	CP002151	Uncultured bacterium plasmid PB5 (activated sludge)	Szczepanowski *et al*., 2011
		tet(C)	99.9	Tetracycline resistance	Y19114	*Escherichia coli* partial plasmid *tetC* gene (NA)	Frech and Schwarz, 1999
RHBC2:15	Riseholme beans 30130	ND	ND	ND	ND	ND	ND
LFWD3:7	Lodge Farm wheat field	*sph*	90.41	Aminoglycoside resistance	HQ424461	*S. maltophilia* strain KJ (*sph*) gene (NA)	Huang *et al*., 2010
		blaL1	98.97	β-lactam resistance	EF126059	*S. maltophilia* strain K1 (L1) β-lactamase gene (NA)	Yang *et al*., 2006

Strain name	Source	ARGs	% Identity	Predicted phenotype resistance	Accession number	Closest related sequence (isolation source)	Reference
LFBF1:10	Lodge Farm beans 142	*sph*	99.38	Aminoglycoside resistance	HQ424461	*S. maltophilia* strain KJ (*sph*) gene (NA)	Huang *et al*., 2010
		blaL1	89.43	β-lactam resistance	EF126059	*S. maltophilia* strain K1 (L1) β-lactamase gene (NA)	Yang *et al*., 2006
		sul1	100	Sulphonamide resistance	CP002151	Uncultured bacterium plasmid PB5 (activated sludge)	Szczepanowski *et al*., 2011
		tet(C)	100	Tetracycline resistance	NC_003213	Uncultured bacterium plasmid pIPO2T (wheat rhizosphere)	Tauch *et al*., 2002
SST-8	Sheffield sputum	*aph(3')-IIc*	97.54	Aminoglycoside resistance	AM743169	*S. maltophilia* strain K279a (blood)	Crossman *et al*., 2008
		blaL1	89.7	β-lactam resistance	EF126059	*S. maltophilia* strain K1 (L1) β-lactamase gene (NA)	Yang *et al*., 2006
YSM-3	York blood	*sph*	99.38	Aminoglycoside resistance	HQ424461	*S. maltophilia* strain KJ (*sph*) gene (NA)	Huang *et al*., 2010
		aac(6')-Iz	97.4	Aminoglycoside resistance	AF140221	*S.maltophilia aac(6')-Iz* gene (sputum)	Lambert *et al*., 1999
		blaL1	89.57	β-lactam resistance	EF126059	*S. maltophilia* strain K1 (L1) β-lactamase gene (NA)	Yang *et al*., 2006

NA: source of sample is not available (unpublished)

To summarize the results obtained by RAST, BLAST and ResFinder, the RAST has classified these different antibiotic resistance genes into their related groups, but it has only illustrated which genes are in which categories without establishing the specific identification of these genes. These ARGs include MDR efflux pump, β-lactamases, fluoroquinolones and aminoglycosides resistance. On the other hand, BLAST identified three different classes of β-lactamases within the isolates, including class A (L2), class B (MBL) and class C (AmpC). However, Res-Finder analysis identified additional ARGs that could be acquired but which were not detected in RAST or in BLAST. These include β-lactamase class B (L1), sulphonamide (*sul1*) and tetracycline (*tet(C)*) resistance. It should be noted that aminoglycoside resistance was detected using all three approaches.

5.2.3.3.3 Overview of heavy metal resistance genes in environmental and clinical isolates of *S. maltophilia*

The genes mediating resistance to heavy metals including mercury, copper, zinc, and cobalt which can be linked to antibiotic resistance, were detected in most of the isolates in variable amounts. For the two clinical isolates (SST-8 and YSM-3), only MRGs related to Gram-negative bacteria were counted and included in the subsequent analysis, whereas those related to Gram-positive bacteria were excluded. The highest numbers of heavy metal resistance genes detected in all of the isolates were those encoding collective resistance to cobalt, zinc, and cadmium. The highest number of these genes were detected in YSM-3 (27), BBS3 (30), LFBF1:10 (34), SST-8 (35), and LFWD3:7 (36). The BBS1:13, BBS4:12, and RHBC2:15 isolates showed the lowest number of these genes (13, 13, and 15) respectively (Figure 5.3). The next most commonly detected heavy metal resistant genes in all isolates were those encoding resistance to arsenic and then copper, with the highest number of genes detected in two clinical isolates (YSM-3 and SST-8). Genes encoding resistance to other heavy metals (e.g., mercury, chromium, and zinc) were less frequently detected, were present only in a few of the isolates, and were also found in lower numbers not exceeding five genes at most (Figure 5.3).

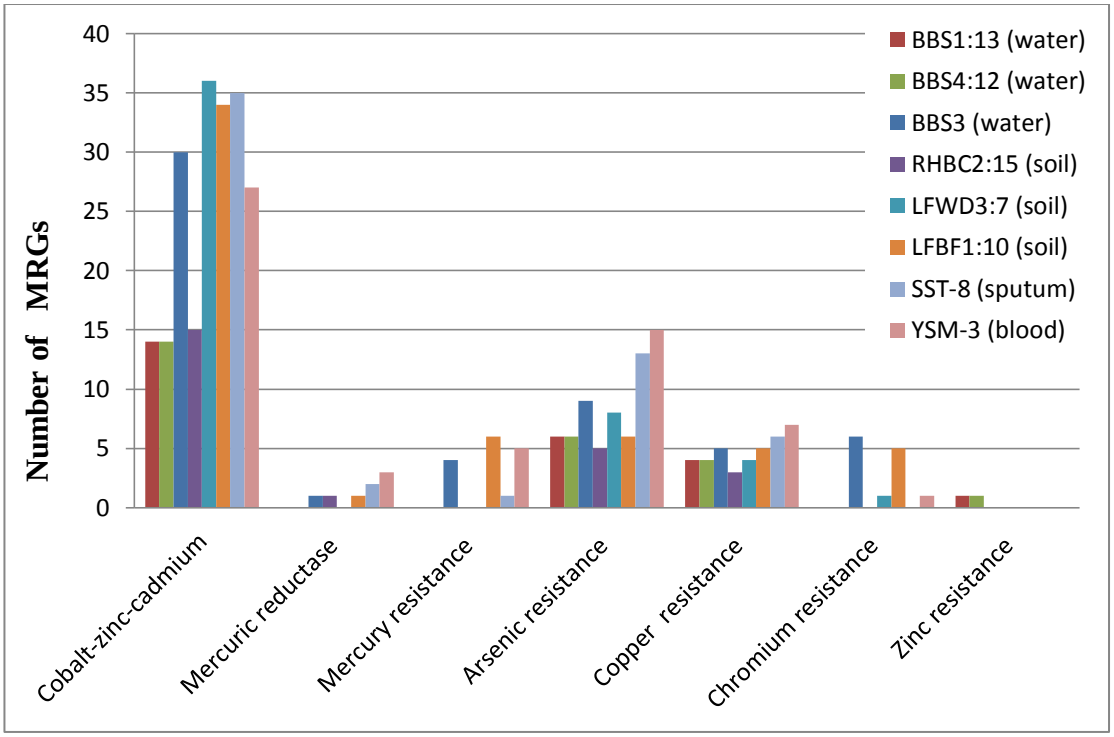


Figure 5.3: Overview of types of heavy metal resistance genes found in environmental and clinical isolates of *S. maltophilia* identified by RAST

5.2.3.4 Estimation of pathogenicity

The potential for the *S. maltophilia* strains to be human pathogens was investigated (Table 5.9) using the PathogenFinder tool from CGE (Centre for Genomic Epidemiology) (http://www.genomicepidemiology.org/). Based on similarities to known pathogenic strains, both of the clinical strains SST8 and YSM-3 showed, as expected, a high probability of being pathogenic, with strain SST-8, isolated from sputum, having the highest potential pathogenicity, matching 378 pathogenicity profiles to the genome of the opportunistic pathogen *Stenotrophomas maltophilia* K279a (Crossman *et al.*, 2008). In addition, four of the environmental strains: BBS3, BBS1:13 and BBS4:12 (isolated from river water) and LFWD3:7 (isolated from non-manured farm soil) were also predicted by PathogenFinder as being potential pathogens for humans but with a lower likelihood, matching pathogenicity profiles of *S. maltophilia* K279a in 27, 9, 20 and 26 families, respectively. The two remaining soil isolates (RHBC2:15 and LFBF1:10) were not predicted to be human pathogens using PathogenFinder.

Table 5.9: Prediction of pathogenicity in environmental and clinical isolates of *S. maltophilia* identified by PathogenFinder

Stenotrophomonas maltophilia strain	Prediction as human pathogen	Probability of being a human pathogen*	Matched Pathogenic Families	Best matched organism	ACCESSION ID
BBS1:13 (water)	Y	0.657	9	*S. maltophilia* K279a	NC_010943.1
BBS4:12 (water)	Y	0.643	20	*S. maltophilia* K279a	NC_010943.1
BBS3 (water)	Y	0.617	27	*S. maltophilia* K279a	NC_010943.1
RHBC2:15 (soil)	N	0.429	8	*S. maltophilia* K279a	NC_010943.1
LFWD3:7 (soil)	Y	0.608	26	*S. maltophilia* K279a	NC_010943.1
LFBF1:10 (soil)	N	0.203	1	*S. maltophilia* K279a	NC_010943.1
SST-8 (sputum)	Y	0.974	378	*S. maltophilia* K279a	NC_010943.1
YSM-3 (blood)	Y	0.975	881	*S. maltophilia* K279a	NC_010943.1

* Probability is on a scale from 0 to 1, where higher scores indicate increased probability of an organism being a human pathogen

5.2.3.5 Multi Locus Sequence Typing of *Stenotrophomonas* spp. isolates

Multi Locus Sequence Typing (MLST) is a technique introduced in 1998 for the identification and typing of different bacterial isolates and strains of individual species, which works by identifying the sequence of different alleles of conserved housekeeping loci (genes) (Maiden *et al*., 1998). MLST was used for typing and identification of antimicrobial resistant isolates of *Stenotrophomonas maltophilia* (Song and Shin, 2012). The Multi Locus Sequence Typing (MLST) web-server (http://cge.cbs.dtu.dk/services/MLST/) was used to conduct *in silico* MLST analysis on seven of these housekeeping genes of the *Stenotrophomonas* spp. isolates (Table 5.10). For *S. maltophilia*, these genes contain between 26 and 56 different known alleles and the technique was considered to be sufficiently rapid and discriminatory to be suitable for the typing of *S. maltophilia* isolates (Song and Shin, 2012). On this basis, one of the clinical isolates (SST-8) was found to belong to the *S. maltophilia* ST-31 MLST class, while the other seven isolates did not exactly match any other known *S. maltophilia* strains on the database, although each of these isolates had genes that were 100% identical to at least one of the alleles of the seven genes (loci).

Table 5.10: MLST profile (allelic profile) for environmental and clinical isolates of *Stenotrophomonas* spp.

Isolate	BBS1:13		BBS4:12		BBS3		RHBC2:15		LFWD3:7		LFBF1:10		SST-8		YSM-3	
Source	Beverley Beck site 1		Beverley Beck site 4		Beverley Beck site 3		Riseholme beans 30130		Lodge Farm wheat 144		Lodge Farm beans 142		Sheffield sputum		York blood	
MLST	Un-ST		Un-ST		Un-ST		Un-ST		Un-ST		Un-ST		ST-31		Un-ST	
Locus	Allele	% ID	Allele	% ID	Allele	% ID	Allele	% ID	Allele	% ID	Allele	% ID	Allele	% ID	Allele	% ID
atpD	*atpd_33*	100	*atpd_33*	99.44	atpd_4	100	*atpd_39*	99.06	*atpd_33*	98.87	*atpd_4*	98.87	*atpd_3*	100	*atpd_5*	100
gapA	*gapa_31*	99.46	*gapa_31*	99.82	gapa_22	99.82	*gapa_6*	91.22	*gapa_53*	100	*gapa_22*	100	*gapa_4*	100	*gapa_22*	99.64
guaA	*guaa_12*	96.25	*guaa_12*	98.55	guaa_14	98.73	*guaa_45*	96.74	*guaa_14*	98.55	*guaa_14*	98.55	*guaa_24*	100	*guaa_2*	98.73
mutM	*mutm_32*	96.34	*mutm_32*	100	mutm_5	100	*mutm_11*	90.11	*mutm_32*	97.20	*mutm_5*	97.20	*mutm_7*	100	*mutm_5*	99.57
nuoD	*nuod_35*	99.77	*nuod_35*	98.87	nuod_9	99.77	*nuod_61*	95.5	*nuod_43*	99.32	*nuod_9*	99.32	*nuod_7*	100	*nuod_9*	99.77
ppsA	*ppsa_46*	100	*ppsa_46*	98.79	ppsa_6	99.19	*ppsa_43*	91.98	*ppsa_45*	97.98	*ppsa_6*	97.98	*ppsa_22*	100	*ppsa_6*	99.19
recA	*reca_12*	100	*reca_12*	97.25	reca_9	100	*reca_35*	94.14	*reca_37*	97.62	*reca_9*	100	*reca_7*	100	*reca_9*	99.45

NB: Un-ST: unknown *Stenotrophomonas* spp sequence type; %ID = % Identity.

5.3 Discussion

Several molecular methodologies are available, including PCR, gene probing, sequencing, and cloning, for the examination and characterisation of microorganisms at the molecular level of study e.g. the study of nucleic acids and genes. Each method has its advantages and disadvantages and the results depend on strict application of the protocols (Moore *et al*., 2004). For genomic DNA (gDNA) sequencing, the preparation of the sample is very important as the optimal quantity and quality of gDNA is crucial.

The genome sequencing in the current study was done to explore the resistance pattern (specifically of β-lactamases and other antibiotic resistance) of *Stenotrophomonas* spp. isolates at molecular level. This was to allow comparison of their genomes and to other strains previously sequenced, and also to enable the comparison of the molecular data obtained here with that observed phenotypically (Chapter 3).

The choice of *Stenotrophomonas* spp. isolates was based upon this being the only genus detected in all of the environmental (water and soil) and clinical samples which showed a high MDR pattern and was detected in reasonable numbers in the current study.

As the quantification of gDNA is an important step before starting the sequencing process, it was measured using the NanoDrop® ND-1000 spectrophotometer and the DNA concentration adjusted as recommended. It is known that if the DNA concentration in the sample is very high this will lead to the overlapping of the produced sequencing clusters. On the other hand, if the gDNA is less than the optimal amount needed, then it will give clusters with low density and both the high and low concentrations of gDNA will affect the sequencing results (Buehler *et al*., 2010).

The GC-contents of the isolates in the current study were between 53.7% and 67%; this is considered a reasonable percentage for obtaining a good sequence without biases occurring due to the variation in GC-contents (Chen *et al*., 2013). However it is known that bacterial genomes have wide variations in GC contents, starting from 17% and rising to 75% (Brocchieri, 2014). The lower the GC contents

the higher the sequence errors, and the higher the GC-contents the higher the fragmentations during sequencing, which can be misread as repetitive elements. However, the threshold value for the high and low GC contents were determined and in some cases the lower limits were less than 25% (Chen *et al*., 2013).

The GC contents of the six environmental isolates in the present study are comparable to each other and similar to those from previously sequenced and documented *Stenotrophomonas maltophilia* isolates in different studies. For example, the GC content for a S208 MDR isolate was 67.07% (Song *et al*., 2012); for strain AU12-09 from an intravenous catheter tip the GC content was 66.5% (Zhang *et al*., 2013) and for strain WJ66 isolated from human blood the GC content was 66.48% (Zhao *et al*., 2015).

The total genome lengths of the six environmental isolates in the current study were found to vary. The shortest one was 4,216,923 bp (in RHBC2:15), while the longest genome was 5,033,008 bp (in LFBF1:10). The lowest possible number of missing genes (66) were detected in the LFBF1:10 isolate, which showed the longest genome size (5,033,008) and the largest number of PEGs (4,609).These findings are comparable to those detected in a clinical strain (D457) from Spain where the total genome contained 4,769,156 bp with 66.8% GC content found to contain 30 pseudogenes and 200 new genes not reported in other isolates. These were mostly encoding hypothetical proteins and transposases, indicating the larger size of the genome of *Stenotrophomonas maltophilia* (Lira *et al*., 2014).

In the current study, RAST analysis of the two clinical isolates, SST-8 and YSM-3 showed that these were derived from mixed isolates (i.e. co-cultures) of both *Stenotrophomonas maltophilia* and *Staphylococcus aureus*. This was apparent from their large genome sizes and lower GC contents, i.e. in comparison to the other *Stenotrophomonas* spp. genomes. However, the detection of this mixed culture had escaped identification as a mixed culture when performing PCR and sequencing of 16S rRNA for these two isolates obtained from hospitals. This could highlight the limitations of the conventional 16S rRNA sequencing in bacterial identification.

Salipante and colleagues (2013) suggest using deep 16S rRNA sequencing (i.e. sequencing of a region in a genome many times) for the primary identification of pathogenic bacteria in many clinical samples, including brain abscess samples,

lymph node samples and sputum from cystic fibrosis patients. Similar to our findings, they reported the miss-identification of some microorganism using this approach and the most commonly miss-identified organism was *Staphylococcus aureus*. However, some studies have reported that using 16S rRNA in organism identification is more accurate than other methods, including phenotypic characterisation (Clarridge, 2004, Petti *et al.*, 2008).

Infection caused by opportunistic organism is frequently polymicrobial. This has been observed in co-infections with *Pseudomonas aeruginosa* and *S. aureus* which had small colony variants (SCVs) which were difficult to detect (Hoffman *et al*., 2006). These SCVs in *S. aureus* have been detected in various infections, e.g. in the airways of more than 50% of cystic fibrosis (CF) patients, and these infections were also found to persist for many years (Proctor *et al*., 2006).

SCVs grow slowly and have uncommon biochemical features, which have gone undetected in mixed cultures and present a challenge as they are difficult for hospital microbiologists to identify phenotypically (Proctor *et al*., 2006).

Although the presence of mixed bacterial cultures in the clinical samples did not affect the research work in the current study, this has a very important application in clinical practice itself. The interpretation of a mixed culture as being a single bacterium would have detrimental effects upon the modality of the selected antimicrobials for the treatment of such infections. The application of different accurate laboratory diagnostic sets is important even if it included both phenotypic and genotypic methodologies to isolate the exact infectious agent. This is our finding, which, along with the other reports, may argue for routine identification of potential non-diagnosed roles of *S. aureus* by genomics when an opportunistic organism is present. Detection and treatment of *S. aureus* can improve the outcomes for the patient (Hoffman *et al*., 2006).

Fortunately, this mixed culture did not affect the results of the genome study, as we extracted only the information from the ARGs that related to *S. maltophilia*, and information about *S. aureus* was ignored. This was confirmed by blasting each of the PEGs, which included only the closest sequences that showed *Stenotrophomonas* identification and features; for example, the identification of class B metallo-β-lactamases (L1) and class A (L2), which are enzymes common in

Stenotrophomonas. However, there have been no reports of its detection in Gram-positive microorganisms (Avison *et al.*, 2002: Hu *et al*., 2008). Also, horizontal transfer of these genes from *S. aureus* to *S. maltophilia* is unlikely (but not impossible) as HGT does not commonly take place from Gram-positive to Gram-negative bacteria, although the reverse has been reported (Musovic *et al*., 2006; Klümper *et al*., 2014).

Strains of *Stenotrophomonas maltophilia* are lacking in distinct phenotypic variations. However, in 1999, genome fingerprinting studies of more than 100 environmental and clinical isolates of *Stenotrophomonas maltophilia*, by the use of amplified fragment length polymorphism (AFLP), detected great genomic diversity in these isolates. The genetic homology between these isolates ranged from 15-30% (Hauben *et al*., 1999).

Based upon this reported genomic diversity of *S. maltophilia*, it was then not regarded as unexpected in the current study to find that genome sequencing revealed similarities in two of the aquatic isolates from Beverley Beck (BBS1:13 and BBS4:12), and one clinical isolate SST8, to the genome of R551-3 isolated from a poplar tree. The R551-3 strain is one of the endophytic bacteria that live within the plant without causing it any harm and is mostly beneficial in helping to promote growth, exclude phytotoxic compounds and fix nitrogen (Taghavi *et al*., 2009). Furthermore, the genome of BBS3 water isolate, RHBC2:15 and LFBF1:10 soil isolates, and YSM-3 clinical isolates in the current study were closely related to K279a isolated from the UK in 1998 (Crossman *et al*., 2008), which may make it difficult to define the characteristics which would identify the clinical or environmental isolates of such genotypically heterogeneous microorganisms.

Genes representing resistance to many classes of antimicrobials were detected by genome study of the isolates using the RAST search tool. These classes of antimicrobials included β-lactams, fluoroquinolones, and aminoglycosides. Resistance to additional classes of antimicrobials, including tetracyclines and sulphonamides, was detected by the application of another search tool (ResFinder) which is used for the detection of potential acquired resistance (Zankari *et al*., 2012).

Trimethoprim-sulphmethoxazole (co-trimoxazole) was considered as the drug of choice in the treatment of infections caused by *Stenotrophomonas maltophilia*

(Nicodemo and Paez, 2007). However, resistance to co-trimoxazole is nowadays reported worldwide (Toleman *et al*., 2007). Resistance to co-trimoxazole was not reported phenotypically in the K279a strain and the gene mediating the sulphonamide resistance was not detected (Crossman *et al*., 2008). However, in the current study, phenotypic resistance to co-trimoxazole was detected in some isolates, and the *sul1* gene mediating resistance to sulphonamide was detected in two of the isolates (BBS3 from water and LFBF1:10 from soil at Lodge farm) by the ResFinder tool, but not by RAST.

β-lactam antimicrobial resistance genes were analysed in detail and were ascribed to their corresponding class in Ambler classification, which is the most widely adopted classification. RAST software did not serve in the identification of the enzymes classes, while the BLAST search was effective in the identification of known classes of β-lactamases.

Fifteen genes encoding β-lactamases were detected in each of the two clinical isolates (SST-8 and YSM-3) of the current study, which is a higher number than that reported in the comparable K279a clinical isolates, where only four L1 and L2 β-lactamase genes were reported (Crossman *et al*., 2008). This was also a higher number than that reported in a clinical isolate (AU12-09) from an intravenous catheter tip, where only 11 genes were detected (Zhang *et al*., 2013). However, these 15 β-lactamase genes in the two clinical isolates represents roughly only half the number detected recently in a clinical isolate (WJ66) obtained from blood, in which 33 β-lactamase genes were reported (Zhao *et al*., 2015). This gradual increase in the numbers of genes encoding resistance to β-lactamases may be due to the selective pressure exerted by the increased use of β-lactam antimicrobials in clinical practice (Safdar and Rolston, 2007) and the increasing use of broad-spectrum antimicrobials and increasing numbers of immuno-compromised patients (Song *et al*., 2012).

Genes encoding β-lactamases were detected in lower numbers in environmental isolates than in the clinical isolates, ranging from eight genes in RHBC2:15 isolated from soil at Riseholme farm to 13 genes in LFWD3:7 from soil at Lodge farm, and also in both of the water isolates BBS1:13 and BBS4:12. In general, the number of ARGs (55-58) within most of our environmental isolates was similar to the number of ARGs (58) found in the closest related sequence of SKA14 isolated from an environmental source (i.e. the Baltic Sea). However, this was not

similar to the number of ARGs in other closest related sequences, including R551-3 (31) and K279a (40) isolated from rhizosphere and blood samples, respectively.

In addition to chromosomally-borne antimicrobial resistance genes in *Stenotrophomonas maltophilia*, the detection of potential acquired antibiotic resistance using ResFinder and also the presence of mobile genetic elements (MGEs) may highlight the important potential role of HGT in the dissemination of this resistance to other bacteria. Avison *et al.* (2000) have detected class A (L2) and class B (L1) β-lactamases in all 10 examined *S. maltophilia* isolates and reported that these enzymes were carried on plasmid-like elements. They also suggested that these elements are likely to disseminate to other Gram-negative bacteria, which could lead to clinical problems. In the current study, the lowest number of these MGEs (4 phage-related genes) were detected in the soil isolate RHBC2:15, while the highest number (42) were detected in the soil isolate LFBF1:10. These MGEs are present in higher quantities than those found by RAST analysis in the related clinical K279a strain (3 phage) (Crossman *et al*., 2008), and the environmental R551-3 strain (1 phage) (Taghavi *et al*., 2009). These phages are integrated in the chromosome via the action of integrase enzymes (Brown-Jaque *et al*., 2015). The absence of plasmid and/or transposon-related genes in either the eight genomes analysed here, or in the genomes of their closest related strains, may reflect the limitations of the RAST annotation (i.e. these MGE genes have not been assigned yet to subsystems). Alternatively, it may also reflect the challenges of co-extracting plasmid DNA, during genomic DNA isolation. For example, only approximately 35% of the ∼3100 complete and published genomes stored on the Genomics Online database (gold.jgi-psf.org) contain plasmid sequences.

Genes encoding MDR efflux pumps, which play a role in resistance to antimicrobials, were detected in higher numbers in all of the sequenced isolates in the current study ranging from 24 to 32 genes. This is comparable to the numbers detected in the K279a isolate, where 31 antibiotic efflux pumps were observed which mediate resistance to chloramphenicol, fluoroquinolones and tetracyclines (Crossman *et al*., 2008). Also, it is similar to the AU32 clinical isolate, in which 24 genes encoding antibiotic resistance efflux pumps were detected (Zhang, 2013) and to the WJ66 clinical isolate, where 32 efflux pump genes were detected (Zhao *et al*., 2015).

Stenotrophomonas maltophilia has been isolated from a variety of environmental contexts, including soil, water and food. It is one of the most common opportunistic pathogens nowadays, with its MDR pattern making treatment a significant problem (Adamek *et al*., 2014). The strains isolated in the current research have been evaluated for human pathogenicity and have been investigated to check whether potential differences between environmental and clinical isolates are significant in causing diseases.

The PathogenFinder tool (Table 5.9) was used to relate our *Stenotrophomonas maltophilia* isolates to pathogenic strains which have already been reported and documented. The results showed that the clinical isolates SST8 and YSM-3 have the highest pathogenic probabilities due to their matching 378 and 991 pathogenic families respectively. This result was as expected, with the highest probabilities on the pathogenicity scale at 0.974 and 0.974, respectively, (probability is on a scale from 0 to 1). Also, interestingly, four isolates (BBS3, BBS1:13 and BBS4:12 isolated from water) and LFWD3:7 (isolated from soil) showed potential pathogenicity matching to between 9 and 27 pathogenic families, with a lower overall rate (0.617 – 0.657) than the two clinical isolates. However, two soil isolates (RHBC2:15 and LFBF1:10) were shown not to be probably pathogenic strains, matching only 1-8 pathogenic families with the lowest overall rate (0.203-0.429). The best matched organism to all the eight isolates, using Pathogen Finder, was the clinical isolate K279a, as reported by Crossman *et al*., (2008).

A study was done to investigate the virulence factors of *Stenotrophomonas maltophilia* both in a clinical (SKK35) and an environmental (RA8) strain, using three known strains as a control (K279a, R551-3, and SKA14). They found that most of the studied genes for virulence (protease, hemolysin and phospholipase) were present in all the studied isolates. Furthermore, they did not detect the gene encoding the toxin Zonula occludens in any of these strains (Adamek *et al*., 2014). These findings may make the differentiation of the virulence between the clinical and environmental isolates of the organism more difficult and demonstrate the need for further investigations.

Genes encoding resistance to heavy metals were detected in all of the sequenced isolates and in different frequencies (Table 5.6), ranging from 24 genes in soil isolate RHBC2:15, and 25 genes in two of the water isolates (BBS1:13 and

BBS4:12), with the highest numbers detected in two of the soil isolates (LFWD3:7, LFBF1:10) and one water isolate (BBS3) which carried 49, 52, and 55 metal resistance genes, respectively. The genes encoding resistance to cobalt-zinc-cadmium (*czc*) were the most abundant metal resistance genes detected in all of the isolates (Figure 5.3), ranging from 14 to 36 copy numbers of *czc* genes. The second most commonly found heavy metal resistance genes in the current study were those encoding resistance to arsenic, followed by copper, ranging from 4 to 15 genes in different isolates. Genes encoding mercury and chromium were the least abundant heavy metal resistance genes to be detected and were only present in a few isolates in lower numbers, ranging from 1-5 genes. These heavy metal genes are comparable to those detected in the K279a strain, where arsenic, mercury, and copper resistance genes were detected, while resistance to cadmium was associated with efflux pumps (Crossman *et al.*, 2008).

Resistance to heavy metals, which are present as contaminants in the environment at levels higher than those of antimicrobials, is often linked with resistance to antibiotics in several mechanisms (Baker-Austin *et al*., 2006). The first mechanism is via the co-resistance to both heavy metals and antimicrobials when the genes encoding both are present on the same genetic elements, e.g. integrons, transposons and/or plasmids. For example, there can be resistance to mercury and ampicillin on the same plasmid (Chapman, 2003) or resistance to copper, macrolides and glycopeptides (Hasman and Aarestrup, 2002). The second mechanism is cross-resistance, when the targets of actions of both metals or antimicrobials are the same; if it is damaged by the metals then a resistance to both the antimicrobials and the metals will develop. This is also exemplified by the MDR efflux pump, which excludes both heavy metals and antibiotics from the cell (Chapman, 2003; Baker-Austin *et al*., 2006). The third mechanism is via the co-regulation of both transcriptional and translational processes for both heavy metals and antibiotics when facing stressful conditions (Lee *et al*., 2005). The last mechanism is via the formation of biofilm by bacteria which affects both the heavy metal and antibiotic actions (Stewart and Costerton, 2001). It is known that *Stenotrophomonas maltophilia* is one of the organisms that forms biofilms (Di Bonaventura *et al*., 2004).

Using the MLST tool (Table 5.10) for the analysis of all eight sequenced isolates showed that only the clinical isolate SST-8 was related to *S. maltophilia* ST-31, while the seven other isolates were not related to known strains. ST-31 was isolated from blood culture in 2002 in Perth, Australia (*S. maltophilia* MLST Isolate Database). This finding, despite being possibly due to the less abundant strains stored in the database of the MLST server (Song and Chen, 2012), may lend support to the previous discussion of the genetic diversity of *S. maltophilia* strains. This genetic diversity was also reported when using the MLST for the typing of 70 different strains of *S. maltophilia* (Kaiser *et al*., 2009).

5.4 Conclusion

Genome sequencing in the current study was done to detect different types of resistance genes which were not detected in the previous chapters by phenotypic and PCR analysis. Results showed the presence of many antimicrobial resistance genes and genes related to heavy metal resistance, which play a role in the resistance to antibiotics. However, the RAST tool was not efficient enough to detect the type of β-lactamase genes, while the BLAST tool provided more detailed characterisation of the ARGs. Although the clinical isolates were more highly matched to pathogenic families than the environmental isolates, the environmental isolates may still cause pathogenicity and the difference in the virulence factors between clinical and environmental isolates of the organism suggests the need for further work and to investigate their potential pathogenicity. Although genomic studies are time-consuming and expensive, they are more accurate and informative about the genetics of bacteria. Efforts are needed to make genomic study, and especially bioinformatics analysis, easier and more convenient for both laboratory and clinical and diagnostic work. The MLST server has been reported as accurate in the typing of *Stenotrophomonas maltophilia* by some studies; however, it is seldom used, which makes it of limited value in the typing and relating of isolates due to the small *S. maltophilia* MLST isolate database. The *Stenotrophomonas maltophilia* genome is highly divergent, occurring in different sizes in different isolates, and it acts as reservoir of antimicrobial resistance genes in both environmental and clinical isolates.

Chapter 6: Concluding Discussion

Infectious bacteria are showing ever-increasing levels of multiple resistance to antibiotics. Antimicrobial resistance has a detrimental effect on human health worldwide and also affects the global economy. Humans, animals and the natural environment, including soil and water, are all potential reservoirs of resistance genes, which are able to move between these sources. In order to limit the impact of bacterial antibiotic resistance, it is important to be able to identify the origin of resistant bacteria and genes as well as the different ways in which these genes are being transferred to clinical isolates of bacteria (Bush *et al*., 2011).

Antimicrobial drug resistance (AMR) is promoted by direct selective processes which are triggered as a response to antimicrobial use in humans or animals. Evolution of AMR also takes place in the natural environment, in which antibiotic-producing bacteria stimulate resistance in bacteria of agricultural soil or in sewage water treatment systems. Once AMR has developed by mutation and selection, the *de novo* resistance genes can then spread to other bacterial isolates by the process of horizontal gene transfer (HGT). A variety of transfer mechanisms have been identified, including conjugation, transduction and transformation. Soil in the natural environment has been found to be a major reservoir of AMR genes, due to the presence of most of the determinants for HGT (Gaze *et al*., 2008).

AMR in bacteria can be achieved by one of four mechanisms: modification of the drug before it reaches the target, exclusion of the drug from the cell by efflux pump, changing the target of antimicrobial action, or production of enzymes which destroy the antimicrobial (Gaze *et al*., 2008). Resistance to antimicrobials can be detected in the laboratory either by phenotypic or genotypic methods.

Although a number of powerful antimicrobials are currently considered to be drugs of 'last resort' for treating resistant bacteria, e.g. vancomycin for methicillin-resistant *Staphylococcus aureus* and imipenem for extended-spectrum β-lactamase producers, both the development and spread of resistance to these antimicrobials are of great concern as they have begun to have a major impact on the efficacy of these drugs.

The primary aim of this research was to investigate the presence and abundance of bacteria resistant to these two important antimicrobials and, in

particular imipenem, in environmental samples. Water samples were collected from the Beverley Beck section of the River Hull, U.K., which was chosen for water sampling because of its proximity to a sewage works which pumps biologically treated effluent into the beck. Soil samples were taken from agricultural soil on two farms in Lincolnshire, U.K. Riseholme (with manure organic fertiliser) and Lodge Farm (with conventional inorganic fertilisers). Isolation of resistant bacteria was performed using a phenotypic method (culture-based isolation). Bacterial resistance to vancomycin in both the river water and the farm soil was higher than resistance to imipenem. This can most likely be attributed to the predominance of Gram-negative bacteria in the samples, which lack the target for this antibiotic. The manured soil contained higher numbers of resistant bacteria than the non-manured soil, which indicates the potential effect of manure in increasing antibiotic-resistant bacteria and their genes (Udikovic-Kolic *et al*., 2014).

The presence of culturable antimicrobial-resistant bacteria in the current study indicates the importance of river water and farm soil environments as a reservoir and potential site for the transport of antibiotic-resistant bacteria. However, culture-based isolation may only identify between 1.5% and 10% of the total bacteria in soil compared to molecular methods (Janssen, 2006). The use of different molecular techniques would be advisable in the future investigation of antimicrobial resistance in environmental samples in order to overcome the limitations of culture-based methods.

The metagenomic approach is a recently introduced technique in culture-independent study entailing sequencing and analysis of whole genomes of bacteria present in environmental samples. The technique involves extracting DNA directly from environmental samples, either for direct shotgun sequencing, or following cloning of metagenomic DNA into a surrogate host. For metagenomic libraries, DNA can be studied by screening the function of the cloned DNA (functional metagenomics) or by direct sequencing of the cloned DNA by random sequencing using vector-based primers (Thomas *et al*., 2012). The advantages of metagenomics are that the details of the composition and function of the genes in microbial communities can be obtained, thus new enzymes can be identified and genes from the uncultured organisms can be linked to function. This is of considerable value in the identification of *de novo* genes and functions (Beja *et al*., 2000; Gilbert *et al*.,

2008). Up until recently, however, cost has been a limitation of this technique, although recently the cost has now fallen closer to that of 16S rRNA gene sequencing (Thomas *et al*., 2012). It would seem likely that the development of more economical techniques will continue to lead to greater advancements in genomic studies.

The spread and distribution of environmental AMR bacteria is known to be affected by a number of different factors, including seasonal variations and human activities, e.g. the use of antimicrobials in animal farming and manure in agriculture (Sahoo *et al*., 2010). Consequently, studying the impacts of such variables in future research could be productive as they may play a role in the development and dissemination of AMR. This also might allow us to suggest some control strategies to reduce AMR spread. The role of soil and water environments in the transfer of resistant bacterial pathogens to humans was not addressed by the current research. However, studying the presence of resistant bacteria in food obtained from these aquatic and soil environments could provide an interesting additional avenue for research (WHO, 2011; Capita and Alonso-Calleja, 2013).

The second phase of the study was the characterisation of imipenem resistance in clinical isolates obtained from different samples from Sheffield, York and Hull hospitals. Although it was found that the highest percentage of resistance among the clinical isolates was in Hull (61.3%) compared to 35.5% and 38.7% in York and Sheffield, respectively, these data are not representative of the actual prevalence of antimicrobial resistance in hospitals or areas of study, as this needs to be related to the total numbers of isolated bacteria of the same and other species of bacteria. It is suggested that patterns of clinical antimicrobial resistance are studied over long time periods in order to compare the effects of different seasonal and geographical factors on the spread of resistance (McCormick *et al.* 2003). It may also be useful to investigate the effect of various infection control measures on the spread of resistance among different healthcare facilities. Several studies have shown that social, behavioural and environmental factors affect the spread of antimicrobial resistance (Heymann, 2006; Sahoo *et al*., 2010).

A recent meta-analysis of 9,748 microbiology reports from 2009 to 2012, reported that carbapenem resistance is increasing yearly and is emerging as a healthcare problem in the U.K. (Freeman *et al*., 2015). The study found high

incidences of resistant isolates in two London hospitals, with increased rates of infections and colonization among the younger age group (16-24 years). The highest percentages of resistance were reported in *Acinetobater baumannii* (65.8%; 129/196), *Pseudomonas aeruginosa* (19%; 591/3119), *Enterobacter* spp. (7.8%; 69/885), *Klebsiella* spp. (5.7%; 49/865) and the lowest percentage of ImR was in *Escherichia coli* (0.9%; 43/4683). These data show the need for more effective control of infections to reduce the increasing spread of ImR (Freeman *et al*., 2015). In another recent study, carbapenem-resistance was found in 60% of *Acinetobacter* spp. isolates in a Korean hospital (Lee *et al*., 2015).

In this study, a suite of PCR primers were used to detect ARGs, but with mixed outcomes. A limitation of PCR technology is that it requires the sequence of ARGs to be known to allow primer design (Riesenfeld *et al*., 2004). However, a selective cloning approach from either metagenomics (or genomic DNA) may provide a better alternative. This has the advantage of detecting new genes by cloning the directly extracted soil (or bacterial) DNA into an applicable host, e.g. *Escherichia coli*, and then sequencing selected cloned strains which express the resistance character of the antimicrobial's soil DNA, which enables the detection of different ARGs in soil samples. Selective cloning is a culture-independent method which has identified resistance to tetracyclines and aminoglycosides in soil, and also detected the possible mechanisms of resistance after sequencing of the implicated genes (Riesenfeld *et al*., 2004).

16S rRNA sequencing is now widely used to identify isolates at the genus and species level (Srinivasan *et al*., 2015), and it is the most frequently used method, even for the identification of the most difficult-to-identify isolates (Chen *et al*., 2014). The use of 16S rRNA genes can also be of help in the primary identification of clinically important bacteria, especially those difficult to grow, such as *Mycobacterium tuberculosis* and *Burkholderia* spp. In the current research, 16S rRNA sequencing was applied to culturable bacteria. Bacterial isolates in the current study were identified using the BLAST alignment approach of 16S rRNA sequencing (Altschul *et al*., 1990). However, the newer Naïve Bayesian (NB) classifier was found in some instances to be superior to the alignment method (16SpathDB). The NB method gave a higher percentage of results than the alignment method at the genus level (96% to 94%, respectively), while at the species level the

percentages were 87.5% and 80% respectively (Srinivasan *et al*., 2015). Thus the application of the NB method in the identification of the sequence products of 16S rRNA may be advantageous in future research as it produces higher rates of identification.

The clinical isolates in the current study showed high level of MICs for imipenem, with 30 out of 42 isolates (71.4%) showing an MIC of 32 mg L^{-1}, while 19 out of 30 (63.4%) of aquatic bacteria showed MICs of 4 mg L^{-1}, and 42.7% of soil isolate MICs were 4 mg L^{-1}. A smaller proportion, i.e. 29 out of the 96 soil isolates (30.2%), showed MICs of 32 mg L^{-1} and only 8 of the 96 isolates (8.3%) had MICs of more than 32 mg L^{-1}. Similar findings were observed in a Brazil hospital, where 17 imipenem-resistant isolates showed a MIC_{50} of 64 mg L^{-1} and MIC_{90} of 256 mg L^{-1}, with the production of SPM-1, VIM-2, and KPC-2 β-lactamases (Rizek et al., 2014). It was also found in a Korean study that imipenem-nonsusceptible clinical isolates showed higher MICs to imipenem, where the MIC_{50} was more than 32 mg L^{-1} (Sung *et al*., 2011). The higher MICs for imipenem in these clinical isolates are most likely due to the selective action of the drug in clinical practice (Alonso *et al*., 1999; Hernandez *et al*., 2011). This is supported by the finding that the number of genes encoding resistance to β-lactamases, identified by genome sequencing, was higher in the clinical isolates of *Stenotrophomonas maltophilia* than in the *S. maltophilia* environmental isolates.

The most common β-lactamases in the environmental isolates in the current study were class B (MBLs) (84.2%). This class has also been reported as having a widespread distribution of MBLs in environmental bacterial isolates. The significance of these enzymes in the environmental isolates lies in their suspected potential ability to transfer to clinical isolates, resulting in the greater spread of resistant β-lactamase encoding genes (Rossolini *et al*., 2001). Whilst PCR-based targeting of ImR genes in the environmental isolates initially suggested detection of these genes, sequencing of resulting PCR products showed that these genes encode unknown protein functions (hypothetical proteins) and so were not proven to be the genes responsible for imipenem resistance in this study. This may indicate that the primers were lacking in specificity and thus led to the amplification of other non-targeted genes. It is recommended, therefore, that many different arrays of primers are used when testing for ImR genes in environmental bacterial isolates, due to the

great diversity of such genes. This may also indicate the presence of a potentially large and divergent gene pool for imipenem resistance within natural environments, and the wider importance of river water and agriculture soil as a reservoir of novel antibiotic resistance (D'Costa *et al*., 2006).

Class A (KPCs) (40.5%) were the most frequent type of β-lactamase observed in the clinical isolates in this study. This finding may raise concerns about the widespread distribution of this class of antimicrobial resistance mechanism in clinical settings. These clinical isolates yielded PCR amplification products with PCR primers targeting ImR genes and the sequence analysis showed that these genes were specific and related to ImR and β-lactamase genes within antibiotic-resistant clinically important species.

Another finding from the current study was the detection of frequencies of multi-drug resistant patterns among the environmental isolates which is mostly attributable to the interensic resistance. It has been claimed that environmental bacteria are the most important source of AR (O'Toole, 2014).

Non-enteric bacteria are considered to be more resistant than Enterobacteria (Chawla *et al*., 2013, Fu and Zheng, 2013). However, the observation of greater multiple resistance patterns to many classes of antimicrobials among Enterobacteria compared to non-enteric bacteria in the clinical isolates is a very important finding of the current study. As they are more resistant and have more than one type of β-lactamase, as previously discussed, and as they are more prevalent in clinical infections, the resistance patterns of the Enterobacteria in the current study could be an important indicator of the ever-increasing spread of resistance genes in clinical settings.

The high frequency of *Stenotrophomonas maltophilia* amongst the York and Sheffield clinical isolates in the current study may reveal how these normal soil-inhabiting microorganisms can cause many types of infection, including pneumonias and septicaemia. Another study indicated that *S. maltophilia* is not a highly virulent organism but that its pathogenicity means that it is one of the most important nosocomial infectious agents nowadays, with mortality rates ranging from 14% to 69% in cases with bacteraemia (Falagas *et al*., 2009). Brooke (2012) reports that this organism has been isolated from a wide range of sources, including soil, animals,

plant roots, water and water treatment systems, lakes, sink holes, hemodialysis water and dialysate samples, tap water, biofilms on different surfaces, hand-washing soaps and even in some antiseptics. Consequently, there is a corresponding wide range of infections associated with *S. maltophilia*, including pneumonia, skin and soft tissue infections, cellulitis, endocarditis, endophthalmitis, UTIs, meningitis, and catheter associated infections (Brooke, 2012).

Genome sequencing was used to characterise a number of *Stenotrophomonas* spp. isolates obtained from the river water, farm soil and clinical samples in this study. The resulting genome analysis was informative not only in terms of the detection of numbers and classes of β-lactamases, but also in identifying the mechanisms which are implicated in bacterial resistance to other antimicrobials. MDR efflux pumps, mediating resistance to many classes of antimicrobials, were identified, as were genes encoding resistance to other non-β-lactam antimicrobials. Genes mediating resistance to heavy metals were also detected, and their role in resistance to antimicrobials has been discussed.

Three different tools, RAST, BLAST and ResFinder, were applied in the genomic analysis of some of *Stenotrophomonas* spp. isolates. RAST classified the different antibiotic resistance genes into their related groups, but was only able to place genes into categories without establishing the specific identification of these genes. These ARGs included MDR efflux pump, β-lactamases, and fluoroquinolone and aminoglycoside resistance. Subsequent BLAST analysis identified three different classes of β-lactamases within the isolates, including class A (L2), class B (MBL) and class C (AmpC). ResFinder analysis identified additional ARGs that could be acquired but which were not detected either by RAST or BLAST. These include β-lactamase class B (L1), sulphonamide (*sul1*) and tetracycline (*tet(C)*) resistance. However, aminoglycoside resistance was detected by all three approaches. In addition to chromosomally-borne antimicrobial resistance genes in *Stenotrophomonas maltophilia*, the detection of potential acquired antibiotic resistance using ResFinder and also the detection of mobile genetic elements (MGEs) using RAST may highlight the important potential role of HGT in the dissemination of this resistance to other bacteria (Poulin-Laprade *et al*., 2015).

6.1 Conclusion

This study has characterised imipenem-resistant (ImR) bacterial isolates found in the natural environment (water and soil) and in clinical settings. The initial methodology used was the culture-dependent isolation of environmental resistant isolates on both PCA and MCA agar plates supplemented with antibiotic imipenem. Using the tests of MIC, MHT, CDST and multiple resistance patterning, Phenotypic resistance to imipenem was observed in 11.2% (75/670 CFU ml^{-1}), 13.3% (145.35 x 10^5/ 109.1 x 10^6 CFU g^{-1}) and 38.5% (42/109) of water, soil and clinical isolates, respectively. β-lactamase activity studies (MHT and CDST) showed that the most common β-lactamases among the environmental isolates were class B metallo β-lactamases (84.2%), while class A *Klebsiella pneumoniae* carbapenemases (KPCs) (40.5%) were the most common β-lactamases observed in the clinical isolates. The low frequency of detection of carbapenamases in the environmental isolates was often associated with corresponding low ImR MICs (4 mgL^{-1}) in these isolates which may indicate the possible absence of carbapenamases in some of these isolates. In contrast, the more frequent detection of carbapenamases in clinical provided isolates was associated with the higher MICs (≥ 8$mgL^{-}1$) observed among these isolates. The observation of frequencies of multi-drug resistant patterns among the environmental isolates is mostly attributable to the interensic resistance. Enterobacteria from clinical isolates showed greater MDR patterns to many classes of antimicrobials compared to non-enteric bacteria. The molecular techniques used in this study were PCR and sequencing for both 16S rRNA and β-lactamases genes. 16S rRNA gene sequencing enabled identification of 30 (17 species), 96 (27 species), and 42 (11 species) ImR bacteria in water, soil and clinical isolates, respectively. The most abundant genera identified were *Caulobacter* (36.7%), *Stenotrophomonas* (44.8%) and *Stenotrophomonas* (40.5%), while the most common species were *Stenotrophomonas* spp. (10%), *S. rhizophila* (21.8%) and *S. maltophilia* (38.1%), in the water, soil and clinical isolates, respectively. PCR products were generated from ImR clinical isolates and some of the environmental isolates using primers targeting β-lactamase genes. Sequence analysis of products from the clinical isolates showed that they were specific and related to β-lactamase genes. However, the PCR products from the environmental isolates were found to be unrelated to known genes characterised from antibiotic resistant clinically important bacteria. There was a high

incidence of *Stenotrophomonas* spp. isolates amongst the environmental and clinical isolates in the current study. These were *Stenotrophomonas* spp. (3 isolates, 10%) in water, *S. rhizophila* (21 isolates, 21.8%) and *S. maltophilia* (19 isolates, 19.8%) in soil, and *S. maltophilia* (16, 38.1%) in clinical isolates. As a significant number of studies have isolated *S. maltophilia* from a wide variety of infection sources, and as *S. maltophilia* is considered to be a normal soil-inhabiting species with low virulence, it was decided to investigate this further. Thus, genome sequencing was performed for eight isolates of *Stenotrophomonas* spp. from water, soil and clinical samples. Bioinformatics analysis, using RAST, BLAST, and ResFinder, detected a number of β-lactamase and other mechanisms mediating resistance to many classes of antimicrobials, including non-β-lactams, aminoglycosides, fluoroquinolones, sulphonamides and MDR efflux pumps. Also detected were genes mediating resistance to heavy metals, which have a role in resistance to antimicrobials. PathogenFinder revealed that three (water) and one (soil) isolates were predicted as being potentially pathogenic to humans. The MLST web-server showed that one of the clinical isolates was found to belong to the *S. maltophilia* ST-31 MLST class, while the other seven isolates showed no exact match with any other known *S. maltophilia* strains on the database.

Overall, this research has demonstrated the presence of imipenem resistant bacteria in environmental and clinical settings that show multiple resistance to other antibiotics. Imipenem resistant *Stenotrophomonas* spp. were present in all environments studied and these bacteria were found to harbour multiple and diverse antibiotic resistance genes, that differed between isolates from environmental and clinical origins.

References

Abaidoo, R.C., Keyser, H.H., Singleton, P.W. and Borthakur, D. (2002) Comparison of molecular and antibiotic resistance profile methods for the population analysis of *Bradyrhizobium* spp. (TGx) isolates that nodulate the new TGx soybean cultivars in Africa. *Journal of Applied Microbiology*, **92**, 109-117.

Adamek, M., Linke, B. and Schwartz, T. (2014) Virulence genes in clinical and environmental *Senotrophomas maltophilia* isolates: A genome sequencing and gene expression approach. *Microbial Pathogenesis* **67–68**, 20–30.

Ahmed, F. and Donaldson, W.A. (2007) Chemistry and biology of the streptogramin A antibiotics. *Mini-Reviews in Organic Chemistry*, **4**, 159-181.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. (2010) Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews*, **8**, 251-259.

Allen, H.K., Moe, L.A., Rodbumrer, J., Gaarder, A. and Handelsman, J. (2009) Functional metagenomics reveals diverse β-lactamases in a remote Alaskan soil. *ISME Journal*, **3**, 243-251.

Alonso, A., Campanario, E. and Martinez, J.L. (1999) Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*. *Microbiology* **145**, 2857–2862.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W.and Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403-10.

Ambler, R. P. (1980) The structure of β-lactamases. *Philosophical Transactions of the Royal Society of London, Series B*, **289**, 321–331.

Ambler, R.P., Coulson, A.F.W., Frère, J.-M., Ghuysen, J.-M., Joris, B., Forsman, M., Levesque, R.C., Tiraby, G. and Waley, S.G. (1991) A standard numbering scheme for the class A β-lactamases. *Biochemical Journal* **276**, 269–272.

Amos, G.C.A., Hawkey, P.M., Gaze, W.H. *et al*. (2014) Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. *Journal of Antimicrobial Chemotherapy*, **69**, 1785-1791.

APHA, AWWA, and WEF (2012) *Standard Methods for the Examination of Water and Wastewater* (22nd Edition). Rice, E.W., Baird, R.B., Eaton, A.D. and Clesceri, L.S. (eds). New York: American Public Health Association.

Aravena-Roman, M., Inglis, T.J.J., Henderson, B., Riley, T.V. and Changa, B.J. (2012) Antimicrobial susceptibilities of *Aeromonas* strains isolated from clinical and environmental sources to 26 antimicrobial agents. *Antimicrobial Agents and Chemotherapy* **56**, 1110–1112.

Arnold, R. S., Thom, K. A., Sharma, S., Phillips, M., Johnson, J. K., and Morgan, D. J. (2011) Emergence of *Klebsiella pneumoniae* carbapenemase (KPC)-producing bacteria. *Southern Medical Journal*, **104**, 40–45.

Ash R.J., Mauck, B. and Morgan, M. (2002) Antibiotic resistance of Gram-negative bacteria in rivers, United States. *Emerging Infectious Diseases* **8**, 713-716.

Aubron, C., Poirel, L., Ash, R.J. and Nordmann, P. (2005) Carbapenemase-producing *Enterobacteriaceae* in U.S. Rivers. *Emerging Infectious Diseases*, **11**.

Avison, M.B., Higgins, C.S., Ford, P.J., von Heldreich, C.J., Walsh, T.R. and Bennett, P.M. (2002) Differential regulation of L1 and L2 β-lactamase expression in *Stenotrophomonas maltophilia. Journal of Antimicrobial Chemotherapy* **49**, 387-389.

Avison, M. B., Higgins, C.S., Von Heldreich, C.J., Bennett, P.M. and Walsh, T.R. (2001) Plasmid location and molecular heterogeneity of the L1 and L2 β-lactamase genes of *Stenotrophomonas maltophilia. Antimicrobial Agents and Chemotherapy* **45**, 413–419.

Avison, M.B., von Heldreich, C.J., Higgins, C.S., Bennett, P.M. and Walsh, T.R. (2000) A TEM-2 β-lactamase encoded on an active Tn1-like transposon in the genome of a clinical isolate of *Stenotrophomonas maltophilia. Journal of Antimicrobial Chemotherapy* **46**, 879-884.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrell, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke A. and Zagnitko, O. (2008) The RAST Server: Rapid Annotations using Subsystem Technology. *BMC Genomics*, **9**, 75.

Baker-Austin, C., Wright, M.S., Stepanauskas, R. and McArthur, J. V. (2006) Co-selection of antibiotic and metal resistance. *Trends in Microbiology* **14**, 176–182.

Baltz, R.H. (2008) Renaissance in antibacterial discovery from actinomycetes. *Current Opinion in Pharmacology*, **8**, 557-563.

Bambeke, F.V., Balzi, E. and Tulkens, P.M. (2000) Antibiotic efflux pumps. *Biochemical Pharmacology*, **60**, 457–470.

Banerjee, M. and Yesmin, L. (2002) *Sulfur-oxidizing plant growth promoting rhizobacteria for enhanced canola performance.* US Patent 07491535.

Barbosa, E.G.V., Aburjaile, F.F., Ramos, R.T.J. *et al.* (2014) Value of a newly sequenced bacterial genome. *World Journal of Biological Chemistry,* **5**, 161-168.

Barkay, T., Miller, S.M. and Summers, A.O. (2003) Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiology Reviews,* **27**, 355-384.

Barker-Reid F., Fox, E.M. and Faggian, R. (2010) Occurrence of antibiotic resistance genes in reclaimed water and river water in the Werribee Basin, Australia. *Journal of Water Health*, **8**, 521-31.

Bates, J. (1997) Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm animals to human infection. *Journal of Hospital Infection*, **39**, 75-77.

Beaz-Hidalgo, R., Magi, G.E., Balboa, S., Barja, J.L. and Romalde, J.L. (2008) Development of a PCR protocol for the detection of *Aeromonas salmonicida* in fish by amplification of the fstA (ferric siderophore receptor) gene. *Veterinary Microbiology*, **128**, 386-394.

Beja, O, Aravind, L., Koonin, E.V., Suzuki, M.T., Hadd, A., Nguyen, L.P., Jovanovich, S.B., Gates, C.M., Feldman, R.A., Spudich, J.L., Spudich, E.N. and DeLong, E.F. (2000) Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* **289**, 1902-1906.

Benassi, F.O., Vergara, M., von Specht, M.H., García, M.A., Quiroga, M.I., Pucciarelli, A.B., Zubreski, E., Laczeski, M., Martin, B.M., Leardini, N. and Gutkind, G. (2001) B-lactam antibiotic sensitivity in *Aeromonas* spp. of clinical, animal, and environmental origin. *Revista Argentina de Microbiología*. **33**, 47-51.

Bennett, P.M. (2008) Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology,* **153**, 347–357.

Benveniste, R. and Davies, J. (1973) Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proceedings of the National Academy of Sciences,* **70**, 2276-2280.

Bhullar K., Waglechner, N., Pawlowski, A., Koreva, K., Banks, E.D., Johnston, M.D., Barton, H.A. and Wright, G.D. (2012) Antibiotic resistance is prevalent in an isolated cave microbiome, *PLoS ONE.* **7**, e34953.

Blaak, H., van Rooijen, S.R. Schuijt, M.S., Docters van Leeuwen A.E., Italiaander, R., van den Berg, H.H.J.L., Lodder-Verschoor, F., Schets, F.M. and de Roda Husman, A.M. (2011) *Prevalence of antibiotic resistant bacteria in the rivers Meuse, Rhine and New Meuse*. RIVM Report 703719071/2011. National Institute for Public Health and the Environment.

Block, M.G. and Ouellette, A. (2012) Genetic identification of eleven aquatic bacteria using the 16S rDNA gene. *Journal of Research across the Discipline* [online]. Available at: **http://www.ju.edu/jrad/documents/block_submission.pdf** [accessed 1 December 2014].

Bogaerts, P., Naas, T., Wybo, I., *et al*. (2006) Outbreak of infection by carbapenem-resistant *Acinetobacter baumannii* producing the carbapenemase OXA-58 in Belgium. *Journal of Clinical Microbiology* **44**, 4189-92.

Bos, K.I., Schuenemann, V.J., Golding, G.B. *et al*. (2011) A draft genome of *Yersinia pestis* from victims of the Black Death. *Nature,* **478**, 506-510.

Bowers, D. (2008) *Medical Statistics from Scratch; An Introduction for Health Professionals* (2nd Ed), JohnWiley and Sons.

Bradford, P.A. (2001) Extended-spectrum β-lactamases in the 21st Century: Characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews* **14**, 933–951.

Bridger, N., Walkty, A., Crockett, M., Fanella, S., Nichol, K. and Karlowsky, J.A. (2012) *Caulobacter* species as a cause of post neurosurgical bacterial meningitis in a pediatric patient. *Canadian Journal of Infectious Diseases and Medical Microbiology* **23**.

British Society for Antimicrobial Chemotherapy (2013) *BSAC Methods for Antimicrobial Susceptibility Testing, Version 12* [online]. Available at: **http://www.bsac.org.uk** [accessed 15 March 2014].

Brocchieri L. The GC content of bacterial genomes. *Journal of Phylogenetic Evolutionary Biology* **2**: e108. doi:10.4172/2329-9002.1000e108.

Brooke, J.S. (2012) *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clinical Microbiology Reviews* **25**, 2-41.

Brown-Jaque, M., Calero-Caceres, W. and Muniesa, M. (2015) Transfer of antibiotic-resistance genes via phage-related mobile elements. *Plasmid* **79**, 1–7.

Buehler, B., Hogrefe, H.H., Scott, G., Ravi, H., Pabon-Pena, C., O'Brien, S., Formosa, R. and Happe, S. (2010) Rapid quantification of DNA libraries for next-generation sequencing. *Methods* **50**, S15–S18.

Bugg, T.D., Wright, G.D., Dutka-Malen, S., Arthur, M., Courvalin, P. and Walsh, C.T. (1991) Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry*, **30**, 10408-10415.

Burdge, D.R., Noble, M.A., Campbell, M.E. *et al*. (1995) *Xanthomonas maltophilia* misidentified as *Pseudomonas cepacia* in cultures of sputum from patients with cystic fibrosis: a diagnostic pitfall with major clinical implications. *Clinical Infectious Diseases*, **20**, 445-448.

Bush, K. (2012) Antimicrobial agents targeting bacterial cell walls and cell membranes. *Scientific and Technical Review of the Office International des Epizooties (Paris)*, **31**, 43-56.

Bush, K. and Jacoby, G.A. (2010) Updated functional classification of β-lactamases: review. *Antimicrobial Agents and Chemotherapy*, **54**, 969–976.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G.A., Kishony, R., Kreiswirth, B.N., Kutter, E., Lerner, S.A., Levy, S., Lewis, K., Lomovskaya, O., Miller, J.H., Mobashery, S.L., Piddock, J.V., Projan, S., Thomas, C.M., Tomasz, A., Tulkens, P.M., Walsh, T.R., Watson, J.D., Witkowski, J., Witte, W., Wright, G., Yeh, P. and Zgurskaya, H.I. (2011) Tackling antibiotic resistance. *Nature Reviews Microbiology*, **9**, 894-896.

Calderón, C.B. and Sabundayo, B.P. (2007) Antimicrobial classifications: drugs for bugs. In: R. Schwalbe, L. Steele-Moore and A.C. Goodwin (eds) *Antimicrobial Susceptibility Testing Protocols*. Boca Raton, London and New York: CRC Press, Taylor and Francis Group, pp.7-48.

Calza, L., Manfredi, R. and Chiodo, F. (2003) *Stenotrophomonas (Xanthomonas) maltophilia* as an emerging opportunistic pathogen in association with HIV infection: a 10-year surveillance study. *Infection*, **31**, 155-161.

Cantón, R. and Coque, T.M. (2006) The CTX-M β-lactamase pandemic. *Current Opinion in Microbiology*, **9,** 466.

Capita, R. and Alonso-Calleja, C. (2013) Antibiotic-resistant bacteria: a challenge for the food industry. *Critical Reviews in Food Science and Nutrition.* **53**, 11-48.

Carattoli, A. (2001) Importance of integrons in the diffusion of resistance. *Veterinary Research*, **32**, 243-259.

Cariello, N.F., Swenberg, J.A. and Skopek, T.R. (1991) Fidelity of *Thermococcus litoralis* DNA polymerase (Vent) in PCR determined by denaturing gradient gel electrophoresis. *Nucleic Acids Research*, **19**, 4193-4198.

Carlet, J., Jarlier, V., Harbarth, S., Voss, A., Goossens, H. and Pittet, D. (2012) Ready for a world without antibiotics? The Pensières antibiotic resistance call to action. *Antimicrobial Resistance and Infection Control* **1**.

Carmody, L.A., Spilker, T. and LiPuma, J.J. (2011) Reassessment of *Stenotrophomonas maltophilia* phenotype. *Journal of Clinical Microbiology*, **49**, 1101-1103.

Carrer A., Poirel, L., Yilmaz, M., Akan, O.A., Feriha, C., Cuzon, G., Matar, G., Honderlick, P. and Nordmann, P. (2010) Spread of OXA-48-encoding plasmid in Turkey and beyond. *Antimicrobial Agents and Chemotherapy*, **54**, 1369-1373.

Carvalhaes, C.G., Picao, R.C., Nicoletti, A.G., Xavier, D.E. and Gales, A.C. (2010) Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results. *Journal of Antimicrobial Chemotherapy* **65**, 249–251.

Carvalho, K.R., Carvalho-Assef, A.P.D., Santos, L.G. dos, Pereira, M.J.F. and Asensi, M.D. (2011) Occurrence of $bla_{\text{OXA-23}}$ gene in imipenem-susceptible *Acinetobacter baumannii*. *Memórios do Instituto Oswaldo Cruz*, Rio de Janeiro, **106**, 505-506

Casewell, M., Friis, C., Marco, E. *et al*. (2003) The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of Antimicrobial Chemotherapy*, **52**, 159-161.

CDC (Centers for Disease Control and Prevention) (2009) Guidance for control of infections with carbapenem-resistant or carbapenemase-producing *Enterobacteriaceae* in acute care facilities. *Morbidity and Mortality Weekly Report* **58**, 256-260 [online]. Available at: **http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5810a4.htm** [accessed 8 January 2015]

CDC (Centers for Disease Control and Prevention) (2013) Vital signs: carbapenem-resistant Enterobacteriaceae. *Morbidity and Mortality Weekly Report* **62**, 165–70 [online].
Available at: **http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6209a3.htm** [accessed 8 January 2015].

Cha, J., Kotra, L.P. and Mobashery, S. (2008) Resistance to β-lactam antibiotics mediated by β-lactamases: structure, mechanism, and evolution. In: R.G. Wax, K. Lewis, A.A. Salyers and H. Taber (eds) *Bacterial Resistance to Antimicrobials* 2nd edition. Florida, USA: CRC Press, Taylor and Francis Group, pp.104-122.

Chambers, H.F. (1999) Penicillin-binding protein-mediated resistance in pneumococci and staphylococci. *Journal of Infectious Diseases*, **179**, S353.

Chapman, J.S. (2003) Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration and Biodegradation*, **51**, 271–276.

Chawla, K., Vishwanath, S. and Munim, F.C. (2013) Nonfermenting Gram-negative bacilli other than *Pseudomonas aeruginosa* and *Acinetobacter* spp. causing respiratory tract infections in a tertiary care center. *Journal of Global Infectious Diseases,* **5**, 144–148.

Chen, Y-C., Liu, T., Yu, C-H., Chiang, T-Y. and Hwang, C-C. (2013) Effects of GC bias in next-generation-sequencing data on de novo genome assembly. *PLoS ONE* **8**, e62856.

Cheng, C., Sun, J., Zheng, F., Wu, K. and Rui, Y. (2014) Molecular identification of clinical "difficult-to-identify" microbes from sequencing 16S ribosomal DNA and internal transcribed spacer 2. *Annals of Clinical Microbiology and Antimicrobials* **13,** 1-7.

Chou, J.H., Greenberg, J.T. and Demple, B. (1993) Posttranscriptional repression of *Escherichia coli* OmpF protein in response to redox stress: positive control of the *micF* antisense RNA by the *soxRS* locus. *Journal of Bacteriology,* **175**, 1026-1031.

Churchward, G. (2002) Conjugative transposons and related mobile elements. In: Craig, N.L., Craigie, R., Gellert, M., Lambowitz, A.M. (eds) *Mobile DNA II.* Washington, DC: ASM Press, pp.177-191.

Clarridge, J. (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology*, **17**, 840–862.

CLSI (2012a) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard* (9^{th} Edition). CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2012b) *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard — Eleventh Edition.* CLSI document M02-A11. Wayne, PA: Clinical and Laboratory Standards Institute.

Corbellini, S., Caccuri, F., Gelmi, M., De Francesco, M.A., Fiorentini, S., Caruso, A. and Giagulli, C. (2014) Emergence of carbapenem-resistant *Klebsiella pneumoniae* strains producing *Kpc-3* in Brescia Hospital, Italy. *New Microbiologica*, **37**, 177-183.

Cox N.A. (1975) Isolation and identification of a genus, *Chromobacterium,* not previously found on processed poultry. *Applied Microbiology* **29**, 864.

Crossman, L.C., Gould, V.C., Dow, J.M., Vernikos, G.S., Okazaki, A., Sebaihia, M., Saunders, D., Arrowsmith, C., Carver, T., Peters, N., Adlem, E., Kerhornou, A., Lord, A., Murphy, L., Seeger, K., Squares, R., Rutter, S., Quail, M.A., Rajandream, M-A., Harris, D., Churcher, C., Bentley, S.D., Parkhill, J., Thomson, N.R. and Avison, M.B. (2008) The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. *Genome Biology*, **9**, 4, R74.

D'Costa, V.M, Griffiths, E. and Wright, G.D. (2007) Expanding the soil antibiotic resistome: exploring environmental diversity. *Current Opinion in Microbiology*, **10**, 481-489.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H.N. and Wright, G.D. (2011) Antibiotic resistance is ancient. *Nature* **477**, 457–461.

D'Costa, V.M., McGrann, K.M., Hughes, D.W. and Wright, G.D. (2006) Sampling the antibiotic resistome. *Science*, **311**, 374-377.

Dantas, G. and Sommer, M.O.A. (2014) How to fight back against antibiotic resistance. *American Scientist*, **102**, 42.

Dark, M.J. (2013) Whole-genome sequencing in bacteriology: state of the art. *Infection and Drug Resistance*, **6**, 115-123.

Datta, N. and Kontomichalou, P. (1965) Penicillinase synthesis controlled by infectious R Factors in *Enterobacteriaceae*. *Nature* **208**, 239–244.

Datta, S., Wattal, C., Goel, N., Oberoi, J.K., Raveendran, R. and Prasad, K.J. (2012) A ten year analysis of multi-drug resistant blood stream infections caused by *Escherichia coli* and *Klebsiella pneumoniae* in a tertiary care hospital. *Indian Journal of Medical Research* **135**, 907–12.

Davies, J. (2006) Are antibiotics naturally antibiotics? *Journal of Industrial Microbiology and Biotechnology* **33**, 496–499.

Davies, J. (2009) Darwin and microbiomes. *EMBO Reports,* **10**, 805.

Davies, J. and Davies, D. (2010) Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews*, **74**, 417–433.

Davies, J., Spiegelman, G.B. and Yim, G. (2006) The world of subinhibitory antibiotic concentrations. *Current Opinion in Microbiology,* **9**, 445-453.

De Carvalho, S.R. (2011) *Screening of antibiotic resistance determinants in Gram-negative bacteria isolated from environmental reservoirs.* Master's dissertation, University of Lisbon [online].
Available at: **http://repositorio.ul.pt/bitstream/10451/6586/1/ulfc092957_tm_sofia_carvalho.pdf** [accessed 10 Dec 2014].

de Figueiredo, D.Q., Santos, K.R.N. dos, Pereira, E.M., Schuenck, R.P., Mendonça-Souza, C.R.V. de, Teixeira, L.M. and Mondino, S.S.B. de (2011) First report of the $bla_{\text{OXA-58}}$ gene in a clinical isolate of *Acinetobacter baumannii* in Rio de Janeiro, Brazil. *Memórios do Instituto Oswaldo Cruz*, **106**, 368-370.

Denton, M., Hall, M.J., Todd, N.J., Kerr, K.G. and Littlewood, J.M. (2000) Improved isolation of *Stenotrophomonas maltophilia* from the sputa of patients with cystic fibrosis using a selective medium. *Clinical Microbiology and Infection*, **6**, 395-396.

Deschepper, R., Grigoryan, L., Lundborg, C.S. *et al*. (2008) Are cultural dimensions relevant for explaining cross-national differences in antibiotic use in Europe? *BMC Health Services Research*, **8**, 123.

Di Bonaventura, D., Spedicato, I., D'Antonio, D., Robuffo, I. and Piccolomini, R. (2004) Biofilm formation by *Stenotrophomonas maltophilia*: modulation by quinolones, trimethoprim-sulfamethoxazole, and ceftazidime. *Antimicrobial Agents and Chemotherapy*, **48**, 151–16.

Donkor, E.S., Dayie, N.T. K. D. and Adiku, T. K. (2014) Bioinformatics with basic local alignment search tool (BLAST) and fast alignment (FASTA). *Journal of Bioinformatics and Sequence Analysis*, **6**, 1-6.

Eaton, A.D., Clesceri, L.S., Rice, E.W. and Greenberg, A.E. (2005) *Standard methods for the examination of water and wastewater* (21st ed.). American Public Health Association, Washington, DC.

ECDC (European Centre for Disease Prevention and Control) (2013) *Carbapenemase-producing bacteria in Europe: interim results from the European Survey on carbapenemase-producing Enterobacteriaceae.* EuSCAPE Project. Stockholm, ECDC.

ECDC (European Centre for Disease Prevention and Control) and (EMEA) European Medicines Agency (2009) *The Bacterial Challenge: Time to React*. Joint Technical Report ECDC–EMEA, Stockholm.

Ehrlich, P. (1907) *Ueber moderne chemotherapie*. Leipzig: Akademische Verlagsgesellschaft mbH, pp.167-202.

Eneroth, A., Ahrné, S. and Molin, G. (2000) Contamination routes of Gram-negative spoilage bacteria in the production of pasteurised milk, evaluated by randomly amplified polymorphic DNA (RAPD). *International Dairy Journal,* **10**, 325-331.

EUCAST (2012) EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and / or epidemiological importance [online]. Available at: **http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_v1.0_20131211.pdf** [accessed 14 December 2014].

Faber, M.S., Heckenbach, K., Velasco, E. and Eckmanns, T. (2010) Antibiotics for the common cold: expectation of Germany's general population. *Eurosurveillance* **15**, 1–7.

Falagas, M.E., Kastoris, A.C., Vouloumanou, E.K., Rafailidis, P. I., Kapaskelis, A.M. and Dimopoulos, G. (2009) Attributable mortality of *Stenotrophomonas maltophilia* infections: a systematic review of the literature. *Future Microbiology* **4**, 1103–1109.

Ferrer-Navarro, M., Planell, R., Yero, D., Mongiardini, E., Torrent, G., Huedo, P., Martinez, P., Roher, N., Mackenzie, S., Gibert, I. and Daura, X. (2013) Abundance of the quorum-sensing factor Ax21 in four strains of *Stenotrophomonas maltophilia* correlates with mortality rate in a New Zebrafish model of infection. *PLoS ONE* **8**, e67207.

Fierer, N., Lauber, C.L., Zhou, N. *et al*. (2010) Forensic identification using skin bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America,* **107**, 6477-6481.

Fleischmann, R.D., Adams, M.D., White, O. *et al*. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science,* **269**, 496-512.

Fleming, A. (1929) On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenza*. *British Journal of Experimental Pathology*, **10**, 226-236.

Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. (2007) *Bailey and Scott's Diagnostic Microbiology (12th Ed.)* St. Louis: Mosby Elsevier.

Franke, A.E. and Clewell, D.B. (1981) Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. *Journal of Bacteriology*, **145**, 494-502.

Freeman, R., Moore, L.S.P., Charlett, A., Donaldson, H. and Holmes, A.H. (2015) Exploring the epidemiology of carbapenem-resistant Gram-negative bacteria in west London and the utility of routinely collected hospital microbiology data. *Journal of Antimicrobial Chemotherapy* **70**, 1212–1218.

Fu, Q. and Zheng, S. (2013) The distribution and antimicrobial resistance of common bacteria of nosocomial infection. *Life Science Journal* **10**, 1658-1661 [online]. Available at: **http://www.lifesciencesite.com** [accessed 14 Dec 2014].

Furushita, M., Okamoto, A., Maeda, T., Ohta, M. and Shiba, T. (2005) Isolation of multidrug-resistant *Stenotrophomonas maltophilia* from cultured yellowtail (*Seriola quinqueradiata*) from a marine fish farm. *Applied and Environmental Microbiology* **71**, 598–5600

Gaibani, P., Colombo, R., Arghittu, M., Cariani, L., Ambretti, S., Bua, G., Lombardo, D., Landini, M.P., Torresani, E. and Sambri,V. (2014) Successful containment and infection control of a carbapenem-resistant *Klebsiella pneumoniae* outbreak in an Italian hospital. *New Microbiologica*, **37**, 87-90.

Galvin, S., Boyle, F., Hickey, P., Vellinga, A., Morris, D. and Cormican, M. (2010) Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. *Applied and Environmental Microbiology*, **76**, 4772-4779.

Garnier, F., Taourit, S., Glaser, P., Courvalin, P. and Galimand, M. (2000) Characterization of transposon Tn*1549*, conferring VanB-type resistance in *Enterococcus* spp. *Microbiology*, **146**, 1481-1489.

Gaze, W., O'Neill, C., Wellington, E. and Hawkey, P. (2008) Antibiotic resistance in the environment, with particular reference to MRSA. *Advances in Applied Microbiology*, **63**, 249-280.

Ghafourian, S., Sadeghifard, N., Soheili, S. and Sekawi Z. (2014) Extended spectrum B-lactamases: definition, classification and epidemiology. *Current Issues in Molecular Biology* **17**, 11-22.

Giaccone V., Alberghini L., Biscotto A., Milandri C. (2008) *Unusual spoilage in rabbit carcasses caused by Janthinobacterium lividum.* The Ninth World Rabbit Congress, Verona, Italy.

Giger, W., Alder, A.C., Golet, E.M., Kohler, H.E., McArdell, C.S., Molnar, E., Siegrist, H. and Suter, J.F. (2003) Occurrence and fate of antibiotics as trace contaminants in wastewaters, sewage, sludges, and surface waters. *Chimia*, **57**, 485–491.

Gilbert, J.A., Field, D., Huang, Y., Edwards, R., Li, W., Gilna, P. and Joint, I. (2008) Detection of large numbers of novel sequences in the metatranscriptomes of complex marine microbial communities. *PLoS One* **3**, e3042.

Girlich, D., Poirel, L. and Nordmann, P. (2010) Letters to the editor; first isolation of the *bla*OXA-23 carbapenemase gene from an environmental *Acinetobacter baumannii* isolate. *Antimicrobial Agents and Chemotherapy*, 578-579.

Girlich, D., Poirel, L. and Nordmann, P. (2010b) Novel Ambler class A carbapenem-hydrolyzing β-lactamase from a *Pseudomonas fluorescens* isolate from the Seine river, Paris, France. *Antimicrobial Agents and Chemotherapy*, **54**, 328–332.

Goossens, H., Ferech, M., Vander Stichele, R., Elseviers, M. and the ESAC Project Group (2005) Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet*, **365**, 579-587.

Goossens, H., Guillemot, D., Ferech, M. *et al*. (2006) National campaigns to improve antibiotic use. *European Journal of Clinical Pharmacology*, **62**, 373-379.

Graham, D.W., Olivares- Rieumont, A., Knapp, C.W., Lima, L., Werner, D. and Bowen, D. (2011) Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. *Environmental Science and Technology*, **45**, 418-424.

Greenwood, D. (2000) Historical introduction. In: D. Greenwood (ed) *Antimicrobial Chemotherapy*, 4th edition. Oxford: Oxford University Press, pp.1-8.

Grundmann, H., Livermore, D.M., Giske, C.G., Canton, R., Rossolini, G.M., Campos, J., Vatopoulos, A., Gniadkowski, M., Toth, A., Pfeifer, Y., Jarlier, V. and Carmeli, Y. (the CNSE Working Group) (2010) Carbapenem-non-susceptible Enterobacteriaceae in Europe: conclusions from a meeting of national experts. *Euro Surveillance* **15** [online].
Available at: **http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19711** [accessed 6 January 2015].

Guardabassi, L. and Dalsgaard, A. (2004) Occurrence, structure, and mobility of Tn*1546*-like elements in environmental isolates of vancomycin resistant enterococci. *Applied and Environmental Microbiology*, **70**, 984-990.

Guardabassi, L., Schwarz, S. and Lloyd, D.H. (2004) Pet animals as reservoirs of antimicrobial-resistant bacteria. *Journal of Antimicrobial Chemotherapy*, **54**, 321-332.

Guenther, S., Ewers, C. and Wieler, L.H. (2011) Extended-spectrum β-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? *Frontiers in Microbiology*, **2**, 246.

Gur, D., Korten, V., Unal, S., Deshpande L.M. and Castanheira, M. (2008) Increasing carbapenem resistance due to the clonal dissemination of oxacillinase (OXA-23 and OXA-58)-producing *Acinetobacter baumannii*: report from the Turkish SENTRY Program sites, *Journal of Medical Microbiology* **57**, 1529–1532

Haniford, D.B. (2002) Transposon Tn*10*. In: N.L. Craig, R. Craigie, M. Gellert and A.M. Lambowitz (eds) *Mobile DNA II*. Washington, DC: ASM Press, 457-483.

Harris, P.N.A., Peleg, A.Y., Iredell, J., Ingram, P.R., Miyakis, S., Stewardson, A.J., Rogers, B.A., McBryde, E.S., Roberts, J.A., Lipman, J., Athan, E., Paul, S.K., Baker, P., Harris-Brown, T. and Paterson, D.L. (2015) Meropenem versus piperacillin-tazobactam for definitive treatment of bloodstream infections due to ceftriaxone non-susceptible *Escherichia coli* and *Klebsiella* spp. (the MERINO trial): study protocol for a randomised controlled trial. *Trials* **16**, DOI 10.1186/s13063-014-0541-9.

Harwood, V., Brownell, M., Perusek, W. and Whitlock, J. (2001) Vancomycin-resistant *Enterococcus* spp. isolated from wastewater and chicken faeces in the United States. *Applied and Environmental Microbiology*, **10**, 4930-4933.

Hasman, H. and Aarestrup, F.M. (2002) *tcrB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. *Antimicrobial Agents and Chemotherapy*, **46**, 1410–1416.

Hauben, L., Vauterin, L., Moore, E.R.B., Hostel, B. and Swings, J. (1999) Genomic diversity of the genus *Stenotrophomonas. International Journal of Systematic Bacteriology* **49**, 1749-1760.

Hawkey, P.M. (2008) The growing burden of antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*, **62**, i1–i9.

Hawkey, P.M. and Jones, A.M. (2009) The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy*, **64**, i3-i10.

Hawkey, P.M. and Livermore, D.M. (2012) Carbapenem antibiotics for serious infections. *British Medical Journal*, **344**, e3236 doi: 10.1136/bmj.e3236.

Hedges, R.W. and Jacob, A.E. (1974) Transposition of ampicillin resistance from RP4 to other replicons. *Molecular and General Genetics*, **132**, 31-40.

Hernandez, A., Ruiz, F.M., Romero, A. and Martínez, J.L. (2011) The binding of triclosan to SmeT, the repressor of the multidrug efflux pump SmeDEF, induces antibiotic resistance in *Stenotrophomonas maltophilia*. *PLoS Pathogens* **7**, e1002103.

Herrero, I.A., Issa, N.C. and Patel, R. (2002) Nosocomial spread of linezolid-resistant, vancomycin-resistant *Enterococcus faecium*. *New England Journal of Medicine*, **346**, 867-869.

Heuer, O. E., Pedersen, K., Andersen, J. S. and Madsen, M. (2002) Vancomycin-resistant enterococci (VRE) in broiler flocks 5 years after the avoparcin ban. *Microbial Drug Resistance* **8**, 133-8.

Heymann, D.L. (2006) *Effects of social, environmental and economic factors on current and future patterns of infectious diseases* [online]. Available at: **www.pas.va/content/dam/accademia/pdf/sv106/sv106-heymann.pdf.** [accessed 21 February 2015].

Higgins, P.G., Dammhayn, C., Hackel, M. and Seifert, H. (2010) Global spread of carbapenem-resistant *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy*, **65**, 233-238.

Hoffman, L.R., Déziel, E., D'Argenio, D.A., Lépine, F., Emerson, J., McNamara, S., Gibson, R.L., Ramsey, B.W. and Miller, S.I. (2006) Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Science* **103**, 19890-5.

Hogg, S. (2005) *Essential Microbiology*, Wiley.

Hong, H.-J., Paget, M.S. and Buttner, M.J. (2002) A signal transduction system in *Streptomyces coelicolor* that activates expression of a putative cell wall glycan operon in response to vancomycin and other cell wall-specific antibiotics. *Molecular Microbiology* **44**, 1199-1211.

Hu, P-M., Huang, K-J., Wu, L-T., Hsiao, Y-J. and Yang, T-C. (2008) Induction of L1 and L2 β-lactamases of *Stenotrophomonas maltophilia. Antimicrobial Agents and Chemotherapy*, **52**, 1198–1200.

Huang, T.D., Bogaerts, P., Berhin, C., Jans, B., Deplano, A., Denis, O. and Glupczynski, Y. (2011) Rapid emergence of carbapenemase-producing *Enterobacteriaceae* isolates in Belgium. *Euro Surveillance* **16** [online]. Available at: **http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19900** [accessed 6 January 2015].

Hugh, R. and Ryschenkow, E. (1961) *Pseudomonas maltophilia*, an *Alcaligenes*-like species. *Journal of General Microbiology* **26**, 123-132.

Hutchison, M.L., Walters, L.D., Moore, A. *et al*. (2004) Effect of length of time before incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural soil. *Applied and Environmental Microbiology*, **70**, 5111-5118.

Iversen, A., Kuhn, I., Franklin, A. and Mollby, R. (2002) High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Applied and Environmental Microbiology*, **68**, 2838-2842.

Jalilian, A.R., Hosseini, M.A., Karimian, A., Saddadi, F. and Sadeghi, M. (2006) Preparation and biodistribution of [201Tl] (III) vancomycin complex in normal rats. *Nukleonika*, **51**, 203-208.

Janssen, P.H. (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA Genes. *Applied and Environmental Microbiology* **72**, 1719–1728.

Jeltsch, A. (2003) Maintenance of species identity and controlling speciation of bacteria: a new function for restriction/modification systems? *Gene,* **317**, 13-16.

Jenkins, C., Ling, C.L., Ciesielczuk, H.L., Lockwood, J., Hopkins, S., McHugh, T.D., Gillespie, S.H. and Kibbler C.C. (2012) Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. *Journal of Medical Microbiology* **6**, 483–488

Jensen, S.E. and Demain, A.L. (1995) B-lactams. *Biotechnology* **28**, 239-68.

Johnson A.P. and Woodford, N. (2013) Global spread of antibiotic resistance: the example of New Delhi metallo-b-lactamase (NDM)-mediated carbapenem resistance. *Journal of Medical Microbiology* **62**, 499–513.

Justesen, U.S., Holt, H.M., Thiesson, H.C., Nielsen, J.B.X C., Dargis, R., Kemp, M. and Christensen, J. J. (2007) Report of the first human case of *Caulobacter* sp. infection. *Journal of Clinical Microbiology* **45**, 1366–1369.

Kaiser, S., Biehler, K. and Jonas, D. (2009) A *Stenotrophomonas maltophilia* multilocus sequence typing scheme for inferring population structure. *Journal of Bacteriology*, **191**, 2934–2943.

Kayser, F.H. (2005) The genetic variability of bacteria. In: F.H. Kayse, K.A. Bienz, J. Eckert and R.M. Zinkernagel (eds) *Medical Microbiology*. Stuttgart and New York: Thieme, pp.170-181.

Khan, E., Ejaz, M., Zafar, A. *et al*. (2010) Increased isolation of ESBL producing *Klebsiella pneumoniae* with emergence of carbapenem resistant isolates in Pakistan: report from a tertiary care hospital. *Journal of the Pakistan Medical Association* **60**, 186–90.

Khawcharoenporn, T., Vasoo, S. and Singh, K. (2013) Urinary tract infections due to multidrug-resistant *Enterobacteriaceae*: prevalence and risk factors in a Chicago emergency department. *Emergency Medicine International.* Article ID 258517.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F. and Hopwood, D.A. (2000) *Practical Streptomyces Genetics*. 1st edn. Norwich: The John Innes Foundation

Kinney, C.A., Furlong, E.T., Zaugg, S.D. et al. (2006) Survey of organic wastewater contaminants in biosolids destined for land application. *Environmental Science and Technology*, **40**, 7207–15.

Kleckner, N. (1981) Transposable elements in prokaryotes. *Annual Review of Genetics*, **15,** 341-404.

Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J. and Smets, B.F. (2014) Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *The ISME Journal* **9**, 934-945.

Knapp, C.W., Dolfing, J., Ehlert, P.A.I. and Graham, D.W. (2010) Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environmental Science and Technology*, **44**, 580-587.

Knapp, C.W., McCluskey, S.M., Singh, B.K., Campbell, C.D. and Hudson, G. (2011) Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. *PLoS ONE*, **6**, e27300.

Kobayashi, I. (2001) Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic Acids Research* **29**, 3742–3756.

Koburger, J.A. and May, S.O. (1982) Isolation of *Chromobacterium* spp. from foods, soil and water. *Applied Environmental Microbiology* **44**, 1463-1465.

Konaklieva, M.I. (2014) Molecular targets of β-lactam-cased antimicrobials: beyond the usual suspects. *Antibiotics,* **3**, 128-142.

Kong, K.-F., Schneper, L. and Mathee, K. (2010) B-lactam antibiotics: from antibiotic to resistance and bacteriology. *APMIS*, **118**, 1-36.

Koren, S., Schatz, M.C., Walenz, B.P. *et al.* (2012) Hybrid error correction and de novo assembly of single-molecule sequencing reads. *Nature Biotechnology*, **30**, 693-700.

Kryndushkin, D.S., Alexandrov, I.M., Ter-Avanesyan, M.D. and Kushnirov, V.V. (2003) Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. *Journal of Biological Chemistry*, **278**, 49636-43.

Kuhn, I., Iversen, A., Finn, M., Greko, C., Burman, L.G., Blanch, A.R., Vilanova, X., Manero, A., Taylor, T., Caplin, J., Dominguez, L., Herrero, I.A., Moreno, M.A. and Mollby, R. (2005) Occurrence and relatedness of vancomycin-resistant enterococci in animals, humans, and the environment in different European regions. *Applied and Environmental Microbiology*, **71**, 5383-5390.

Kumar, K.P. and Chatterji, D. (1992) Differential inhibition of abortive transcription initiation at different promoters catalysed by *E. coli* RNA polymerase. Effect of rifampicin on purine or pyramidine-initiated phosphodiester synthesis. *FEBS Letters*, **306**, 46-50.

Kümmerer, K. (2009) Antibiotics in the aquatic environment: a review, part II. *Chemosphere* **75**, 435–441.

Lakshmi, R., Nusrin, K.S., Georgy, S.A. and Sreelakshmi, K.S. (2014) Role of β lactamases in antibiotic resistance: a review. *International Research Journal of Pharmacy*, **5**, 37-40.

Lauretti, L., Riccio, M.L., Mazzariol, A., Cornaglia, G., Amicosante, G., Fontana, R. and Rossolini, G.M. (1999) Cloning and characterization of *bla*$_{VIM}$, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate, *Antimicrobial Agents and Chemotherapy*, **43**, 1584-1590.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A.K.M., Wertheim, H.F.L., Sumpradit, N., Vlieghe, E., Hara, G.L., Gould, I.M., Goossens, H., Greko, C., So, A.D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A.Q., Qamar, F.N., Mir, F., Kariuki, S., Bhutta, Z.A., Coates, A., Bergstrom, R., Wright, G.D., Brown, E.D. and Cars, O. (2013) Antibiotic resistance: the need for global solutions. *Lancet Infectious Diseases* **13**, 1057–98.

Leclercq, R., Derlot, E., Duval, J. and Courvalin, P. (1988) Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *New England Journal of Medicine.* **319**, 157–161.

Lederberg, J. and Lederberg, E.M. (1952) Replica plating and indirect selection of bacterial mutants. *Journal of Bacteriology*, **63**, 399-406.

Lee, I.W., Livrelli, V., Park, S.J., Totis, P.A. and Summers, A.O. (1993) In vivo DNA-protein interactions at the divergent mercury resistance (*mer*) promoters. II. Repressor/activator (MerR)-RNA polymerase interaction with merOP mutants. *Journal of Biological Chemistry*, **268**, 2632-2639.

Lee, L. *et al.* (2005) Genome wide transcriptional response of chemostat-cultured *Escherichia coli* to zinc. *Journal of Bacteriology* **187**, 1124–1134.

Lee, Y., Kim, C-K., Chung, H-S., Yong, D., Jeong, S.H., Lee, K. and Chong, Y. (2015) Increasing carbapenem-resistant Gram-negative bacilli and decreasing metallo-β-lactamase producers over eight years from Korea. *Yonsei Medical Journal* **56**, 572-577.

Levinson, W. (2008) Antimicrobial drugs: mechanism of actions. In: *Review of Medical Microbiology and Immunology*, 10th edition. USA: McGraw-Hill Companies, pp.114-136.

Levy, S.B. (2002) Factors impacting on the problem of antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, **49**, 25-30.

Levy, S.B. and O'Brien, T.F. (2005) Global antimicrobial resistance alerts and implications. *Clinical Infectious Diseases,* **41**, S219-S220.

Lewis K. (2013) Platforms for antibiotic discovery. *Nature Reviews Drug Discovery,* **12,** 371-387.

Lin, M.F., Chang, K.C., Lan, C.Y., Chou, J., Kuo, J.W., Chang, C.K. and Liou, M.L. (2011) Molecular epidemiology and antimicrobial resistance determinants of multidrug resistant Acinetobacter baumannii in five proximal hospitals in Taiwan. *Japanese Journal of Infectious Diseases*, 64, 222-227.

Lira, F., Hernandez, A. Belda, E., Sanchez, M.B., Moya, A., Silva, F.J. and Martinez J.L. (2012) Whole-genome sequence of *Stenotrophomonas maltophilia* D457, a clinical isolate and a model strain. *Journal of Bacteriology* **194**, 3563-3564.

Liu, C.M., Osborne, B.J.W., Hungate, B.A., Shahabi, K., Huibner, S., Lester, R., Dwan, M.G., Kovac, C., Contente-Cuomo, T.L., Benko, E., Aziz, M., Price, L.B. and Kaul, R. (2014) The semen microbiome and its relationship with local immunology and viral load in HIV infection. *PLoS Pathogens* **10**.

Livermore, D.M. (2009) Has the era of untreatable infections arrived? *Journal of Antimicrobial Chemotherapy* **64**, i29-36.

Llarrull L.I., Fisher, J.F. and Mobashery, S. (2009) Molecular basis and phenotype of methicillin resistance in *Staphylococcus aureus* and insights into new β-lactams that meet the challenge. *Antimicrobial Agents and Chemotherapy*, **53**, 4051-4063.

Lo, Y.M.D. and Chan, K.C.A. (2006) Introduction to the polymerase chain reaction. In: Y.M.D. Lo, R.W.K. Chiu and K.C.A. Chan (eds) Book Series *Methods in Molecular Biology: Clinical Applications of PCR*, 2nd edn. Totowa, NJ, USA: Humana Press, pp.1-11.

Lu, S.Y., Zhang, Y.L., Geng, S.N., Li, T.Y., Ye, Z.M., Zhang, D.S., Zou, F. and Zhou, H.W. (2010) High diversity of extended-spectrum β-lactamase-producing bacteria in an urban river sediment habitat. *Applied and Environmental Microbiology*, **76**, 5972-5976.

Mabilat, C., Lourencaovital, J., Goussard, S. and Courvalin, P. (1992) A new example of physical linkage between Tn*1* and Tn*21*—the antibiotic multiple-resistance region of plasmid Pcff04 encoding extended-spectrum β-lactamase TEM-3. *Molecular and General Genetics,* **235**, 113-121.

Maestro. B., Sanz, J.M., Faelen, M., Couturier, M., Díaz-Orejas, R. and Fernández-Tresguerres, E. (2002) Modulation of pPS10 host range by DnaA. *Molecular Microbiology,* **46**, 223-234.

Mahillon, J., Leonard, C. and Chandler, M. (1999) IS elements as constituents of bacterial genomes. *Research in Microbiology*, **150**, 675-687.

Maiden, M.C.J., Bygraves, J.A., Feilm E., Morelli, G., Russell, J.E., Urwin, R. *et al*. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Science USA* **95**, 3140–3145.

Majewski, P., Sacha, P., Wieczorek, P., Ojdana, D., Michalska, A. and Tryniszewska, E. (2012) New Delhi metallo-β-lactamases – the dawn of a post-antibiotic era? *Progress in Health Sciences* **2**, 163-160.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Dymock, D. and Wade, W.G. (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology*, **64,** 795-799.

Marshall, C.G., Lessard, I. A., Park, I. and Wright G. D. (1998) Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrobial Agents and Chemotherapy* **42**, 2215-2220.

Martel, J.-L., Tardy, F, Sanders, P. and Boisseau, J. (2001) New trends in regulatory rules and surveillance of antimicrobial resistance in bacteria of animal origin. *Veterinary Research*, **32,** 381-392.

Martinez, J.L., Baquero, F. and Andersson, D.I. (2007) Predicting antibiotic resistance. *Nature Reviews Microbiology*, **5**, 958-965.

Mathers, A. J., Carroll, J., Sifri, C. D., and Hazen, K. C. (2013) Modified Hodge test versus indirect carbapenemase test: prospective evaluation of a phenotypic assay for detection of *Klebsiella pneumoniae* carbapenemase (KPC) in *Enterobacteriaceae*. *Journal of Clinical Microbiology*, **51**, 1291–1293.

Matthews, D.E. and Farewell, V.T. (2007) *Using and Understanding Medical Statistics* (4th Ed), Karger.

McCaig, A.E., Glover, L.A. and Prosser, J.I. (1999) Molecular analysis of eubacterial community structure and diversity in unimproved and improved upland grass pastures. *Applied Environmental Microbiology* **65**, 1721-1730.

McCaig, A.E., Grayston, S.J., Prosser, J. I. and Glover, L. A. (2001) Impact of cultivation on characterisation of species composition of soil bacterial communities. *FEMS Microbiology Ecology* **35**, 37-48.

McCormick, A.W., Whitney, C.G., Farley, M.M., Lynfield, R., Harrison, L.H., Bennett, N.M., Schaffner, W., Reingold, A., Hadler, J., Cieslak, P., Samore, M.H. and Lipsitch, M. (2003) Geographic diversity and temporal trends of antimicrobial resistance in *Streptococcus pneumoniae* in the United States. *Nature Medicine*. **9**, 424-30.

McLaughlin, M., Advincula, M.R., Malczynski, M., Qi, C., Bolon, M. and Scheetz, M.H. (2013) Correlations of antibiotic use and carbapenem resistance in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy* **57**, 5131–3.

Medeiros, A. (1997) Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. *Clinical Infectious Diseases*, **24**, S19-45.

Mishra, M., Patel, A.K. and Behera, N. (2013) Prevalence of multidrug resistant *E. coli* in the river Mahanadi of Sambalpur. *Current Research in Microbiology and Biotechnology*, **1**, 239-244.

Moore E, Arnscheidt, A., Kruger, A., Strompl, C. and Mau, M. (2004) Simplified protocols for the preparation of genomic DNA from bacterial cultures. *Molecular Microbial Ecology Manual, Second Edition* **1**, 3–18.

Moore, J.E., Xu, J., Millar, B.C., Courtney, J. and Elborn, J.S. (2003) Development of a Gram-negative selective agar (GNSA) for the detection of Gram-negative microflora in sputa in patients with cystic fibrosis. *Journal of Applied Microbiology*, **95**, 160 –166.

Morris, M., Boyle, F., Morris, C., Condon, I., Delannoy-Vieillard, A.-S., Power, L., Khan, A., Morris-Downes, M., Finnegan, C., Powell, J., Monahan, R., Burns, K., O'Connell. N., Boyle, L., O'Gorman, A., Humphreys, H., Brisse, S., Turton, J., Woodford, N. and Cormican, M. (2012) Inter-hospital outbreak of *Klebsiella pneumoniae* producing KPC-2 carbapenemase in Ireland. *Journal of Antimicrobial Chemotherapy*, **67**, 2367–2372.

Muniesa, M., Imamovic, L. and Jofre, J. (2011) Bacteriophages and genetic mobilization in sewage and faecally polluted environments. *Microbial Biotechnology*, **4**, 725–734.

Munoz-Price, L. and Jacoby, G. (2014) *Extended-spectrum β-lactamases* [online]. Available at: **http://www.uptodate.com/contents/extended-spectrum-β-lactamases** [accessed 24 September 2014].

Murray, I.A., Clark, T.A., Morgan, R.D. *et al*. (2012) The methylomes of six bacteria. *Nucleic Acids Research*, **40**, 11450-11462.

Musovic, S., Oregaard, G., Kroer, N. and Sørensen, S.J. (2006) Cultivation-independent examination of horizontal transfer and host range of an incP-1 plasmid among Gram-positive and Gram-negative bacteria indigenous to the barley rhizosphere. *Applied and Environmental Microbiology*, **72**, 6687–6692.

Muthu, S.E., Aberna, R.A., Mohan, V., Premalatha, G., Srinivasan, R.S., Thyagarajan, S.P. and Rao, U.A. (2006) Phenotypes of isolates of *Pseudomonas aeruginosa* in a diabetes care center. *Archives of Medical Research,* **37**, 95-101.

Naas, T. and Nordmann, P. (1994) Analysis of a carbapenem-hydrolyzing class A β-lactamase from *Enterobacter cloacae* and of its LysR-type regulatory protein. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 7693–7697.

Naito, T., Kusano, K. and Kobayashi, I. (1995) Selfish behavior of restriction-modification systems. *Science*. **267,** 897-899.

Nakamura, A., Sugimoto, Y., Ohishi, K. *et al*. (2010) Diagnostic value of PCR analysis of bacteria and fungi from blood in empiric-therapy-resistant febrile neutropenia. *Journal of Clinical Microbiology*, **48**, 2030-2036.

Nakamura, Y., Itoh, T., Matsuda, H. and Gojobori, T. (2004) Biased function of horizontally transferred genes in prokaryotic genomes. *Nature Genetics*, **36**, 760-766.

Neidhardt, F.C. (2004) Bacterial Genetics. In: K.J. Ryan and C.G. Ray (eds) *Sherris Medical Microbiology: an Introduction to Infectious Diseases*. USA: McGraw-Hill Companies, Inc., Chapter 4, pp.53-75.

Nicodemo, A.C. and Paez, J.I. (2007) Antimicrobial therapy for *Stenotrophomonas maltophilia* infections. *European Journal of Clinical Microbiological Infectious Diseases* **26**, 229-237.

Nicolaou, K.C., Chen, J.S., Edmonds, D.J. and Estrada, A.A. (2009) Recent advances in the chemistry and biology of naturally occurring antibiotics. *Angewandte Chemie International Edition* **48**, 660-719.

Nordmann P., Naas T. and Poirel L. (2011) Global spread of carbapenemase-producing *Enterobacteriacea*. *Emerging Infectious Diseases*, **17**, 1791-1798.

Nordmann, P., Gniadkowski, M., Giske, C. G., Poirel, L., Woodford, N., Miriagou, V. and the European Network on Carbapenemases (2012) Identification and screening of carbapenemase-producing *Enterobacteriaceae*. *Clinical Microbiology and Infection* **18**, 432–438.

Novais, C., Coque, T.M., Ferreira, H., Sousa, J.C. and Peixe, L. (2005) Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. *Applied and Environmental Microbiology*, **71**, 3364-3368.

Nucleo, E., Spalla, M., Piazza, A., Caltagirone, M.S., Asticcioli, S., Debiaggi, M., Matti, C., Daturi, R., Navarra, A., Labonia, M. and Migliavacca, R. (2013) Emergence of a VIM-1 MBL and CTX-M-15 ESbL-producing *Klebsiella pneumoniae* clone from acute and rehabilitation hospitals in Italy. *New Microbiologica*, **36**, 279-282.

Nunvar, J., Elhottova, D., Chronakova, A., Schneider, B. and Licha, I. (2014) Draft genome sequence of *Stenotrophomonas maltophilia* strain 5BA-I-2, a soil isolate and a member of a phylogenetically basal lineage. *Genome Announcements* **2**, e00134-14.

O'Toole, D.K. (2014) The natural environment may be the most important source of antibiotic resistance genes. *mBio* **5**, e01285-14.

Ochiai, K., Yamanaka, T., Kimura, K. and Sawada, O. (1959) Inheritance of drug resistance (and its transfer) between *Shigella* strains and between *Shigella* and *E. coli* strains. *Nihon Iji Shimpo* (in Japanese), **1861**, 34-46.

Osborn A.M. and Boltner, D. (2002) When phage, plasmids, and transposons collide: genomic islands, and conjugative- and mobilizable-transposons as a mosaic continuum. *Plasmid*, **48**, 202-212.

Osborn, A.M., Moore, E.R.B. and Timmis, K.N. (2000) An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology* **2**, 39-50.

Osterblad, M., Norrdahl, K., Korpimaki, E. and Huovinen, P. (2001) Antibiotic resistance —how wild are wild mammals? *Nature*, **409**, 37–38.

Özdemir, Z. (2009) Development of a multiplex PCR assay for the simultaneous detection of *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. tomato and *Xanthomonas axonopodis* pv. *vesicatoria* using pure cultures. *Journal of Plant Pathology*, **91**, 495-497.

Ozen, N., Ergani, A., Naas, T., Ögünç, D., Gültekin, M., Colak, D. and Nordmann, P. (2009) Outbreak of carbapenem-resistant *Acinetobacter baumannii* producing the carbapenemase *OXA-58* in Turkey. *Antimicrobial Agents Journal*, **1**, 1-8.

Pagani, I., Liolios, K., Jansson, J., Chen, I-M. A., Smirnova, T., Nosrat, B. Markowitz,, V. M. and Kyrpides, N.C. (2012) The genomes online database (GOLD) v.4: status of genomic and metagenomic projects and their associated metadata. *Nucleic Acids Research*, **40**, D571–D579.

Pages, D., Rose, J., Conrod, S., Cuine, S., Carrier, P., Heulin, T. and Achouak, W. (2008) Heavy metal tolerance in *Stenotrophomonas maltophilia*. *PLoS One* **3**, e1539.

Pallecchi L., Lucchetti, C., Bartoloni, A., Bartalesi, F., Mantella, A., Gamboa, H., Carattoli, A., Paradisi, F. and Rossolini, G.M. (2007) Population structure and resistance genes in antibiotic-resistant bacteria from a remote community with minimal antibiotic exposure. *Antimicrobial Agents and Chemotherapy*, **51**, 1179-1184.

Pantanella, F., Berlutti, F., Passariello, C., Sarli, S., Morea, C. and Schippa, S. (2006) Violacein and biofilm production in *Janthinobacterium lividum*. *Journal of Applied Microbiology* **102**, 992-999.

Pareek, C.S., Smoczynski, R. and Tretyn, A. (2011) Sequencing technologies and genome sequencing. *Journal of Applied Genetics*, **52**, 413-435.

Park, M. *et al*. (2005) Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microbiology Research* **160**, 127–133.

Park-Wyllie, L.Y., Juurlink, D.N., Kopp, A., *et al.* (2006) Outpatient gatifloxacin therapy and dysglycemia in older adults. *New England Journal of Medicine* **354**, 1352 – 61.

Paul, M., Shani, V., Muchtar, E., Kariv, G., Robenshtok, E. and Leibovici, L. (2010) Systematic review and meta-analysis of the efficacy of appropriate empiric antibiotic therapy for sepsis. *Antimicrobial Agents and Chemotherapy* **54**, 4851–63

Perichon, B. and Courvalin, P. (2009) VanA-type vancomycin-resistant *Staphylococcus aureus*: minireview. *Antimicrobial Agents And Chemotherapy*, **53**, 4580–4587.

Permina, E.A., Kazakov, A.E., Kalinina, O.V. and Gelfand, M.S. (2006) Comparative genomics of regulation of heavy metal resistance in eubacteria. *BMC Microbiology*, **6**, 49-59.

Peter-Getzlaff, S., Polsfuss, S., Poledica, M., Hombach, M., Giger, J., Böttger, E.C. and Bloemberg, G.V. (2011) Detection of AmpC Beta-Lactamase in *Escherichia coli*: comparison of three phenotypic confirmation assays and genetic analysis. *Journal of Clinical Microbiology*, **49**, 2924–2932.

Petridou, N., Wharton, S. J., Lotfipour, A., Gowland, P. and Bowtell R. (2010) Investigating the effect of blood susceptibility on phase contrast in the human brain. *Neuroimage* **50**, 491–498.

Petti, C.A. (2007) Detection and identification of microorganism gene amplification and sequencing. *Clinical Infectious Diseases* **44**, 1108–1114.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R. and Waddell, J. (2004) Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. In: *Journal of Antimicrobial Chemotherapy*, **53**, 28-52.

Poirel, L. and Nordmann, P. (2006) Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clinical Microbiology and Infectious Disease* **12**, 826–836.

Poole, K. (2005) Efflux-mediated antimicrobial resistance. *Journal of Antimicrobiology and Chemotherapy*, **56**, 20-51.

Poole, K. and Srikumar, R. (2001) Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. *Current Topics in Medical Chemistry* **1**, 59-71.

Poulin-Laprade, D., Matteau, D., Jacques, P-E., Rodrigue, S. and Burrus, V. (2015) Transfer activation of SXT/R391 integrative and conjugative elements: unraveling the SetCD regulon. *Nucleic Acids Research* **1**. doi: 10.1093/nar/gkv071.

Prado, T., Pereira, W.C., Silva, D.M., Seki, L.M., Carvalho, A.P.D. and Asensi, M.D. (2008) Detection of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant. *Letters in Applied Microbiology*, **46**, 136-141.

Proctor, R.A., von Eiff, C., Kahl, B.C., Becker, K., McNamara, P., Herrmann, M. and Peters, G. (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Reviews Microbiology* **4**, 295-305.

Pudełko, K. (2013) The occurrence of soft rot (*Janthinobacterium agaricidamnosum*) in mushroom (*Agaricus bisporus*) crops. *Journal of Plant Protection Research* **53**.

Queenan, A.M. and Bush, K. (2007) Carbapenemases: the versatile β-lactamases. *Clinical Microbiology Rev*iew **20**, 440–58.

Rathnayake, I., Hargreaves, M. and Huygens, F. (2011) SNP diversity of *Enterococcus faecalis* and *Enterococcus faecium* in a South East Queensland waterway, Australia, and associated antibiotic resistance gene profiles. *BMC Microbiology*, **11**, 201.

Ravatn, R., Studer, S., Springael, D., Zehnder, A.J.B. and Meer, J.R. van der (1998) Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. strain B13. *Journal of Bacteriology*, **180**, 4360-4369.

Reasoner, D.J. and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology*, **49**, 1-7.

Reuter, S., Ellington, M.J., Cartwright, E.J. *et al*. (2013) Rapid bacterial whole-genome sequencing to enhance diagnostic and public health microbiology. *JAMA Internal Medicine*, **173**, 1397-1404.

Reynolds, P.E., Depardieu, F., Dutka-Malen, S., Arthur, M. and Courvalin, P. (1994) Glycopeptide resistance mediated by enterococcal transposon Tn*1546* requires production of VanX for hydrolysis of D-alanyl-D-alanine. *Molecular Microbiology*, **13**, 1065-1070.

Reznikoff, W.S. (2002) Tn*5* transposition. In: N.L. Craig, R. Craigie, M. Gellert and A.M. Lambowitz (eds) *Mobile DNA II*. Washington, DC: ASM Press, pp.403-422.

Riesenfeld, C.S., Goodman, R. M. and Handelsman, J. (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environmental Microbiology* **6**, 981–989.

Rizek, C., Fu, L., dos Santos, L.C., Leite, G., Ramos, J., Rossi, F., Guimaraes, T., Levin, A.S. and Costa, S.F. (2014) Characterization of carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates, carrying multiple genes coding for this antibiotic resistance. *Annals of Clinical Microbiology and Antimicrobials* **13**, 43-47.

Rocco, F., De Gregorio, E., Colonna, B. and Di Nocera, P.P. (2009). *Stenotrophomonas maltophilia* genomes: a start-up comparison. *International Journal of Medical Microbiology*, **299**, 535-546.

Rossolini, G., Condemi, M.A., Pantanella, F., Docquier, J.-D., Amicosante, G. and Thaller, M.C. (2001) Metallo-β-lactamase producers in environmental microbiota: new molecular Class B enzyme in *Janthinobacterium lividum. Antimicrobial Agents and Chemotherapy* **45**, 837-44.

Ryan K.J. (2004) Antibacterial Resistance. In: K.J. Ryan and C.G. Ray (eds) *Sherris Medical Microbiology: an Introduction to Infectious Diseases*. USA: McGraw-Hill Companies, Inc., Chapter 14, pp.215-228.

Ryan, K.J. and Drew, W.L. (2004) Antibacterial and Antiviral Agents. In: K.J. Ryan and C.G. Ray (eds) *Sherris Medical Microbiology: an Introduction to Infectious Diseases*. USA: McGraw-Hill Companies, Inc., Chapter 13, pp.193-214.

Ryan, R.P. and Dow, J.M. (2008) Diffusible signals and interspecies communication in bacteria. *Microbiology*, **154**, 1845-1858.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van der Lelie, D. and Dow, J. M. (2009) The versatility and adaptation of bacteria from the genus *Stenotrophomonas. Nature Reviews Microbiology* **7**, 514-525.

Sader, H.S. and Jones, R.N. (2005) Antimicrobial susceptibility of uncommonly isolated non-enteric Gram-negative bacilli. *International Journal of Antimicrobial Agents* **25**, 95-109.

Safdar, A., Rolston, K.V. (2007) *Stenotrophomonas maltophilia*: changing spectrum of a serious bacterial pathogen in patients with cancer. *Clinical Infectious Diseases* **45**,1602–1609.

Sahoo, K.C., Tamhankar, A.J., Johansson, E.C. and Lundborg, S. (2010) Antibiotic use, resistance development and environmental factors: a qualitative study among healthcare professionals in Orissa, India. *BMC Public Health* **10**, 629.

Saidel-Odes, L. and Borer, A. (2013) Limiting and controlling carbapenem-resistant *Klebsiella pneumonia*. *Infection and Drug Resistance*, **7**, 9-14.

Saino, Y., Inoue, M. and Mitsuhashi, S. (1984) Purification and properties of an inducible cephalosporinase from *Pseudomonas maltophilia* GN12873. *Antimicrobial Agents and Chemotherapy* **25**, 362−365.

Saino, Y., Kobayashi, F., Inoue, M. and Mitsuhashi, S. (1982) Purification and properties of inducible penicillin β-lactamase isolated from *Pseudomonas maltophilia*. *Antimicrobial Agents and Chemotherapy* **22**, 564−570.

Saleem, A.F., Ahmed, I., Mir, F., Ali, S.R. and Zaidi, A.K. (2010) Pan-resistant *Acinetobacter* infection in neonates in Karachi, Pakistan. *Journal of Infection in Developing Countries* **4**, 30–37.

Salipante, S.J., Sengupta, D.J., Rosenthal, C., Costa, G., Spangler, J., *et al.* (2013) Rapid 16S rRNA next-generation sequencing of polymicrobial clinical samples for diagnosis of complex bacterial infections. *PLoS ONE* **8**, e65226.

Sambrook, J. and Russell, D.W. (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sánchez, M., Hernández, A. and Martinez, J. (2009) *Stenotrophomonas maltophilia* drug resistance. *Future Microbiology*, **4**, 655-660.

Schuenemann, V.J., Singh, P., Mendum, T.A. *et al*. (2013) Genome-wide comparison of medieval and modern *Mycobacterium leprae*. *Science*, **341**, 179-183.

Schwartz, T., Kohnen, W., Jansen, B. and Obst, U. (2003) Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology* **43**, 325-335.

Schweizer, H.P. (2003) Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. *Genetics and Molecular Research* **2**, 48-62.

Seah, C., Low, D.E., Patel, S.M. *et al*. (2011) Comparative evaluation of a chromogenic agar medium, the modified Hodge test, and a battery of meropenem-inhibitor discs for detection of carbapenemase activity in Enterobacteriacae. *Journal of Clinical Microbiology* **49,** 1965–1969.

Senda, K., Arakawa, Y., Ichiyama, S., Nakashima, K., Ito, H., Ohsuka, S., Shimokata, K., Kato, N. and Ohta, M. (1996) PCR detection of metallo-β-lactamase gene (bla_{IMP}) in Gram-negative rods resistant to broad-spectrum β-lactams. *Journal of Clinical Microbiology*, **34**, 2909-2913.

Shah, P.M. (2008) Parenteral carbapenems: A Review. European Society of Clinical Microbiology and Infectious Diseases. *Clinical Microbiology and Infection*, **14** (Suppl. 1), 175-180.

Shlaes, D.M. (2010) The Miracle. In: D.M. Shlaes (ed), *Antibiotics: The Perfect Storm*. London and New York: Springer Dordrecht Heidelberg, pp.9-14.

Silver, L.L. (2011) Challenges of antibacterial discovery. *Clinical Microbiology Reviews*, **24**, 71–109.

Skurnik, D., Ruimy, R., Ready, D., Ruppe, E., Berne` de-Bauduin, C., Djossou, R., Guillemot, D., Pier, G.B. and Andremont, A. (2010) Is exposure to mercury a driving force for the carriage of antibiotic resistance genes? *Journal of Medical Microbiology*, **59**, 804-807.

Song, W. and Shin, J.H. (2012) Multilocus sequence typing for clonality analysis of antimicrobial-resistant *Stenotrophomonas maltophilia* strains. *Annals of Laboratory Medicine*, **32**, 3-4.

Šeputienė, V., Povilonis, J., Armalytė, J., Sužiedėlis, K., Pavilonis, A. and Sužiedėlienė, E. (2010) Tigecycline – how powerful is it in the fight against antibiotic-resistant bacteria? *Medicina (Kaunas)* **46**, 240-8.

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S., Nadarajan, R., Brodie, E.L. and Lynch, S.V. (2015) Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS ONE* **10**, e0117617.

Stewart, P.S. and Costerton, J.W. (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135–138.

Stokes H.W. and Hall R.M. (1989) A novel family of potentially mobile DNA elements encoding site specific gene-integration functions: integrons. *Molecular Microbiology*, **3**, 1669-1683.

Stokes, H.W. and Hall, R.M. (1992) The integron In1 in plasmid R46 includes 2 copies of the Oxa2 gene cassette. *Plasmid*, **28**, 225-234.

Streit, J.M., Jones, R.N. and Sader, H.S. (2004) Daptomycin activity and spectrum: A worldwide sample of 6737 clinical gram-positive organisms. *Journal of Antimicrobiology and Chemotherapy*, **53**, 669-674.

Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K. and Wallace, R.B. (1981) Use of synthetic oligonucleotides for the isolation of specific cloned DNA sequences. In: D. Brown and C.F. Fox (eds) *Developmental Biology Using Purified Genes*. New York: Academic Press, pp.663-693.

Summers, W.C. (2008) Microbial drug resistance, a historical perspective. In: Richard G. Wax, Kim Lewis, Abigail A. Salyers and Harry Taber (eds) *Bacterial resistance to antimicrobials*, 2nd edition. Boca Raton, London and New York: CRC Press, Taylor and Francis Group, pp.1-10.

Sung, J.Y., Kwon, K.C., Cho, H.H. and Koo, S.H. (2011) Antimicrobial resistance determinants in imipenem-nonsusceptible *Acinetobacter calcoaceticus-baumannii* complex isolated in Daejeon, Korea. *Korean Journal of Laboratory Medicine* **31**, 265-270.

Taghavi, S., Garafola, C., Monchy, S., Newman, L., Hoffman, A., Weyens, N., Barac, T., Vangronsveld, J. and van der Lelie, D. (2009) Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. *Applied Environmental Microbiology* **75**, 748–757.

Tanimoto K. (2013) *Stenotrophomonas maltophilia* strains isolated from a university hospital in Japan: genomic variability and antibiotic resistance. *Journal of Medical Microbiology* **62**, 565–570.

Teke, T.A., Oz, F. N., Metin, O., Bayhan, G. I., Aydin, Z.G.G., Oguz, M. and Tanir, G. (2014) *Chryseobacterium indologenes* septicemia in an infant. Hindawi Publishing Corporation. *Case Reports in Infectious Diseases* 2014, Article ID 270521.

Thomas, C.M. and Nielsen, K.M. (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews*, **3,** 711-721.

Thomas, T., Gilbert, J. and Meyer, F. (2012) Metagenomics - a guide from sampling to data analysis. *Microbial Informatics and Experimentation* **2**.

Tissera, S. and Lee, S.M. (2013) Isolation of extended spectrum ß-lactamase (ESBL) producing bacteria from urban surface waters in Malaysia. *Malaysian Journal of Medical Sciences* **20**, 14-22.

Tofteland, S., Naseer, U., Lislevand, J.H., Sundsfjord, A. and Samuelsen, Ø. (2013) A long-term low-frequency hospital outbreak of KPC-producing *Klebsiella pneumoniae* involving intergenus plasmid diffusion and a persisting environmental reservoir. *PLoS ONE* **8**, e59015.

Toleman, M.A., Bennett, P.M., Bennett, D.M., Jones, R.N., Walsh, T.R. (2007) Global emergence of trimethoprim/sulfamethoxazole resistance in *Stenotrophomonas maltophilia* mediated by acquisition of *sul* genes. *Emerging Infectious Diseases* **13**, 559-565.

Török, M.E. and Peacock, S.J. (2012) Rapid whole-genome sequencing of bacterial pathogens in the clinical microbiology laboratory - pipe dream or reality? *Journal of Antimicrobial Chemotherapy*, **67**, 2307-2308.

Torres, J.A., Villegas, M.V. and Quinn, J.P. (2007) Current concepts in antibiotic resistant Gram-negative bacteria. *Expert Review of Anti-Infective Therapy* **5**, 833-843.

Towner, K.J. (2000) The problem of resistance. In: D. Greenwood (ed) *Antimicrobial Chemotherapy*, 4th edition. Oxford: Oxford University Press.

Tseng, S.-P., Tsai, J.-C., Teng, L.-J. Hsueh, P.-R. (2009) Dissemination of transposon Tn6001 in carbapenem-non-susceptible and extensively drug-resistant *Pseudomonas aeruginosa* in Taiwan. *Journal of Antimicrobial Chemotherapy*, **64**, 1170-1174.

Tzouvelekis, L.S., Markogiannakis, A., Psichogiou, M., Tassios, P.T. and. Daikos, G.L. (2012) Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an evolving crisis of global dimensions. *Clinical Microbiology Reviews* **25**, 682–707.

Udikovic-Kolic, N., Wichmann, F., Broderick, N.A. and Handelsman, J. (2014) Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proceedings of the National Academy of Sciences*, **111**, 15202-15207.

Uttley, A.H., Collins, C.H., Naidoo, J. and George R.C. (1988) Vancomycin resistant enterococci. *The Lancet* **332**, 57-58.

Van den Bogaard, A. E. and Stobberingh, E. E. (1999) Antibiotic usage in animals: impact on bacterial resistance and public health. *Drugs* **58**, 589-607.

Vaz-Moreira, I., Nunes, O.C. and Manaia, C.M. (2011) Diversity and antibiotic resistance patterns of *Sphingomonadaceae* isolates from drinking water. *Applied and Environmental Microbiology,* **77**, 5697–5706.

Vien, L.T.M., Baker, S., Thao, L.T.P., Tu, L.T.P.,Thuy, C.T., Nga, T.T.T., Hoang, N.V.M., Campbell, J.I., Yen, L.M., Hieu, N.T., Chau, N.V.V., Farrar, J. and Schultsz, C. (2009) High prevalence of plasmid-mediated quinolone resistance determinants in commensal members of the *Enterobacteriaceae* in Ho Chi Minh City, Vietnam. *Journal of Medical Microbiology*, **58**, 1585-1592.

Walsh, C. (2003) Where will new antibiotics come from? *Nature Review*, **3**, 65-69.

Wang, Y.C., Yeh, K-M., Chiu, S-K., Shang, S-T., Kan, L-P., Yu, C-M. and Lin, J-C. (2011) *Chryseobacterium indologenes* peritonitis in a patient with malignant ascites. *International Medical Case Reports Journal* **4**, 13–15.

Watanabe, T. (1963) Infective heredity of multiple drug resistance in bacteria. *Bacteriology Reviews,* **27**, 87-115.

Wegener, H.C., Aarestrup, F.M., Jensen, L.B., Hammerum, A.M. and Bager, F. (1999) Use of antimicrobial growth promoters in food animals and *Enterococcus faecium*: resistance to therapeutic antimicrobial drugs in Europe. *Emerging Infectious Diseases*. **5**, 29-335.

Wehr, H.M. and Frank, J.F. (eds.) (2004) *Standard methods for the examination of dairy products,* 17th ed. American Public Health Association [online] Available at: **http://ajph.aphapublications.org/doi/pdf/10.2105/9780875530024fm01** [accessed 10 Dec 2014].

Weigel, L.M., Clewell, D.B., Gill, S.R., Clark, N.C., McDougal, L.K., Flannagan, S.E., Kolonay, J.F., Shetty, J., Killgore, G.E. and Tenover, F.C. (2003) Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* **302**, 1569-71.

Wellington, E.M.H., Boxall, A.B.A., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D., Lee, N.M., Otten, W., Thomas, C.M. and Williams, A.P. (2013) The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases*, **13**, 155–65.

Wichmann, F., Udikovic-Kolic, N., Andrew, S., Handelsman, J. (2014) Diverse antibiotic resistance genes in dairy cow manure. *mBio* **5**, e01017-13.

Witte, W. (1997) How great is the potential danger from vancomycin-resistant enterococci? *Dtsch Med Wochenschr* **122**, 1161-3.

Witte, W. (2000) Ecological impact of antibiotic use in animals on different complex microflora: environment. *International Journal of Antimicrobial Agents*, **14**, 321 – 325.

Wolf, A., Fritze, A., Hagemann, M.and Berg, G. (2002) *Stenotrophomonas rhizophila* sp.*nov*., a novel plant-associated bacterium with antifungal properties. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1937–1944.

Woodford, N. (2012) *Fighting the Rising Tide of Carbapenemases in Enterobacteriaceae. Be S.M.A.R.T. with Resistance* [online]. Available at: **http://www.biomerieuxdiagnostics.com/sites/clinic/files/be_smart_newsletter_8p_num_5_final.pdf** [accessed 10 December 2014].

Woodford, N., Wareham, D.W., Guerra, B. and Teale, C. (2014) Carbapenemase-producing *Enterobacteriaceae* and non-*Enterobacteriaceae* from animals and the environment: an emerging public health risk of our own making? *Antimicrobial Chemotherapy* **69**, 287–291.

World Health Organization (2011) *Tackling antibiotic resistance from a food safety perspective in Europe* [online]. Available at: **http://www.euro.who.int/en/publications/bibliographical-databases/contact-us/request-forms** [accessed 19 March 2015].

World Organisation for Animal Health. (2014) *OIE List of Antimicrobials of Veterinary Importance* [online]. Available at: **http://web.oie.int/downld/Antimicrobials/OIE_list_antimicrobials.pdf** [accessed 10 December 2014].

Woyke, T., Tighe, D., Mavromatis, K. *et al*. (2010) One bacterial cell, one complete genome. *PLoS One*, **5**, e10314.

Wright P.M., Seiple, I.B. and Myers, A.G. (2014) The evolving role of chemical synthesis in antibacterial drug discovery. *Angewandte Chemie International Edition*, **53**, 8840 – 8869.

Wright, G.D. (2007) The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Reviews Microbiology* **5**, 175-186.

Xu, X., Lin, D., Yan, G., Ye, X., Wu, S., Guo, Y., Zhu, D., Hu, F., Zhang, Y., Wang, F., Jacoby, G.A. and Wang, M. (2010) *vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium. Antimicrobial Agents and Chemotherapy*, **54**, 4643-464.

Yim, G., Wang, H.H. and Davies, J. (2007) Antibiotics as signaling molecules. *Philosophical Transactions of the Royal Society B*, **362**, 1195-1200.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M. and Larsen, M. V. (2012) Identification of acquired antimicrobial resistance genes. *Antimicrobial Chemotherapy* **67**, 2640–44.

Zhang, L., Morrison, M.Ó., Cuív, P., Evans, P. and Rickard, C.M. (2013) Genome sequence of *Stenotrophomonas maltophilia* strain AU12-09, isolated from an intravascular catheter. *Genome Announcements* **1**, e00195-13.

Zhao, Q., Li, X-Zhi., Mistry, A., Srikumar,R., Zhang, L., Lomovskaya, O. and Poole, K. (1998) Influence of the *tonB* energy-coupling protein on efflux-mediated multidrug resistance in *Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy* **42**, 2225–2231.

Zhao, Y., Niu, W., Sun, Y. Hao, H. Yu, D., Xu, G., Shang, X., Tang, X., Lu, S., Yue, J. and Li, Y. (2015) Identification and characterization of a serious multidrug resistant *Stenotrophomonas maltophilia* strain in China. *BioMedical Research International* **2015** Article ID 580240 [online]. Available at: **http://dx.doi.org/10.1155/2015/580240** [accessed 12 March 2015].

Appendices

Appendix A1

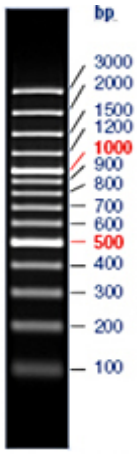


Figure A1.1: 100 bp DNA marker used in electrophoresis (Fermentas Life Science genetic marker)

Appendix A2

A2.1 Isolation and enumeration of antibiotic resistant bacteria in river water

After performing cultures for the water samples from all sites, using 100 µl from each dilution upon PCA and MCA media, with and without antibiotic, for 24 (MCA) and 72 (PCA) hours incubation, the resulting colonies were counted and reported (Table A2.1). No growth was observed in the 10^{-3} dilutions or lower. Mean numbers of viable and antibiotic resistant bacteria are shown in Table A2.2.

Table A2.1: Numbers of bacterial colonies and antibiotic resistant bacteria isolated from river water using different media and series dilutions

Sample site	Dilution	MCA	MCA+Vm	MCA+Im	PCA	PCA+Vm	PCA+Im
S-1	10^{0}	38	6	1	69	13	4
	10^{-1}	7	1	ND	13	2	2
	10^{-2}	1	ND	ND	2	1	ND
S-2	10^{0}	33	15	ND	67	42	8
	10^{-1}	4	3	ND	27	19	2
	10^{-2}	ND	ND	ND	3	1	ND
S-3	10^{0}	32	20	1	65	44	7
	10^{-1}	8	4	ND	17	8	1
	10^{-2}	ND	ND	ND	4	1	ND
S-4	10^{0}	29	17	ND	67	46	11
	10^{-1}	4	11	ND	29	18	1
	10^{-2}	ND	ND	ND	3	3	ND

(**MCA** = MacConkey, **Vm**= vancomycin, **Im** = imipenem, **PCA** = plate count agar, **SD** = Standard Deviation, ND: not determined).

NB. No growth was observed on the 10^{-3} dilution or lower on all media used.

Table A2.2: Mean numbers of colonies forming units and antibiotic resistant bacteria (CFU ml^{-1}) isolated from river water after incubation for between 24 hours (MCA) and 72 hours (PCA)

Sample site	Dilution	MCA	MCA+Vm	MCA+Im	PCA	PCA+Vm	PCA+Im
S-1	10^0	380	60	10	690	130	40
S-2	10^0	330	150	ND	670	420	80
S-3	10^0	320	200	10	650	440	70
S-4	10^0	290	170	ND	670	460	110
Total		1320	580	20	2680	1450	300
Mean* (%)		330	145 (43.9%)	5 (1.5%)	670	362.5 (54.1%)	75 (11.2%)
± SD		+37.4	+60.3	+5.8	+16.3	+155.9	+28.9

(**MCA** = MacConkey, **Vm**= vancomycin, **Im** = imipenem, **PCA** = plate count agar, **SD** = Standard Deviation, ND: not determined).

* Values are calculated as the % of resistant bacteria per media type e.g. (mean number of VmR bacteria on MCA /total viable count on MCA) X 100.

A2.2 Isolation and enumeration of antibiotic resistant bacteria in farm soil

After performing cultures for the soil samples from all fields using 100µl from each dilution upon PCA media with and without antibiotic for 24 (MCA) and 72 (PCA) hours incubation, the resulting colonies were counted (Table A2.3).

Table A2.3: Numbers of bacterial colonies and imipenem resistant bacteria isolated from farm soil expressed as colony forming units (CFU) g^{-1} soil at each dilution.

Site	Media	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
RA1	PCA	TMTC	TMTC	900000	ND	ND
RA1	PCA+Im	TMTC	TMTC	230000	ND	ND
RA1	PCA+Vm	TMTC	TMTC	850000	ND	ND
RA2	PCA	TMTC	TMTC	910000	ND	ND
RA2	PCA+Im	TMTC	TMTC	180000	ND	ND
RA2	PCA+Vm	TMTC	TMTC	880000	ND	ND
RA3	PCA	ND	ND	ND	ND	ND
RA3	PCA+Im	ND	ND	ND	ND	ND
RA3	PCA+Vm	TMTC	TMTC	ND	ND	ND
RB1	PCA	TMTC	TMTC	TMTC	1600000	ND
RB1	PCA+Im	9500	12000	ND	ND	ND
RB1	PCA+Vm	TMTC	TMTC	440000	ND	ND
RB2	PCA	TMTC	TMTC	TMTC	2200000	ND
RB2	PCA+Im	7000	13000	ND	ND	ND
RB2	PCA+Vm	TMTC	TMTC	460000	ND	ND
RB3	PCA	TMTC	TMTC	TMTC	2800000	ND
RB3	PCA+Im	TMTC	15000	ND	ND	ND
RB3	PCA+Vm	TMTC	TMTC	1230000	ND	ND
RC1	PCA	TMTC	TMTC	TMTC	1400000	ND
RC1	PCA+Im	TMTC	22000	NA	ND	ND
RC1	PCA+Vm	TMTC	TMTC	240000	ND	ND
RC2	PCA	TMTC	TMTC	TMTC	1300000	ND
RC2	PCA+Im	TMTC	44000	ND	ND	ND
RC2	PCA+Vm	TMTC	TMTC	420000	ND	ND
RC3	PCA	TMTC	TMTC	TMTC	1900000	ND
RC3	PCA+Im	TMTC	7000	ND	ND	ND
RC3	PCA+Vm	TMTC	TMTC	430000	ND	ND

Site	Media	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
LA1	PCA	TMTC	TMTC	TMTC	1900000	ND
LA1	PCA+Im	TMTC	24000	ND	ND	ND
LA1	PCA+Vm	TMTC	TMTC	800000	ND	ND
LA2	PCA	TMTC	TMTC	TMTC	1600000	ND
LA2	PCA+Im	TMTC	69000	ND	ND	ND
LA2	PCA+Vm	TMTC	TMTC	380000	ND	ND
LA3	PCA	TMTC	TMTC	TMTC	2100000	ND
LA3	PCA+Im	9500	12000	ND	ND	ND
LA3	PCA+Vm	TMTC	TMTC	1020000	ND	ND
LB1	PCA	TMTC	TMTC	980000	1500000	ND
LB1	PCA+Im	2200	ND	ND	ND	ND
LB1	PCA+Vm	TMTC	75000	150000	ND	ND
LB2	PCA	TMTC	TMTC	TMTC	2300000	ND
LB2	PCA+Im	4300	ND	ND	ND	ND
LB2	PCA+Vm	TMTC	TMTC	840000	ND	ND
LB3	PCA	TMTC	TMTC	TMTC	3300000	ND
LB3	PCA+Im	5000	ND	ND	ND	ND
LB3	PCA+Vm	TMTC	TMTC	750000	ND	ND
LC1	PCA	TMTC	TMTC	TMTC	2100000	ND
LC1	PCA+Im	TMTC	15000	ND	ND	ND
LC1	PCA+Vm	TMTC	TMTC	310000	ND	ND
LC2	PCA	TMTC	TMTC	TMTC	2400000	ND
LC2	PCA+Im	2200	ND	ND	ND	ND
LC2	PCA+Vm	TMTC	TMTC	170000	ND	ND
LC3	PCA	TMTC	TMTC	TMTC	1900000	ND
LC3	PCA+Im	700	ND	ND	ND	ND
LC3	PCA+Vm	TMTC	73000	ND	ND	ND

N.B: RA: winter wheat- Riseholme farm, RB: sugar beet- Riseholme farm, RC: Spring beans- Riseholme farm, LA: winter wheat- Lodge farm, LB: sugar beet- Lodge farm,LC: Spring beans- Lodge farm, TMTC: Too Many To Count, ND: not determined.

Table A2.4: Species characterisation of water isolates including MIC levels, CDST and MDR profile testing results arranged alphabetically according to species names

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Acidovorax spp.	BBS4:15	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Brevundimonas lenta	BBS1:14	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Caulobacter segnis	BBS1:15	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Caulobacter spp.	BBS4:5	4	-	+	-	-	R	R	R	R	R	R	R	R	R	R	R	S
Caulobacter spp.	BBS2:5	16	-	+	-	-	R	R	R	R	R	R	R	R	R	R	R	S
Caulobacter spp.	BBS2:6	16	-	+	-	-	R	R	R	S	R	R	R	R	R	S	R	S
Caulobacter spp.	BBS1:21	16	-	+	-	-	R	R	R	S	R	R	R	R	R	S	R	S
Caulobacter spp.	BBS1:5	16	-	+	+	-	R	R	R	R	R	R	S	R	R	S	R	S
Caulobacter spp.	BBS4:18	16	-	-	-	-	R	R	R	S	R	R	S	R	R	R	R	S
Caulobacter spp.	BBS4:11	16	-	-	-	-	R	R	R	S	R	R	S	R	R	S	R	S
Caulobacter spp.	BBS3:20	32	-	-	-	-	R	R	R	S	R	R	S	R	R	S	R	S
Caulobacter spp.	BBS2:8	16	-	-	-	-	S	R	S	S	R	R	S	R	R	S	R	S
Caulobacter vibrioides	BBS2:7	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Chitinophaga spp.	BBS1:7	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Chryseobacterium spp.	BBS2:12	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Epilithonimonas lactis	BBS3:3	4	-	-	-	-	R	R	R	S	S	R	R	S	R	S	S	S

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Epilithonimonas lactis	BBS3:1	4	-	-	-	-	R	R	R	S	R	R	R	R	R	R	S	R
Kinneretia spp.	BBS4:6	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Pedobacter alluvionis	BBS4:2	4	-	+	-	-	S	R	S	R	R	R	R	R	R	R	S	S
Pedobacter koreensis	BBS4:7	4	-	+	-	-	S	R	R	S	R	R	R	R	R	R	S	S
Pedobacter spp.	BBS2:2	4	-	+	-	-	S	R	R	S	R	R	R	R	R	S	S	S
Pedobacter spp.	BBS2:4	4	-	+	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Pelomonas spp.	BBS4:4	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Proteus spp.	BBS1	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Proteus spp.	BBS1:20	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Sphingomonas spp.	BBS4:1	4	-	-	-	-	S	R	R	S	R	R	R	S	R	R	S	S
Sphingomonas spp.	BBS4:10	4	-	-	-	-	S	R	R	S	R	R	R	S	R	S	S	S
Stenotrophomonas spp.	BBS3	>32	-	+	+	-	R	R	R	S	R	R	R	R	R	R	R	S
Stenotrophomonas spp	BBS1:13	32	-	+	+	-	R	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas spp	BBS4:12	32	-	+	-	-	R	R	R	S	R	R	R	R	R	S	R	S

+: positive, -: negative, R: resistant, S: susceptible

Table A2.5: Species characterisation of soil isolates including MIC levels, CDST and MDR profile testing results arranged alphabetically according to species names

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Acetobacter pasteurianus	RHWA3:7	4	-	-	-	-	S	R	S	S	R	R	R	S	R	S	S	S
Acetobacter pasteurianus	RHSB2:14	4	-	-	-	-	S	R	S	S	R	R	R	S	R	S	S	S
Acetobacter pasteurianus	RHBC2:8	4	-	-	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Acetobacter pasteurianus	RHBC2:9	4	-	-	-	-	S	R	S	S	R	R	R	S	R	S	S	S
Acetobacter pasteurianus	RHBC2:11	4	-	-	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Acetobacter pasteurianus	LFWD1:1	4	-	-	-	-	S	R	S	S	R	R	R	S	R	S	S	S
Acetobacter pasteurianus	LFWD2:1	4	-	-	-	-	R	R	R	S	R	R	R	S	R	R	S	S
Acetobacter pasteurianus	LFWD3:20	4	-	-	-	-	S	R	S	S	R	R	R	S	R	S	S	S
Acidovorax facilis	LFSE2:3	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Acidovorax facilis	LFSE2:4	4	-	-	-	-	S	R	S	S	S	R	R	S	R	R	S	S
Chryseobacterium hominis	LFBF1:16	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Chryseobacterium soldanellicola	LFSE1:13	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Chryseobacterium spp.	LFWD1:5	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Duganella zoogloeoides	LFBF1:8	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Epilithonimonas lactis	LFWD3:10	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Flavobacterium johnsoniae	RHBC1:11	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Flavobacterium pectinovorum	RHBC3:7	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Flavobacterium saccharophilum	RHSB3:8	4	-	-	-	-	S	R	S	S	S	R	R	S	R	R	S	S
Janthinobacterium lividum	RHWA2:1	32	-	-	-	-	S	R	R	R	S	R	S	S	R	S	R	S
Janthinobacterium lividum	RHWA2:5	16	-	-	+	-	R	R	R	R	S	R	S	S	R	S	S	S
Janthinobacterium lividum	RHWA3:1	32	-	+	+	-	R	R	R	R	S	R	S	S	R	S	S	S
Janthinobacterium lividum	RHWA3:13	16	+	+	-	-	R	R	R	R	R	R	S	R	R	S	R	S
Janthinobacterium lividum	RHSB2:15	32	-	+	-	-	R	R	R	R	S	R	S	R	R	S	R	S
Janthinobacterium lividum	RHSB2:16	16	-	+	-	-	R	R	R	R	R	R	S	S	R	S	S	S
Janthinobacterium lividum	RHSB2:17	8	-	+	-	-	R	R	R	R	S	R	S	R	R	S	S	S
Janthinobacterium lividum	RHBC1:14	8	-	+	-	-	R	R	R	R	S	R	S	S	R	R	S	S
Janthinobacterium lividum	LFWD3:5	16	-	+	-	-	R	R	R	S	S	R	R	R	R	S	S	S

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Janthinobacterium lividum	LFWD3:15	32	-	+	+	-	R	R	R	S	S	R	S	S	R	R	S	S
Janthinobacterium lividum	LFBF2:8	16	-	+	+	-	R	R	R	S	S	R	R	R	R	R	S	S
Microbacterium foliorum	RHWA1:19	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Microbacterium oxydans	RHWA3:6	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Mucilaginibacter mallensis	LFSE1:9	>32	+	+	-	-	R	S	R	R	R	R	R	R	R	S	R	R
Pedobacter agri	RHSB1:1	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Pedobacter suwonensis	LFSE1:7	4	-	-	-	-	S	R	S	S	S	R	R	S	R	R	S	S
Pedobacter terrae	LFSE2:1	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Pedobacter wanjuense	LFSE3:8	4	-	-	-	-	S	R	S	S	S	R	R	S	R	R	S	S
Pseudomonas geniculata	RHWA1:18	4	-	-	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Pseudomonas geniculata	RHBC2:1	4	-	-	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Pseudomonas geniculata	LFWD1:10	4	-	-	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Pseudomonas geniculata	LFWD3:9	4	-	-	-	-	R	R	R	S	R	R	R	S	R	S	S	S
Pseudomonas geniculata	LFBF1:5	4	-	-	-	-	S	R	R	S	R	R	R	S	R	S	S	S

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Pseudomonas geniculata	LFBF1:12	4	-	-	-	-	S	R	R	S	R	R	R	S	R	S	S	S
Pseudomonas poae	RHBC2:2	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Pseudomonas veronii	LFBF1:1	4	-	-	-	-	S	R	S	S	S	R	R	S	R	R	S	S
Sporocytophaga spp.	RHBC3:13	4	-	-	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Stenotrophomonas maltophilia	RHWA1:8	16	-	+	-	-	S	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	RHWA1:11	16	-	+	+	-	S	R	R	S	R	R	R	R	R	R	R	S
Stenotrophomonas maltophilia	RHWA3:5	32	-	+	+	-	R	R	S	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	RHWA3:8	16	-	+	+	-	S	R	R	R	R	R	R	S	R	S	R	S
Stenotrophomonas maltophilia	RHWA3:9	16	-	+	-	-	S	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	RHWA3:14	32	-	+	+	-	R	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	RHBC2:7	16	-	+	-	-	S	R	R	S	S	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	LFWD1:2	16	-	+	+	-	S	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	LFWD1:4	32	-	+	+	-	R	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	LFWD3:14	16	-	+	+	-	S	R	R	S	R	R	R	R	R	S	R	S

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Stenotrophomonas maltophilia	LFSE3:4	32	-	+	-	-	S	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	LFSE3:6	32	-	+	-	-	S	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	LFBF1:10	>32	-	+	+	-	R	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	LFBF1:14	32	-	+	+	-	S	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	LFBF2:2	>32	-	+	-	-	S	S	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	LFBF3:8	16	-	+	+	-	S	S	R	S	S	R	R	S	R	R	R	S
Stenotrophomonas maltophilia	LFBF3:20	32	-	-	+	-	R	R	S	R	R	R	R	R	R	R	R	S
Stenotrophomonas maltophilia	LFBF3:21	16	-	-	+	-	S	R	R	R	R	R	R	R	R	R	R	S
Stenotrophomonas maltophilia	RHBC2:15	32	-	-	-	-	S	R	R	R	R	R	R	R	R	R	R	R
Stenotrophomonas rhizophila	RHWA1:7	>32	+	+	-	-	S	R	R	S	R	R	R	R	R	S	R	R
Stenotrophomonas rhizophila	RHWA2:2	32	-	+	-	-	R	R	R	S	R	R	R	R	R	S	R	S
Stenotrophomonas rhizophila	RHWA2:8	>32	+	+	-	-	S	R	R	S	R	R	R	R	R	R	R	S
Stenotrophomonas rhizophila	RHWA3:10	32	-	+	+	-	S	R	R	S	R	R	R	S	R	R	R	S
Stenotrophomonas rhizophila	RHWA3:11	32	-	+	-	-	S	R	S	S	R	R	R	S	R	S	S	S

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Stenotrophomonas rhizophila	RHWA3:12	32	-	+	-	-	S	R	R	R	R	R	R	S	R	S	R	S
Stenotrophomonas rhizophila	RHSB3:9	32	-	+	-	-	S	R	R	R	R	R	S	S	R	S	S	S
Stenotrophomonas rhizophila	RHBC2:3	32	-	+	-	-	S	R	S	S	R	R	R	S	R	S	S	S
Stenotrophomonas rhizophila	LFWD3:3	>32	-	+	+	-	S	R	R	S	R	R	R	S	R	S	R	R
Stenotrophomonas rhizophila	LFWD3:4	>32	+	+	-	-	R	R	R	R	R	R	R	R	R	R	R	R
Stenotrophomonas rhizophila	LFWD3:13	32	-	+	-	-	R	R	R	S	S	R	R	S	R	S	R	S
Stenotrophomonas rhizophila	LFSE1:3	>32	+	+	-	-	R	S	R	R	R	R	R	R	R	R	R	R
Stenotrophomonas rhizophila	LFSE1:12	32	+	+	-	-	R	R	R	R	R	R	R	R	R	R	R	R
Stenotrophomonas rhizophila	LFBF1:2	32	-	+	-	-	S	R	R	S	R	R	S	S	R	S	S	S
Stenotrophomonas rhizophila	LFBF1:3	32	-	+	-	-	S	R	R	S	R	R	R	S	R	S	S	S
Stenotrophomonas rhizophila	LFBF1:7	>32	-	+	-	-	R	R	R	S	R	R	S	S	R	R	R	R
Stenotrophomonas rhizophila	LFBF1:9	32	-	+	-	-	R	R	R	R	R	R	R	R	R	R	R	R
Stenotrophomonas rhizophila	LFBF1:11	>32	-	+	-	-	S	R	R	S	R	R	S	R	R	S	R	S
Stenotrophomonas rhizophila	LFBF2:1	32	-	+	+	-	R	R	R	S	R	R	R	S	R	R	S	S

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Stenotrophomonas rhizophila	LFBF2:3	32	-	+	-	-	S	R	R	S	R	R	R	S	R	S	R	S
Stenotrophomonas rhizophila	LFBF2:7	16	-	+	-	-	S	R	R	S	R	R	R	S	R	S	R	S
Stenotrophomonas spp.	LFWD3:6	32	+	-	-	-	R	R	R	R	R	R	R	R	R	S	R	S
Stenotrophomonas spp.	LFWD3:7	32	+	-	-	-	R	R	R	R	R	R	R	R	R	S	R	R
Stenotrophomonas spp.	LFBF1:4	4	-	+	-	-	S	S	S	S	R	R	R	S	R	R	S	S
Xanthomonas oryzae pv. *oryzae*	RHWA1:12	4	-	+	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Xanthomonas oryzae pv. *oryzae*	RHBC1:5	4	-	+	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Xanthomonas oryzae pv. *oryzae*	LFWD2:5	4	-	+	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Xanthomonas oryzae pv. *oryzae*	LFBF1:6	4	-	+	-	-	R	R	R	S	R	R	R	R	R	R	S	S
Xanthomonas oryzae pv. *oryzae*	LFBF1:21	4	-	-	-	-	R	R	R	S	R	R	R	R	R	S	S	S
Xanthomonas oryzae pv. *oryzae*	LFBF1:22	4	-	-	-	-	R	R	R	S	R	R	R	R	R	S	S	S
Xanthomonas retroflexus	RHWA2:10	4	-	+	-	-	S	R	S	S	S	R	R	S	R	S	S	S
Xanthomonas retroflexus	LFBF1:20	4	-	-	-	-	S	S	S	S	R	R	R	S	R	S	S	S

+: positive, -: negative, R: resistant, S: susceptible

Table A2.6: Species characterisation of hospital isolates including MIC levels, CDST and MDR profile testing results arranged alphabetically according to species names

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Acinetobacter spp.	SAT-1	16	-	+	-	-	S	S	S	S	R	R	S	S	R	S	S	S
Enterobacter aerogenes	HENT-1	>32	+	-	-	-	R	S	R	S	R	R	R	R	R	S	S	S
Enterobacter aerogenes	HENT-2	>32	+	-	-	-	R	S	R	S	S	R	R	R	R	S	R	R
Enterobacter aerogenes	HENT-3	32	+	-	-	-	S	R	S	S	S	R	R	R	R	R	R	R
Enterobacter aerogenes	HENT-4	32	+	-	-	-	S	R	R	R	R	R	S	R	R	R	S	S
Enterobacter cloacae	HENT-5	>32	+	-	-	-	R	S	R	S	R	R	S	R	R	R	R	S
Escherichia coli	YEC-1	>32	-	+	-	-	S	R	S	R	R	R	R	R	R	S	R	R
Escherichia coli	HEC-4	>32	-	+	-	-	R	R	R	S	S	R	S	R	R	R	S	S
Escherichia spp	HEC-2	>32	+	-	-	-	R	S	S	S	R	R	S	R	R	S	S	R
Escherichia spp	HEC-3	>32	+	-	-	-	R	R	R	R	R	S	R	R	R	R	R	S
Klebsiella pneumoniae	YKP-1	>32	-	+	-	-	R	R	R	R	R	R	R	R	R	S	R	S
Klebsiella pneumoniae	HKP-2	>32	+	-	-	-	S	S	R	S	R	R	R	R	R	S	R	R

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Klebsiella pneumoniae	HKP-3	>32	+	-	-	-	R	R	S	R	S	R	R	R	R	R	R	R
Klebsiella pneumoniae	HKP-4	>32	+	-	-	-	R	R	S	R	S	R	S	R	R	S	S	S
Klebsiella pneumoniae	HKP-5	>32	+	-	-	-	R	R	S	R	S	R	S	R	R	R	R	R
Klebsiella pneumoniae	HKP-6	>32	+	-	-	-	R	R	R	R	R	R	R	R	R	R	R	R
Klebsiella pneumoniae	HKP-7	>32	+	-	-	-	R	S	S	S	R	R	R	R	R	R	S	R
Klebsiella pneumoniae	HKP-8	>32	+	-	-	-	R	R	R	S	S	R	R	R	R	S	S	R
Klebsiella pneumoniae	HKP-9	>32	+	-	-	-	S	S	R	R	R	R	R	R	R	R	R	S
Klebsiella pneumoniae	HKP-10	>32	+	-	-	-	S	S	R	R	R	R	R	R	R	R	R	S
Klebsiella pneumoniae	HKP-11	>32	+	-	-	-	S	S	R	R	R	R	R	R	R	R	S	S
Pseudomonas aeruginosa	SPS-1	4	-	+	-	-	R	S	R	S	S	R	S	R	R	S	S	S
Pseudomonas aeruginosa	SPS-2	4	-	+	-	-	S	R	S	S	S	R	S	R	R	S	R	R
Pseudomonas aeruginosa	SPS-3	4	-	-	-	-	S	R	S	R	R	R	R	R	R	R	R	S
Pseudomonas geniculata	YPSG-1	>32	-	-	-	-	S	S	S	S	S	R	S	S	R	R	S	S
Stenotrophomonas maltophilia	SST-2	>32	-	+	-	-	S	S	S	S	R	R	S	R	R	S	R	R

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Stenotrophomonas maltophilia	SST-3	4	-	-	-	-	S	S	S	S	S	R	S	R	R	S	S	S
Stenotrophomonas maltophilia	SST-4	4	-	-	-	-	S	S	R	S	R	R	R	R	R	R	S	S
Stenotrophomonas maltophilia	SST-6	>32	-	+	+	-	S	S	S	S	S	R	S	R	R	S	R	R
Stenotrophomonas maltophilia	SST-7	16	-	+	+	-	S	S	S	S	S	R	S	R	R	S	S	S
Stenotrophomonas maltophilia	SST-8	16	-	+	+	-	S	S	S	S	S	R	S	R	R	S	S	S
Stenotrophomonas maltophilia	SST-9	16	-	-	-	-	S	R	R	R	R	R	R	R	R	S	S	S
Stenotrophomonas maltophilia	YSM-1	>32	-	+	-	-	S	S	R	R	R	R	S	R	R	R	R	S
Stenotrophomonas maltophilia	YSM-2	>32	-	+	-	-	S	S	S	S	S	R	R	R	R	S	R	S
Stenotrophomonas maltophilia	YSM-3	>32	-	+	-	-	R	R	S	S	S	R	R	R	R	S	S	S
Stenotrophomonas maltophilia	YSM-4	>32	-	+	-	-	S	S	S	S	S	R	S	R	R	S	S	S
Stenotrophomonas maltophilia	YSM-5	>32	-	+	-	-	S	S	S	S	S	R	S	R	R	R	S	S
Stenotrophomonas maltophilia	YSM-6	>32	-	+	+	-	S	S	S	S	R	R	S	R	R	S	S	S
Stenotrophomonas maltophilia	YSM-7	>32	-	-	+	-	R	R	R	R	S	R	R	R	R	S	S	R
Stenotrophomonas maltophilia	YSM-8	>32	-	-	+	-	R	R	R	R	S	R	R	R	R	R	S	R

Closest database sequence	Strain Name	ImR MIC (µg/ml)	Phenotypic Activity				Multi-resistance											
			Class A (KPC)	Class B (MBL)	Class C (AMPC)	Class D (OXA)	TIM	CN	CAZ	LEV	ATM	IPM	CIP	MEM	W	SXT	TE	MH
Stenotrophomonas maltophilia	YSM-9	>32	-	-	+	-	R	S	S	R	S	R	S	R	R	R	R	S
Stenotrophomonas spp.	SST-5	8	-	-	-	-	S	S	S	S	S	R	S	S	R	R	S	S

+: positive, -: negative, R: resistant, S: susceptible

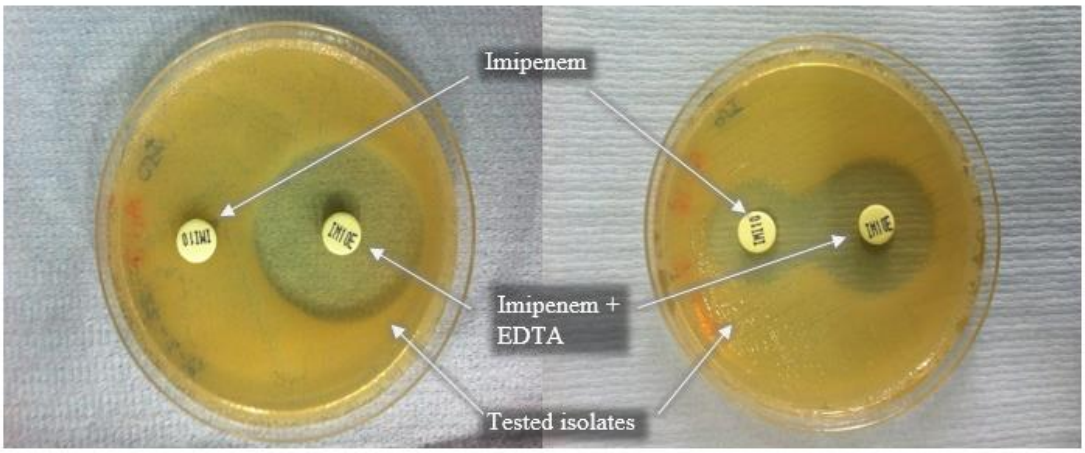


Figure A2.1: Imipenem-EDTA combined disc synergy test (CDST) for *Stenotrophomonas maltophilia* isolated from farm soil and for *Pseudomonas geniculate* isolated from a clinical setting

The left image shows an example of the determination of Class B (MBLs) carbapenamase production by *Stenotrophomonas maltophilia* (i.e. there is a difference in zone size (≥5 mm) between imipenem 10 µg alone and imipenem 10 µg + EDTA 750 µg 'MBLs inhibitor'). The image on the right shows an example of the non-production of MBLs by *Pseudomonas geniculate* (i.e. imipenem-EDTA zone < 5 mm).

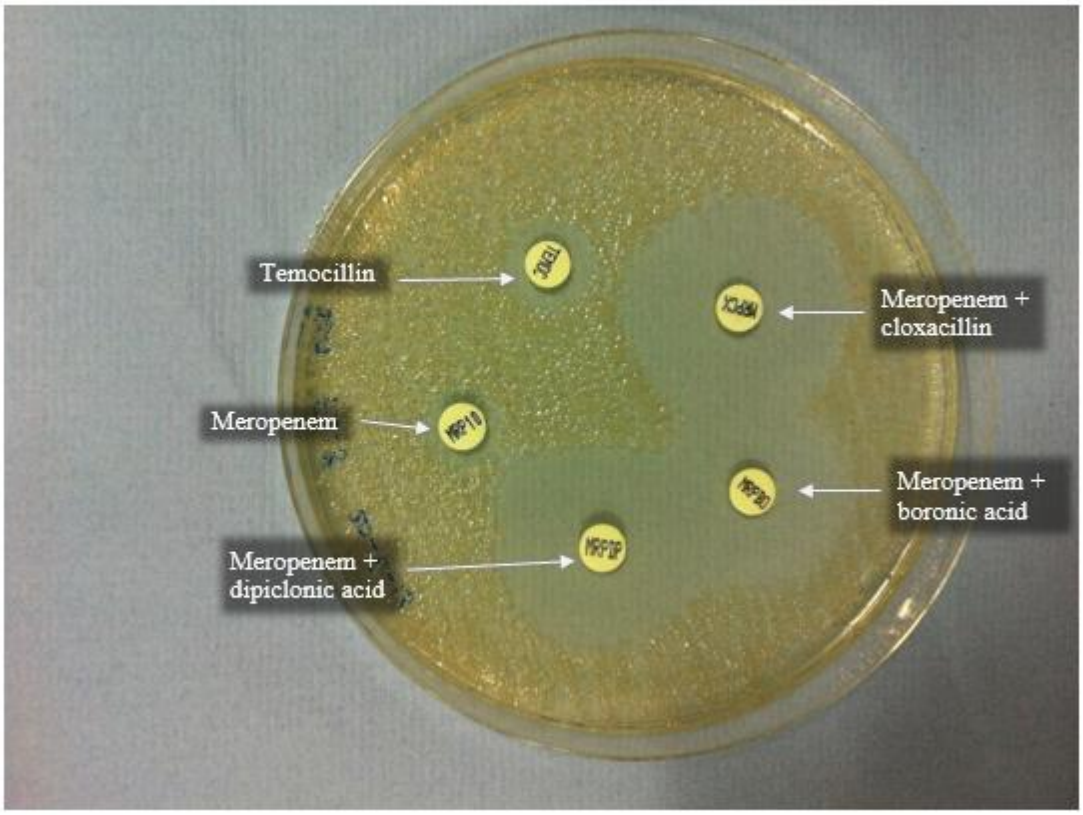


Figure A2.2: Combined disc synergy test (CDST) for β-lactamase identification of *Janthinobacterium lividum* isolated from farm soil

This figure shows an example of inhibition of different β-lactamases, indicating production of AmpC and Class B (MBL) enzymes by *Janthinobacterium lividum* [the difference (≥5mm) in zones between meropenem 10 µg (MRP10) and meropenem + cloxacillin (MRPCX) for AmpC, and meropenem + DPA (MRPDP) for Class B (MBL)]. Temocillin 30ug (TEMOC) was used for Class D (OXA-48 or similar), however no production of this enzyme was observed.

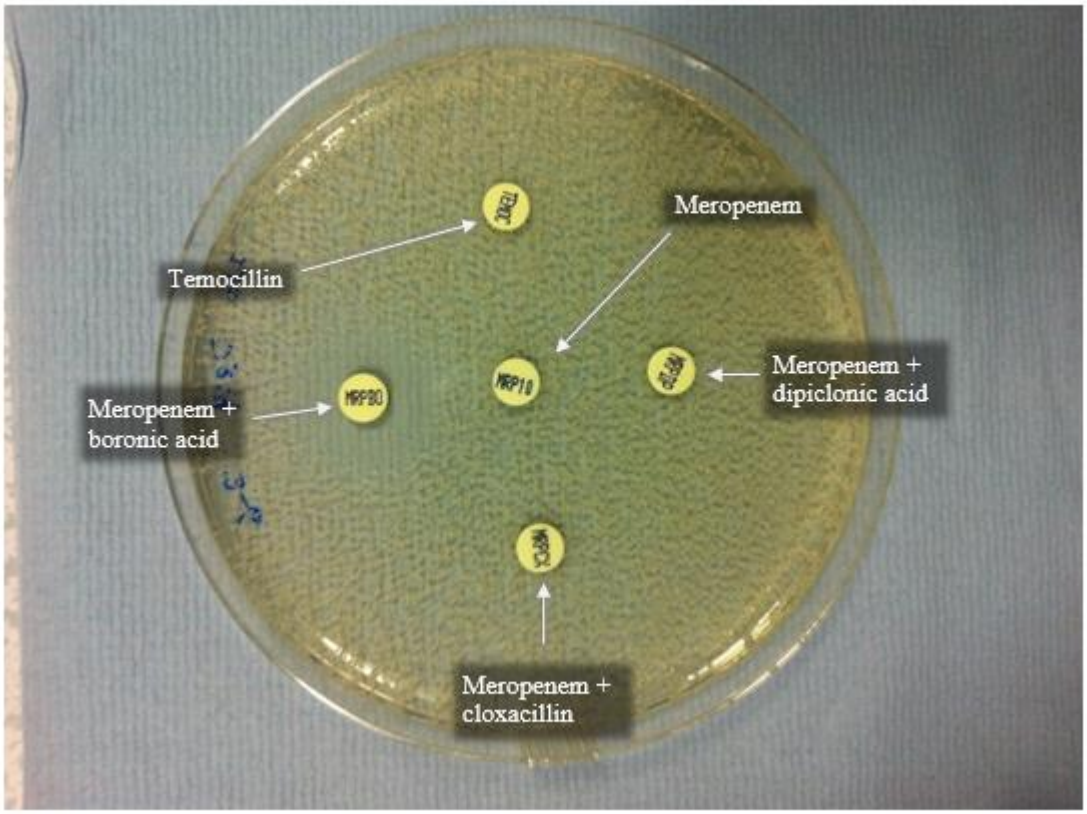


Figure A2.3: Combined disc synergy test (CDST) for β-lactamase identification of *Stenotrophomonas rhizophila* isolated from farm soil

The figure shows an example of inhibition of different β-lactamases indicating production of Class A (KPC) by *Stenotrophomonas rhizophila* [the difference (≥5mm) in zones between meropenem 10 µg (MRP10) and meropenem + boronic acid (MRPBO) for Class A (KPC). However, there was no production for other β-lactamases.

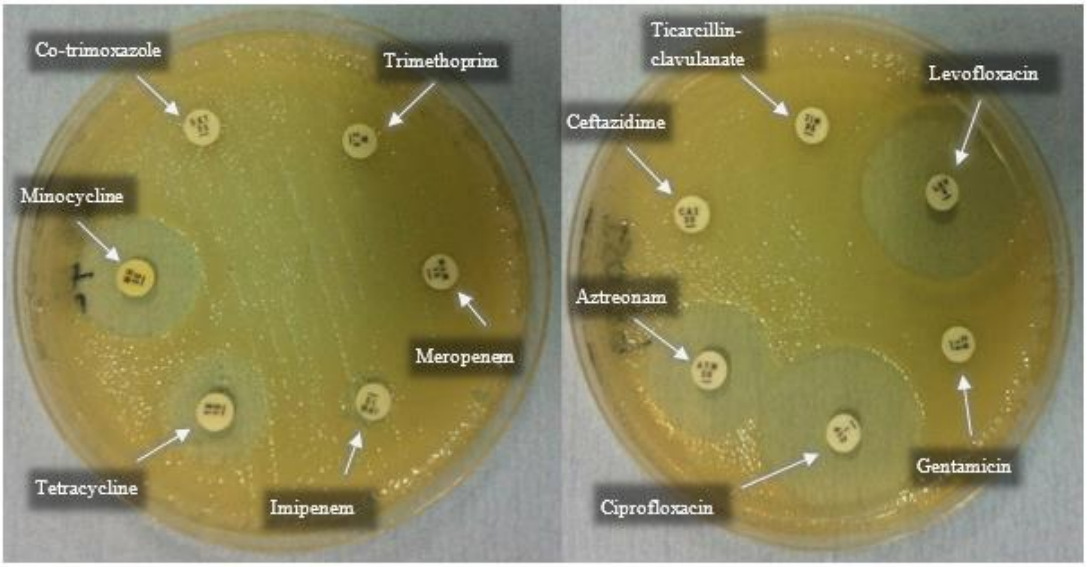


Figure A2.4: Multiple resistance profiling for *Janthinobacterium lividum* isolated from soil

This figure shows an example of an isolate of *Janthinobacterium lividum*, isolated from soil, showing a MDR pattern to seven antimicrobials. These are ticarcillin-clavulanate (TIM 85 µg), gentamicin (CN 10 µg), ceftazidime (CAZ 30 µg), imipenem (IPM 10 µg), meropenem (MEM 10 µg), trimethoprim (W 2.5 µg) and co-trimoxazole (SXT 25 µg). This isolate also showed susceptibility to five antimicrobials including aztreonam (ATM 30 µg), ciprofloxacin (CIP 1 µg), levofloxacin (LEV 5 µg), tetracycline (TE 10 µg) and minocycline (MH 30 µg).

Appendix A3

Table A3.1: 16S rRNA gene identification of ImR bacteria isolated from agricultural soil

Genus and species identification	Phylum (and class)	Number of isolates	Identity %	Strain name	Accession number of closest related sequence
Stenotrophomonas rhizophila	Proteobacteria (Gammaproteobacteria)	21	99%	RHWA1:7 RHWA2:2 RHWA2:8 RHWA3:10 RHWA3:11 RHWA3:12 RHSB3:9 RHBC2:3 LFWD3:3 LFWD3:4 LFWD3:13 LFSE1:3 LFSE1:12 LFBF1:2 LFBF1:3 LFBF1:7 LFBF1:9 LFBF1:11 LFBF2:1 LFBF2:3 LFBF2:7	JX908718.1 JX908718.1 JX908718.1 JX908718.1 JX908718.1 JX908718.1 JX908718.1 JQ890538.1 JQ890538.1 JQ890538.1 JX908718.1 JX862180.1 JQ890538.1 JX908718.1 HF545317.1 JX908718.1 JX908718.1 HF545317.1 JX908718.1 JX908718.1 JX908718.1
Stenotrophomonas maltophilia		19	100%	RHWA1:8 RHWA1:9 RHWA1:10 RHWA1:11 RHWA1:12 RHWA1:13 RHWA1:14 RHWA1:15 RHWA1:16 RHWA1:17 RHWA1:18 RHWA1:19 RHWA1:20 RHWA1:21 RHWA1:22 RHWA1:23 RHWA1:24 RHWA1:25 RHBC2:15 LFBF1:10	DQ862553.1 HQ224661.1 HQ224658.1 HQ224661.1 HQ224661.1 HQ224661.1 JN650549.1 HQ224661.1 AB680321.1 JN176584.1 AB661774.1 AB661774.1 AB661774.1 HQ224661.1 HQ224661.1 HQ224661.1 HQ224661.1 HQ224661.1 DQ862553.1 HQ224658.1
Stenotrophomonas spp.		3	99%	LFWD3:6 LFWD3:7 LFBF1:4	JX899633.1 KC618445.1 KF202769.1

Genus and species identification	Phylum (and class)	Number of isolates	Identity %	Strain name	Accession number of closest related sequence
Janthinobacterium lividum	Proteobacteria (Betaproteobacteria)	11	99%	RHWA2:1 RHWA2:5 RHWA3:1 RHWA3:13 RHSB2:15 RHSB2:16 RHSB2:17 RHBC1:14 LFWD3:5 LFWD3:15 LFBF2:8	EF111116.1 AB680301.1 JX971548.1 JX971548.1 JX971548.1 JX429049.1 JX971548.1 JX971548.1 AB680301.1 JX429049.1 JX429049.1
Mucilaginibacter mallensis	Bacteroidetes (Sphingobacteriia)	1	98%	LFSE1:9	FN400859.1
Xanthomonas oryzae pv. *oryzae*	Proteobacteria (Gammaproteobacteria)	6	99%	RHWA1:12 RHBC1:5 LFWD2:5 LFBF1:6 LFBF1:21 LFBF1:22	HM989021.1 HM989021.1 HM989021.1 HM989021.1 HM989021.1 HM989021.1
Xanthomonas retroflexus		2	99%	RHWA2:10 LFBF1:20	JQ890537.1 JQ890537.1
Microbacterium foliorum	Actinobacteria (Actinobacteria)	1	99%	RHWA1:19	KC139419.1
Microbacterium oxydans		1	99%	RHWA3:6	JX869578.1
Acetobacter pasteurianus	Proteobacteria (Alphaproteobacteria)	8	99%	RHWA3:7 RHSB2:14 RHBC2:8 RHBC2:9 RHBC2:11 LFWD1:1 LFWD2:1 LFWD3:20	KC122706.1 KC122706.1 KC122706.1 KC122706.1 FM178869.1 KC122706.1 KC122706.1 KC122706.1
Pedobacter agri	Bacteroidetes (Sphingobacteriia)	1	98%	RHSB1:1	JQ342863.1
Pedobacter suwonensis		1	98%	LFSE1:7	DQ297951.1
Pedobacter terrae		1	99%	LFSE2:1	GU385862.1
Pedobacter wanjuense		1	98%	LFSE3:8	AM279217.1

Genus and species identification	Phylum (and class)	Number of isolates	Identity %	Strain name	Accession number of closest related sequence
Flavobacterium saccharophilum	Bacteroidetes (Flavobacteriia)	1	99%	RHSB3:8	HM278518.1
Flavobacterium johnsoniae		1	98%	RHBC1:11	EU984151.1
Flavobacterium pectinovorum		1	99%	RHBC3:7	AM230490.1
Chryseobacterium spp.		1	99%	LFWD1:5	AY599655.1
Chryseobacterium soldanellicola		1	99%	LFSE1:13	EU834270.1
Chryseobacterium hominis	Bacteroidetes (Flavobacteriia)	1	96%	LFBF1:16	AM423081.1
Sporocytophaga spp.	Bacteroidetes (Cytophagia)	1	99%	RHBC3:13	AM179866.1
Epilithonimonas lactis	Bacteroidetes (Flavobacteriia)	1	98%	LFWD3:10	EF204460.2
Acidovorax facilis	Proteobacteria (Betaproteobacteria)	2	99%	LFSE2:3 LFSE2:4	JQ342846.1 JQ236816.1
Pseudomonas poae	Proteobacteria (Gamma-proteobacteria)	1	99%	RHBC2:2	JQ782898.1
Pseudomonas veronii		1	99%	LFBF1:1	JQ317806.1
Pseudomonas geniculata		6	99%	RHWA1:18 RHBC2:1 LFWD1:10 LFWD3:9 LFBF1:5 LFBF1:12	HQ857772.1 JF460769.1 HQ857772.1 HQ857772.1 HQ857772.1 JF460769.1
Duganella zoogloeoides	Proteobacteria (Betaproteobacteria)	1	98%	LFBF1:8	JQ689172.1

Table A3.2: Results of PCR amplification for genes encoding β-lactamase enzymes in water isolates

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla IMP-1	*Bla* OXA23	*Bla* L1	*Bla*NDM-1	*Bla*IND-1	*Bla* B-1	*Bla* CAU-1	*Bla*Mb1b	*Bla* IMP	*Bla*VIM-1	*Bla*OXA-48	*Bla*NDM	*Bla*KPC-1
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Acidovorax spp.	BBS4:15	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Brevundimonas lenta	BBS1:14	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Caulobacter segnis	BBS1:15	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Caulobacter spp.	BBS4:5	N	N	NT	N	N	NT	Y(788)	N	N	N	N	N	N
Caulobacter spp.	BBS4:6	N	N	NT	N	N	NT	N	Y(439)	N	N	N	N	N
Caulobacter spp.	BBS4:7	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Caulobacter spp.	BBS4:8	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Caulobacter spp.	BBS4:9	N	N	NT	N	N	NT	Y(654)	N	N	N	N	N	N
Caulobacter spp.	BBS4:10	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Caulobacter spp.	BBS4:11	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Caulobacter spp.	BBS3:20	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Caulobacter spp.	BBS4:13	N	N	NT	N	N	NT	Y(600)	N	N	N	N	N	N
Caulobacter spp.	BBS2:8	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Caulobacter vibrioides	BBS2:7	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Chitinophaga spp.	BBS1:7	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Chryseobacterium spp.	BBS2:12	N	N	NT	N	N	Y(260)	NT	NT	N	N	N	N	N
Epilithonimonas lactis	BBS3:3	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Epilithonimonas lactis	BBS3:1	Y(700)	N	NT	N	N	NT	NT	NT	N	N	N	N	N

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla $_{IMP-1}$	*Bla* $_{OXA23}$	*Bla* $_{L1}$	*Bla*$_{NDM-1}$	*Bla*$_{IND-1}$	*Bla* $_{B-1}$	*Bla* $_{CAU-1}$	*Bla*$_{Mb1b}$	*Bla* $_{IMP}$	*Bla*$_{VIM-1}$	*Bla*$_{OXA-48}$	*Bla*$_{NDM}$	*Bla*$_{KPC-1}$
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Kinneretia spp.	BBS4:6	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Pedobacter alluvionis	BBS4:2	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Pedobacter koreensis	BBS4:7	Y(439)	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Pedobacter spp	BBS2:2	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Pedobacter spp.	BBS2:4	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Pelomonas spp.	BBS4:4	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Proteus spp.	BBS1	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Proteus spp.	BBS1:20	Y(439)	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Sphingomonas spp.	BBS4:1	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Sphingomonas spp.	BBS4:2	N	N	NT	N	N	NT	NT	NT	N	N	N	N	N
Stenotrophomonas spp.	BBS4:12	N	N	NT	N	N	NT	N	N	N	N	N	N	N
Stenotrophomonas spp.	BBS3	Y(439)	N	N	N	N	NT	N	N	N	N	N	Y(350)	N
Stenotrophomonas spp.	BBS1:13	N	N	Y(730)	N	N	NT	NT	NT	N	N	N	N	N

NB:ARGs: antibiotic resistance genes Y: PCR product, N: no PCR product, NT: not tested. Primer details are given in Table 2.5

Table A3.3: Results of PCR amplification for genes encoding β-lactamase enzymes in soil isolates

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla IMP-1	*Bla* OXA23	*Bla* L1	*Bla*NDM-1	*Bla*IND-1	*Bla* B-1	*Bla* CAU-1	*Bla*Mbl1b	*Bla* IMP	*Bla*VIM-1	*Bla*OXA-48	*Bla*NDM	*Bla*KPC-1
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Acetobacter pasteurianus	RHWA3:7	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acetobacter pasteurianus	RHSB2:14	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acetobacter pasteurianus	RHBC2:8	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acetobacter pasteurianus	RHBC2:9	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acetobacter pasteurianus	RHBC2:11	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acetobacter pasteurianus	LFWD1:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acetobacter pasteurianus	LFWD2:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acetobacter pasteurianus	LFWD3:20	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acidovorax facilis	LFSE2:3	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Acidovorax facilis	LFSE2:4	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Chryseobacterium hominis	LFBF1:16	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Chryseobacterium soldanellicola	LFSE1:13	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Chryseobacterium spp.	LFWD1:5	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Duganella zoogloeoides	LFBF1:8	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Epilithonimonas lactis	LFWD3:10	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Flavobacterium johnsoniae	RHBC1:11	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Flavobacterium pectinovorum	RHBC3:7	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Flavobacterium saccharophilum	RHSB3:8	N	N	N	N	N	N	NT	NT	N	N	N	N	N

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla IMP-1	*Bla* OXA23	*Bla* L1	*Bla*NDM-1	*Bla*IND-1	*Bla* B-1	*Bla* CAU-1	*Bla*Mb1b	*Bla* IMP	*Bla*VIM-1	*Bla*OXA-48	*Bla*NDM	*Bla*KPC-1
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Janthinobacterium lividum	RHWA2:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Janthinobacterium lividum	RHWA2:5	N	N	N	N	N	N	NT	NT	Y(301)	N	N	N	N
Janthinobacterium lividum	RHWA3:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Janthinobacterium lividum	RHWA3:13	N	N	N	N	Y(499)	N	NT	NT	N	N	N	N	N
Janthinobacterium lividum	RHSB2:15	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Janthinobacterium lividum	RHSB2:16	N	N	N	N	N	N	NT	NT	Y(277)	N	N	N	N
Janthinobacterium lividum	RHSB2:17	N	N	N	N	N	Y(234)	NT	NT	N	N	N	N	N
Janthinobacterium lividum	RHBC1:14	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Janthinobacterium lividum	LFWD3:5	N	N	N	N	N	Y(244)	NT	NT	N	N	N	N	N
Janthinobacterium lividum	LFWD3:15	N	N	N	N	N	N	NT	NT	Y(291)	N	N	N	N
Janthinobacterium lividum	LFBF2:8	N	N	N	N	Y(537)	N	NT	NT	N	N	N	N	N
Microbacterium foliorum	RHWA1:19	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Microbacterium oxydans	RHWA3:6	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Mucilaginibacter mallensis	LFSE1:9	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pedobacter agri	RHSB1:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pedobacter suwonensis	LFSE1:7	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pedobacter terrae	LFSE2:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pedobacter wanjuense	LFSE3:8	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pseudomonas geniculata	RHWA1:18	N	N	N	N	Y(612)	N	NT	NT	N	N	N	N	N
Pseudomonas geniculata	RHBC2:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla IMP-1	*Bla* OXA23	*Bla* L1	*Bla*NDM-1	*Bla*IND-1	*Bla* B-1	*Bla* CAU-1	*Bla*Mbl1b	*Bla* IMP	*Bla*VIM-1	*Bla*OXA-48	*Bla*NDM	*Bla*KPC-1
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Pseudomonas geniculata	LFWD1:10	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pseudomonas geniculata	LFWD3:9	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pseudomonas geniculata	LFBF1:5	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pseudomonas geniculata	LFBF1:12	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pseudomonas poae	RHBC2:2	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Pseudomonas veronii	LFBF1:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Sporocytophaga spp.	RHBC3:13	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas maltophilia	RHWA1:8	N	N	N	N	N	N	NT	NT	N	Y(321)	N	N	N
Stenotrophomonas maltophilia	RHWA1:9	N	N	N	N	N	N	NT	NT	N	Y(411)	N	N	N
Stenotrophomonas maltophilia	RHWA1:10	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas maltophilia	RHWA1:11	N	N	N	N	N	N	NT	NT	N	Y(490)	N	N	N
Stenotrophomonas maltophilia	RHWA1:12	N	N	N	N	N	N	NT	NT	N	Y(502)	N	N	N
Stenotrophomonas maltophilia	RHWA1:13	N	N	N	N	N	N	NT	NT	N	Y(496)	N	N	N
Stenotrophomonas maltophilia	RHWA1:14	N	N	N	N	N	N	NT	NT	N	Y(296)	N	N	N
Stenotrophomonas maltophilia	RHWA1:15	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas maltophilia	RHWA1:16	N	N	N	N	N	N	NT	NT	N	Y(453)	N	N	N
Stenotrophomonas maltophilia	RHWA1:17	N	N	N	N	N	N	NT	NT	N	Y(433)	N	N	N
Stenotrophomonas maltophilia	RHWA1:18	N	N	N	N	N	N	NT	NT	N	Y(511)	N	N	N
Stenotrophomonas maltophilia	RHWA1:19	N	N	N	N	N	N	NT	NT	N	Y(317)	N	N	N
Stenotrophomonas maltophilia	RHWA1:20	N	N	N	N	N	N	NT	NT	N	Y(497)	N	N	N

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla IMP-1	*Bla* OXA23	*Bla* L1	*Bla*NDM-1	*Bla*IND-1	*Bla* B-1	*Bla* CAU-1	*Bla*Mbl1b	*Bla* IMP	*Bla*VIM-1	*Bla*OXA-48	*Bla*NDM	*Bla*KPC-1
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Stenotrophomonas maltophilia	RHWA1:21	N	N	N	N	N	N	NT	NT	N	Y(518)	N	N	N
Stenotrophomonas maltophilia	RHWA1:22	N	N	N	N	N	N	NT	NT	N	Y(438)	N	N	N
Stenotrophomonas maltophilia	RHWA1:23	N	N	N	N	N	N	NT	NT	N	Y(420)	N	N	N
Stenotrophomonas maltophilia	RHWA1:24	N	N	N	N	N	N	NT	NT	N	Y(522)	N	N	N
Stenotrophomonas maltophilia	LFBF1:10	N	N	N	N	N	N	NT	NT	N	Y(416)	N	N	N
Stenotrophomonas maltophilia	RHBC2:15	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	RHWA1:7	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	RHWA2:2	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	RHWA2:8	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	RHWA3:10	N	N	Y(290)	N	N	N	NT	NT	N	Y(350)	N	N	N
Stenotrophomonas rhizophila	RHWA3:11	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	RHWA3:12	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	RHSB3:9	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	RHBC2:3	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	LFWD3:3	N	N	N	N	N	N	NT	NT	N	Y(355)	N	N	N
Stenotrophomonas rhizophila	LFWD3:4	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	LFWD3:13	N	N	N	N	N	N	NT	NT	N	Y(357)	N	N	N
Stenotrophomonas rhizophila	LFSE1:3	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	LFSE1:12	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	LFBF1:2	N	N	N	N	N	N	NT	NT	N	N	N	N	N

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla IMP-1	*Bla* OXA23	*Bla* L1	*Bla*NDM-1	*Bla*IND-1	*Bla* B-1	*Bla* CAU-1	*Bla*Mbl1b	*Bla* IMP	*Bla*VIM-1	*Bla*OXA-48	*Bla*NDM	*Bla*KPC-1
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Stenotrophomonas rhizophila	LFBF1:3	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	LFBF1:7	N	N	N	N	N	N	NT	NT		Y(354)	N	N	N
Stenotrophomonas rhizophila	LFBF1:9	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	LFBF1:11	N	N	N	N	N	N	NT	NT	N	Y(354)	N	N	N
Stenotrophomonas rhizophila	LFBF2:1	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas rhizophila	LFBF2:3	N	N	N	N	N	N	NT	NT		Y(354)	N	N	N
Stenotrophomonas rhizophila	LFBF2:7	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas spp.	LFWD3:6	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Stenotrophomonas spp.	LFWD3:7	N	N	N	N	N	N	NT	NT	N	Y(561)	N	N	N
Stenotrophomonas spp.	LFBF1:4	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Xanthomonas oryzae pv.oryzae	RHWA1:12	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Xanthomonas oryzae pv. oryzae	RHBC1:5	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Xanthomonas oryzae pv. oryzae	LFWD2:5	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Xanthomonas oryzae pv. oryzae	LFBF1:6	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Xanthomonas oryzae pv. oryzae	LFBF1:21	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Xanthomonas oryzae pv. oryzae	LFBF1:22	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Xanthomonas retroflexus	RHWA2:10	N	N	N	N	N	N	NT	NT	N	N	N	N	N
Xanthomonas retroflexus	LFBF1:20	N	N	N	N	N	N	NT	NT	N	N	N	N	N

NB: ARGs: antibiotic resistance genes Y: PCR product, N: no PCR product, NT: not tested. Primer details are given in Table 2.5.

Table A3.4: Results of PCR amplification for genes encoding β-lactamase enzymes in clinical isolates

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla $_{IMP-1}$	*Bla* $_{OXA23}$	*Bla* $_{L1}$	*Bla*$_{NDM-1}$	*Bla*$_{IND-1}$	*Bla* $_{B-1}$	*Bla* $_{CAU-1}$	*Bla*$_{Mbl1b}$	*Bla* $_{IMP}$	*Bla*$_{VIM-1}$	*Bla*$_{OXA-48}$	*Bla*$_{NDM}$	*Bla*$_{KPC-1}$
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Acinetobacter spp.	SAT-1	N	Y(473)	N	N	N	N	NT	NT	N	N	N	N	NT
Enterobacter aerogenes	HENT-1	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(757)
Enterobacter aerogenes	HENT-2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(757)
Enterobacter aerogenes	HENT-3	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(760)
Enterobacter aerogenes	HENT-4	N	NT	NT	N	N	NT	NT	NT	N	N	NT	N	Y(757)
Enterobacter cloacae	HENT-5	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(757)
Escherichia coli	YEC-1	N	N	N	Y(674)	N	N	NT	NT	N	N	N	Y(596)	N
Escherichia coli	HEC-4	N	NT	NT	N	N	NT	NT	NT	N	N	NT	Y(594)	N
Escherichia spp	HEC-2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(759)
Escherichia spp	HEC-3	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(762)
Klebsiella pneumoniae	YKP-1	N	N	N	N	Y(468)	N	NT	NT	N	Y(361)	N	N	N
Klebsiella pneumoniae	HKP-2	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(757)
Klebsiella pneumoniae	HKP-3	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(758)
Klebsiella pneumoniae	HKP-4	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(757)
Klebsiella pneumoniae	HKP-5	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(757)
Klebsiella pneumoniae	HKP-6	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(759)
Klebsiella pneumoniae	HKP-7	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(756)
Klebsiella pneumoniae	HKP-8	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(757)

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla IMP-1	*Bla* OXA23	*Bla* L1	*Bla*NDM-1	*Bla*IND-1	*Bla* B-1	*Bla* CAU-1	*Bla*Mbl1b	*Bla* IMP	*Bla*VIM-1	*Bla*OXA-48	*Bla*NDM	*Bla*KPC-1
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Klebsiella pneumoniae	HKP-9	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	Y(757)
Klebsiella pneumoniae	HKP-10	N	NT	NT	N	N	NT	NT	NT	N	N	NT	N	Y(762)
Klebsiella pneumoniae	HKP-11	N	NT	NT	N	N	NT	NT	NT	N	N	NT	Y(607)	N
Pseudomonas aeruginosa	SPS-1	N	N	N	N	N	N	NT	NT	N	N	N	Y(617)	NT
Pseudomonas aeruginosa	SPS-2	N	N	N	N	N	N	NT	NT	N	N	N	Y(454)	NT
Pseudomonas aeruginosa	SPS-3	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Pseudomonas geniculata	YPSG-1	N	N	N	Y(341)	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	SST-2	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	SST-3	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	SST-4	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	SST-6	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	SST-7	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	SST-8	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	SST-9	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	YSM-1	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	YSM-2	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	YSM-3	N	N	N	N	N	N	NT	NT	N	Y(355)	N	N	NT
Stenotrophomonas maltophilia	YSM-4	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	YSM-5	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	YSM-6	N	N	N	N	N	N	NT	NT	N	N	N	N	NT

Closest database sequence	Isolate Name	ARGs PCRs (product sizes in bp in brackets)												
		Bla IMP-1	*Bla* OXA23	*Bla* L1	*Bla*NDM-1	*Bla*IND-1	*Bla* B-1	*Bla* CAU-1	*Bla*Mbl1b	*Bla* IMP	*Bla*VIM-1	*Bla*OXA-48	*Bla*NDM	*Bla*KPC-1
		[587]	[501]	[888]	[350]	[580]	[205]	[870]	[850]	[232]	[390]	[438]	[621]	[798]
Stenotrophomonas maltophilia	YSM-7	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	YSM-8	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas maltophilia	YSM-9	N	N	N	N	N	N	NT	NT	N	N	N	N	NT
Stenotrophomonas spp.	SST-5	N	N	N	N	N	N	NT	NT	N	N	N	N	NT

NB: ARGs: antibiotic resistance genes Y: PCR product, N: no PCR product, NT: not tested. Primer details are given in Table 2.5.