16S rRNA methyltransferases: the end of aminoglycosides?

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2020

Declaration of originality

I, Emma Louise Taylor, confirm that the work presented in this thesis is entirely my own, and if otherwise, is referenced accordingly.

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Abstract

16S rRNA methyltransferases (16S RMTases) confer high-level aminoglycoside resistance (MICs >256 mg/L) to Gram-negative bacteria and are an emerging threat. The prevalence of 16S RMTases in the UK is entirely unknown as are the drivers behind their emergence.

The occurrence of 16S RMTases (ArmA, RmtA-RmtH and NpmA) in the UK was identified by screening bacteria from Public Health England's Antimicrobial Resistance and Healthcare Associated Infections Reference Unit from 2003-2015 and whole-genome sequences from the British Society for Antimicrobial Chemotherapy Resistance Surveillance Project from 2001-2013, with positivity rates of 83.2% (1,312/1,576) and 0.3% (4/1,566), respectively. A prospective surveillance study, where 14 NHS Trusts sent amikacin-resistant bacterial isolates from May 1st to October 31st 2016, determined a period prevalence of 16S RMTases of 0.1% (79/71,063). Potential risk factors for acquisition of 16S RMTase-producing bacteria, were identified using anonymised case questionnaires and included age (≥65 years), being male, an inpatient or a non-UK resident and receiving medical treatment abroad.

16S RMTases were frequently associated with carbapenemases, which were identified in 94.3% (1,237/1,312), 87.3% (69/79) and 50.0% (2/4) 16S RMTase-producing isolates in these isolate collections. 16S RMTases were frequently carried by 'high-risk' bacterial clones such as *Klebsiella pneumoniae* ST14. Analysis of genome sequence data identified mobile genetic elements such as Tn*1548* (*armA*), Tn*2* (*rmtB*), IS*Ecp1* (*rmtC*) and IS*91* (*rmtC* and *rmtF*) were associated with 16S RMTases. Analysis of plasmids identified genetic linkage of 16S RMTases with the carbapenemase NDM-1 and the circulation of novel plasmids within the UK. Although currently rare in the UK, 16S RMTases appear to be emerging through clonal expansion, and potentially through association with carbapenemases and mobile genetic elements. Given the critical therapeutic role of aminoglycosides in combatting the challenge of antimicrobial resistance in Gram negative pathogens, the risk of future emergence is high, underlining a need for ongoing surveillance.

Acknowledgements

Firstly, I would like to thank the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Healthcare Associated Infections and Antimicrobial Resistance at Imperial College London in partnership with Public Health England (PHE) for funding my PhD project.

Special thanks to my supervisors Dr Katie Hopkins, Professor Neil Woodford and Professor Shiranee Sriskandan for supporting me throughout my PhD and offering their invaluable advice to help me write my thesis and help me through my assessments and conference presentations.

I want to thank Claire Perry, Amy Coward, Alice Fuller and Noshin Sajedi from the AMRHAI Reference Unit at PHE for their assistance with strain characterization, especially Dr Jane Turton for going above and beyond to help me whenever I was in need. Your kindness and support was very much appreciated. Many thanks go to Dr Rachel Pike, Aiysha Chaudry and Rachael Adkin from the AMRHAI Reference Unit for their help with antibiotic susceptibility testing; Daniel Godoy and Nazim Mustafa from the AMRHAI Reference Unit for their help with carbapenemase screening and Neville Verlander from the Statistics, Modelling and Economics Department at PHE for guidance during the statistical analysis. Special thanks to all of the laboratory staff and Consultant Microbiologists who participated in the prospective surveillance study and Professor Alan Johnson at PHE and Dr Michael Lockhart from Health Protection Scotland for their help with hospital recruitment.

I would also like to thank Professor Julian Parkhill and Professor Sharon Peacock for allowing me to work at the Wellcome Trust Sanger Institute and for giving me access to data from the British Society for Antimicrobial Chemotherapy Resistance Surveillance Project. Additional thanks go to the Wellcome Trust Sanger Institute for carrying out Illumina sequencing on the bacterial isolates from this project.

Many thanks to Professor Bruno González-Zorn, Dr Laurent Poirel and Dr Yohei Doi for providing bacterial positive controls for *rmtA* + *rmtE* + *rmtF*, *rmtG* and *rmtH*, respectively.

I'm extremely grateful to Dr Elita Jauneikaite for supporting me throughout my PhD and for teaching me how to use bioinformatics software. Thank you for encouraging me when times were tough and for always being there for me when I was in need.

Thank you to all of my family and friends for their love and support throughout the last four years.

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Abbreviations

16S RMTase	16S rRNA methyltransferase
95% CI	95% confidence interval
µg	Microgram
µl	Microlitre
A-site	Aminoacyl-site
AAC	Acetyltransferase
AME	Aminoglycoside-modifying enzyme
AMP	Adenosine monophosphate
AMRHAI	Antimicrobial Resistance and Healthcare Associated Infections
ANT	Nucleotidyltransferase
APH	Phosphotransferase
ARG-ANNOT	Antibiotic Resistance Gene-ANNOTation
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BRIG	BLAST Ring Image Generator
BSAC	British Society for Antimicrobial Chemotherapy
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CLED	Cystine–lactose–electrolyte-deficient agar
CLSI	Clinical Laboratory Standards Institute
CPE	Carbapenemase-producing Enterobacterales
CS	Conserved segments
Ct	Cycle threshold
DDD	Daily defined doses
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOS	2-deoxystreptamine
E-site	Exit-site
EARS-Net	European Antimicrobial Resistance Surveillance Network

EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum β-lactamase
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GNAT	GCN5-related N-acetyltransferases
GP	General practice
HAMP	Histidine kinase, adenylyl cyclases, methyl-binding proteins and phosphatases
HPLC	High-performance liquid chromatography
Inc	Incompatibility
IS	Insertion sequence
kb	Kilobase
LB	Luria-Bertani
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
Mb	Megabase
MBC	Minimum bactericidal concentration
MDR	Multidrug-resistant
MGE	Mobile genetic element
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
ml	Millilitre
MLST	Multilocus sequence typing
mM	Millimolar
ng	Nanogram
NTC	No-template control
P-site	Peptidyl-site
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PHE	Public Health England
PMQR	Plasmid-mediated quinolone resistance
QRDR	Quinolone resistance determining regions
RNA	Ribonucleic acid

RND	Resistance-nodulation cell-division
rtPCR	Real-time PCR
SAM	S-adenosyl-$_L$-methionine
SE	Sodium chloride/EDTA
SGSS	Second Generation Surveillance System
SLV	Single locus variant
SMAC	Standing Medical Advisory Committee
SMRT sequencing	Single-molecule, real-time sequencing
SNP	Single nucleotide polymorphism
SOC	Super-optimal broth with catabolite repression
SRST2	Short Read Sequence Typing for Bacterial Pathogens
ST	Sequence type
TA system	Toxin-antitoxin system
TAT	Twin-arginine translocation
TBE	Tris-Borate EDTA
TE	TRIS-EDTA
tRNA	Transfer rRNA
UPEC	Uropathogenic *Escherichia coli*
UPGMA	Unweighted pair group method with arithmetic mean
USA	United States of America
VNTR	Variable-number tandem-repeat
WGS	Whole-genome sequencing
WHO	World Health Organisation
XDR	Extensively-drug resistant

Chapter 1: Introduction

1.1 Antibiotic resistance

1.1.1 Discovery of antibiotics

In 1928 Alexander Fleming discovered the first antibiotic, penicillin [1], and revolutionised the treatment of infectious diseases. The discovery of antibiotics has enabled the treatment of bacterial infections in humans and animals, and, through prophylaxis, has allowed surgical procedures to be performed with low risk of infection and protected those with medical conditions that make them vulnerable to infection [2]. There are multiple classes of antibiotics with different mechanisms of action including inhibition of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or protein synthesis, prevention of cell wall synthesis or damage to the cell wall itself, as well as disruption of microbial metabolism [3] (Figure 1.1). However, Fleming himself warned about the consequences of misusing penicillin as using too little, or using penicillin over a period of time that was too short, could result in resistance [4]. Now, only 90 years following the discovery of penicillin, we are approaching a 'post-antibiotic era' through misuse.

1.1.2 Antibiotic resistance

Antibiotic resistance is now a global health issue and it has been predicted by the O'Neill Commission that by 2050 as many as 10 million deaths will occur each year as the result of antibiotic resistance [5], with 63,000 and 25,000 patients currently being reported to die per year in the United States of America (USA) [4] and Europe [6], respectively. Antibiotic resistance has been predicted to cost the European Union (EU) €1.5 billion and the USA $20 billion annually through additional healthcare costs and productivity losses [5-6].

The main driving factor of antibiotic resistance is the misuse or overuse of antibiotics in both healthcare and community settings; it has been estimated that around 50% of prescribed antimicrobials in human medicine are unnecessary [7]. In addition, a study conducted in the UK identified that 11.3% patients did not finish their last course of antibiotics with the main reasoning being that patients felt better or had forgotten to take their dose on time [8].

Antibiotics are available over the counter in many developing countries meaning there is no control of their distribution. Counterfeit antibiotics, which according to the World Health Organisation (WHO) account for 28% of all counterfeited drugs worldwide [9], can lead to treatment failure and development of antibiotic resistance [10]. Another factor driving resistance is that many classes of antibiotics that are used in agriculture (as growth promoters for example) and veterinary medicine are the same as those used in clinical medicine, meaning that resistance can spread between companion animals, farmers, and livestock, or the food chain to humans [11,12]. Finally, antibiotic resistance genes have also been found in the environment, especially in countries with poor sanitation where sewage can spread to aquatic environments [13] (Figure 1.2).

Several mechanisms of antibiotic resistance have been identified in Gram-negative and Gram-positive bacteria including membrane impermeability, the use of porins or efflux pumps, antibiotic-modifying enzymes as well as modification of the antibiotic target, such as the bacterial 70S ribosome, through either genetic mutation or modifying enzymes and metabolic bypass [3,14] (Figure 1.1). Genes encoding certain resistance mechanisms can be found on mobile genetic elements (MGEs) such as plasmids, which enables these genes to spread between bacteria via horizontal gene transfer, promoting antibiotic resistance. This has enabled the generation of multidrug-resistant (MDR) bacteria, where the bacteria are resistant to a minimum of three classes of antibiotics [15], as well as extensively-drug resistant (XDR) bacteria, with susceptibility to only one or two antibiotic classes [15].

Another mechanism of antibiotic resistance is the use of biofilms, which are communities of bacteria adhered to a biological or non-biological surface (such *Pseudomonas aeruginosa* on the surface of a cystic fibrosis patient's lungs or uropathogenic *Escherichia coli* (UPEC) on the surface of a urinary catheter) [16-19]. The bacteria within biofilms are held together by an extracellular matrix containing extracellular DNA, lipids, polysaccharides and proteins [17,18]. Biofilms are able to promote antibiotic resistance due to factors such as poor antibiotic penetration through the biofilm as well decreased levels of oxygen and nutrients throughout

the biofilm (where the concentration is lowest in the centre) [16-18]. This results in metabolically-inactive bacteria or slow-growing bacteria being present in the centre of the biofilm [16-18], which can avoid being targeted by antibiotics that target duplicating bacteria (such as β-lactams) due to their inactivity or those that are oxygen-dependent (such as aminoglycosides) due to the anaerobic conditions they live in [17]. Furthermore, horizontal gene transfer frequently occurs within biofilms where MGEs, including plasmids, that carry antibiotic resistance genes are exchanged between bacteria meaning multidrug resistance can develop [17,18].

Toxin-antitoxin (TA) systems can also be used as an antibiotic resistance mechanism. TA systems, such as kacAT in *Klebsiella pneumoniae*, encode a toxin (e.g. KacT) that can inhibit cell growth and promote dormancy in bacterial cells (which are called “persister cells”) in the presence of environmental stress such as antibiotic pressure [20,21]. Toxins can promote dormancy by altering key cellular functions such as DNA replication and protein synthesis [22]. This enables the bacteria to become tolerant of the antibiotic and survive. However, when the environmental pressure is gone, the antitoxin (e.g. KacA), is expressed, which binds to the toxin and enables the bacterial cell to become active again [20,22]. Furthermore, TA systems are used to kill bacterial cells that have lost plasmids, such as those that contain antibiotic resistance genes [23]. Here, the plasmid encodes the TA system but once the plasmid is lost the toxin encoded by the TA system cannot be neutralised as the antitoxin is no longer encoded [23,24]. This kills the bacterial cell and promotes the survival of bacterial cells that harbour the plasmid in a process known as post-segregational killing [23,25]. Therefore, this would promote the survival of antibiotic-resistant bacteria and maintenance of the plasmid encoding antibiotic resistance genes.

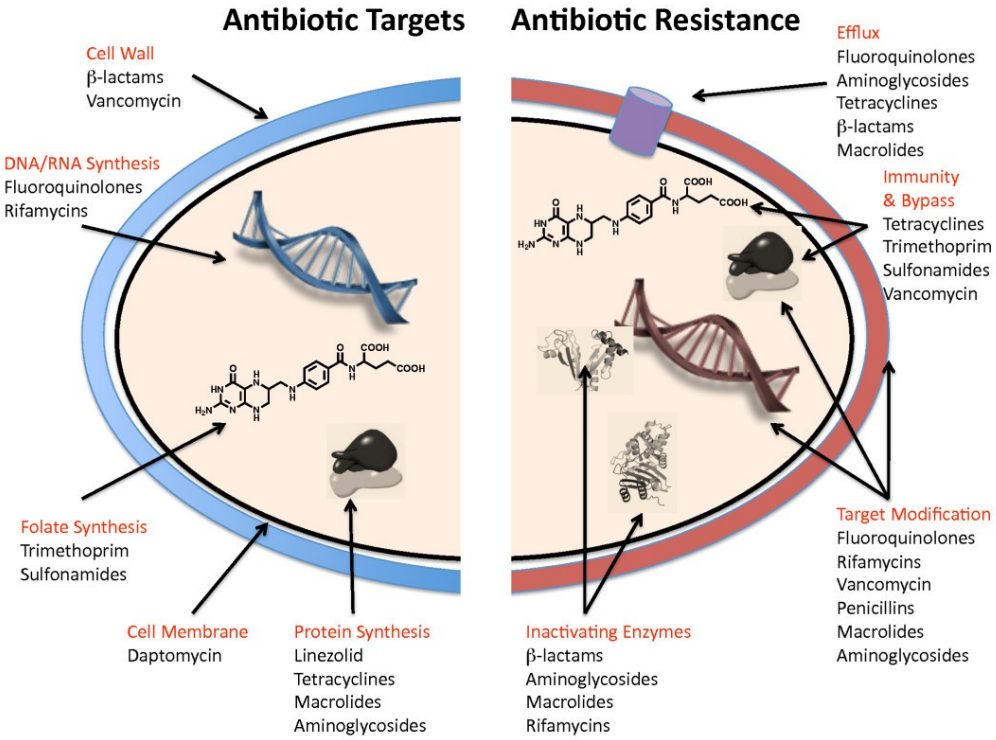


Figure 1.1: The main antibiotic mechanisms of action and mechanisms of resistance. This figure was obtained from Zhivich (2017) [26] and is licenced under the Creative Commons Attribution 4.0 International Licence. To view a copy of this licence, visit https://creativecommons.org/licenses/by-nc-sa/4.0/.

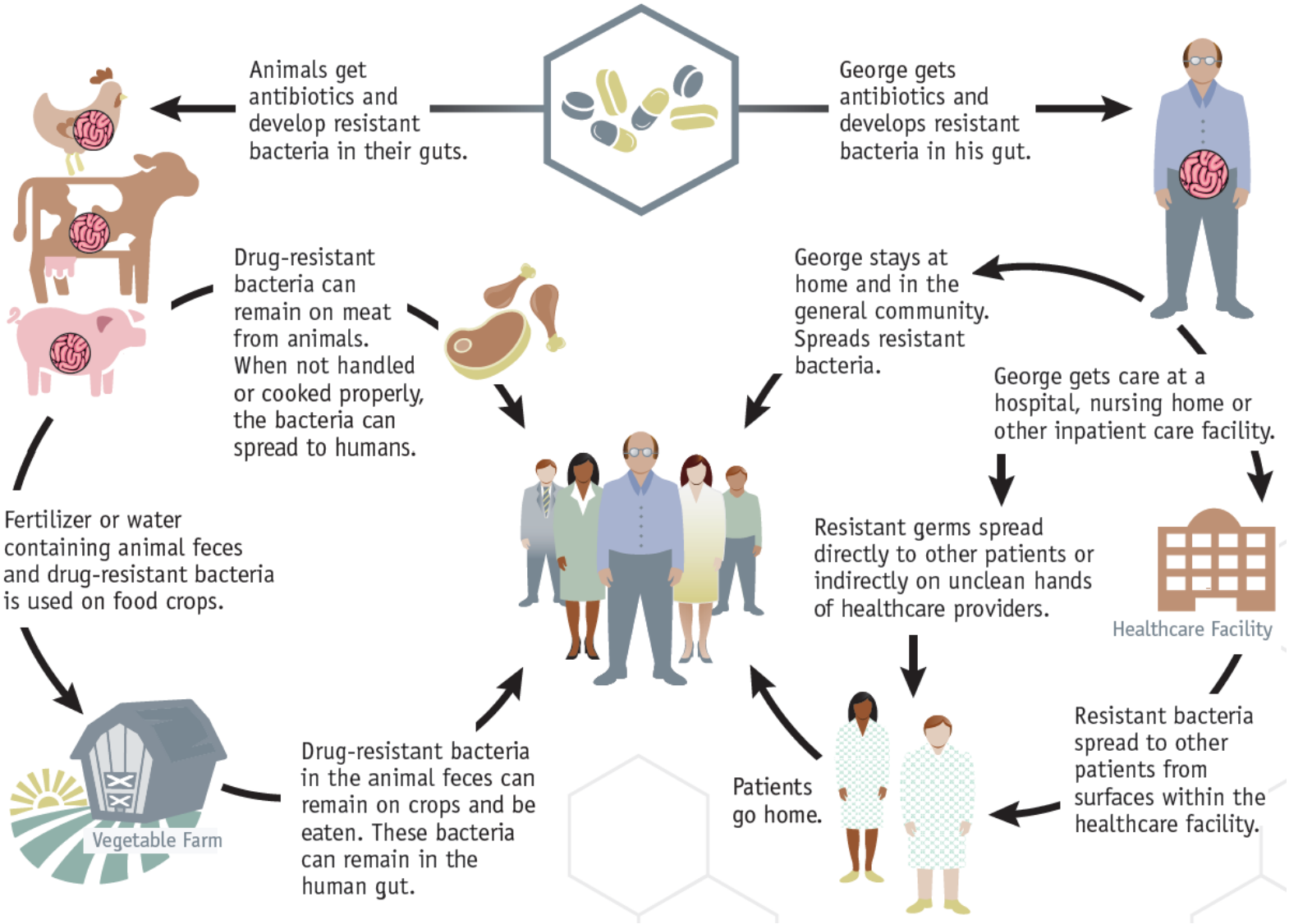


Figure 1.2: Driving factors of antibiotic resistance. This figure was obtained from the Centers for Disease Control and Prevention [27].

1.1.3 Microbiological investigation of antibiotic-resistant bacteria

1.1.3.1 Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic that inhibits bacterial growth [28-30]. MICs differ from the minimum bactericidal concentration (MBC), which is the lowest concentration of an antibiotic to cause bacterial death [28-30]. However, MBCs are rarely used due to the additional effort required by diagnostic laboratories to determine them [28,29].

MICs are used to govern the prescription of antibiotics, to monitor trends in antibiotic resistance over time for a bacterial species, and to investigate the effectiveness of newly designed antibiotics [28]. There are a range of antibiotic susceptibility testing methods used to determine MICs, where bacteria are deemed to be either susceptible, intermediate or resistant to antibiotics based on breakpoints, which differ per antibiotic, provided by organisations such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI) [28,29,31]. Bacterial isolates are deemed to be susceptible to an antibiotic when they are inhibited at concentrations equal to or lower than the specified breakpoint for susceptibility; those that are only inhibited by concentrations over the specified breakpoint for resistance are known as resistant; and those that are inhibited at a concentration between the breakpoints are deemed to be susceptible with increased exposure (formerly known as 'intermediate') according to EUCAST [32]. Those that are deemed susceptible to an antibiotic are likely to respond to treatment using the standard dosage. Those that are susceptible with increased exposure are likely to respond to treatment when the dosage or concentration of antibiotic at the infection site is increased [32]. Resistant bacteria are those where therapeutic failure is highly likely to occur when using the standard dosage, or with increased exposure [31,32].

Antibiotic susceptibility testing methods include disk diffusion and the gradient strip test (Figure 1.3), where paper disks containing a known concentration of antibiotic (disk diffusion) or a paper/plastic strip containing a continuous gradient of antibiotic (gradient strip test), are placed

onto an agar plate that has been spread with a bacterial suspension of a specified cell density [28-30,31]. Additionally, the agar dilution or broth dilution methods can be used where a pure bacterial culture is grown on agar or in broth containing two-fold dilutions of the antibiotic [28,29,31]. Alternatively, automated testing methods such as the VITEK-2 can be used to determine MICs [28,29,31].

Other techniques that are currently used to identify antibiotic-resistant bacteria without the need for conventional phenotypic testing include the polymerase chain reaction (PCR), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or whole-genome sequencing (WGS) [33]. PCR has been used to identify antibiotic resistance genes encoding β-lactamases [34] and carbapenemases [35]; MALDI-TOF MS has been used to identify the production of β-lactamases [36] as well as bacterial growth in the presence of antibiotics [37] and WGS can be used to detect antibiotic resistance genes through the use of read-mapping based tools such as Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) [38], ResFinder [39] and Short Read Sequence Typing for Bacterial Pathogens (SRST2) [40]. Even though these methods are unable to calculate MICs, they can provide results in several hours compared to phenotypic testing, which can take several days [28]. However, these assays cannot guide treatment regimens in the manner of phenotypic assays due to the inability to calculate MICs, which can give guidance on the prescription of antibiotics based on the results and can indicate the presence of novel resistance mechanisms. Furthermore, PCR or WGS, could detect the presence of antibiotic resistance genes that are not being expressed. This means that antibiotics may be wrongfully removed from a patient's treatment regime.

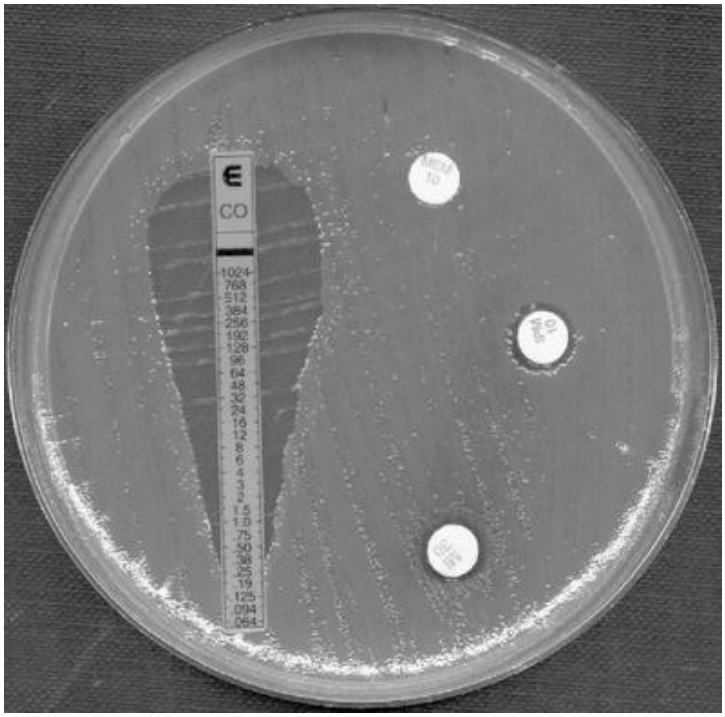


Figure 1.3: Gradient strip (left-hand side) and disk diffusion (right-hand side). The gradient strip is measuring resistance to colistin (MIC 0.50 µg/ml) and the disks (from top to bottom) are measuring meropenem, imipenem and minocycline resistance. Reproduced from Gilad *et al.* [41] with permission from Springer Nature.

1.1.3.2 Bacterial typing

DNA-based bacterial typing methods such as pulsed-field gel electrophoresis (PFGE), variable-number tandem-repeat (VNTR) analysis, multilocus sequence typing (MLST) or WGS are now the most commonly performed to determine if a bacterial isolate is an emerging pathogenic strain, belongs to a clone, or is involved in an outbreak [42,43]. Clones are bacteria that are genetically indistinguishable due to sharing a common ancestor, but they may have been isolated independently from different sources and locations [42,43]. Some clones are deemed to be 'high risk' due to their virulence and/or multidrug resistance, enabling them to spread across the globe with ability to disseminate antibiotic resistance or virulence through MGEs such as plasmids [44].

1.1.3.2.1 PFGE

In PFGE, large DNA fragments (from 20-600 kb) are produced using restriction enzymes to digest bacterial DNA, that are specific to the species tested e.g. *Xba*I for Enterobacterales or *Apa*I for *Acinetobacter baumannii* [42,45]. The fragments are then separated within an agarose gel using PFGE, where the direction of the electric field is altered periodically in three different directions to separate large DNA fragments (up to 10 Mb) [42,45,46]. This differs from standard gel electrophoresis, where DNA fragments of up to ~50 kb can be separated [46]. PFGE generates a profile of bands, representing a DNA fingerprint of a bacterial isolate. This can be used to compare the bacterial isolates with others from the same species, as differences (or similarities) in band patterns can be observed. PFGE is highly discriminatory but it is a time-consuming process and can take several days to complete [42].

1.1.3.2.2 VNTR analysis

VNTR analysis utilises PCR to amplify tandem repeats, which are short sequences of DNA repeated in tandem, found within the bacterial genome at multiple loci (Figure 1.4) [43]. Targeted areas of the genome have a high mutation rate due to strand slippage and the resulting variation in the number of tandem repeats is used to type bacterial isolates through comparison of the size of amplicons produced from PCR of these regions [42,43,47]. The size

of the amplicons is determined through agarose gel electrophoresis, where DNA-size markers are used as a reference, capillary electrophoresis on a DNA sequencer, or by DNA sequencing [42,43,47,48]. This results in a numerical barcode that can be used to compare bacterial isolates. VNTR analysis is highly discriminatory and has a shorter turnaround time compared to PFGE, as results are available within 24-48 hours, whereas PFGE can take up to five days. However, laboratories may use different VNTR schemes so data may not be comparable and not all species have a VNTR scheme [43].

1.1.3.2.3 MLST

MLST is a technique that involves PCR amplification and DNA sequencing of 450-500 bp internal fragments of five to ten housekeeping genes, which are highly conserved due to their role in encoding essential proteins [43]. Alleles within these genes are assigned a unique number [43,47-49], generating a numerical barcode that is translated into a sequence type (ST) according to the appropriate MLST scheme, which analyses the combination of alleles to generate the ST. STs and allele sequences are publicly available in online databases such as https://pubmlst.org/ and http://www.mlst.net. MLST has been used to identify bacterial clones and clonal complexes [44,49]. Clonal complexes are groups of STs that share identical alleles at a specified number of loci (such as five out of seven housekeeping genes for *Staphylococcus aureus* [50]). The main disadvantage of MLST is its high cost and it can be less discriminatory than other methods such as PFGE due to the focus on conserved housekeeping genes only [43,44,49]. However, MLST is useful for global epidemiology and evolutionary studies as it is based on a system that is internationally standardised, with low mutation rate of housekeeping genes and high reproducibility of results [48].

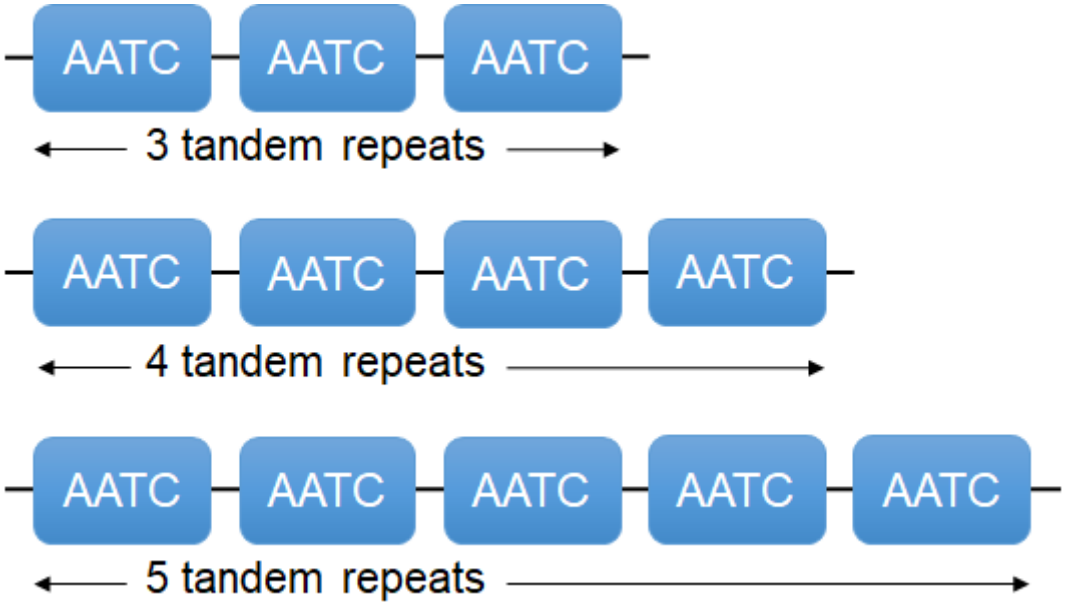


Figure 1.4: Example of tandem repeats, which are short sequences of DNA repeated in tandem within a genome.

1.1.3.2.4 WGS

WGS can also be used to identify genetic relatedness between bacterial isolates. Genome sequences can be used for MLST and analysis of complete genomes can lead to the identification of single nucleotide polymorphisms (SNPs), which are mutations in single nucleotides [48,51]. Therefore, WGS provides the highest level of discrimination compared to other bacterial typing methods and, unlike the other typing methods, WGS can also be used to identify antibiotic resistance genes and virulence factors. Limitations of WGS include the high cost, although it is becoming cheaper; the need for knowledge in bioinformatic techniques for in-depth analysis of the data and the inability of short-read sequencing technologies (which are currently the most commonly used in research and public health laboratories) to successfully sequence regions containing long tandem repeats or MGEs due to short-read length (<1000 bp) [52-55].

1.1.3.2.5 Uses of bacterial typing

Bacterial typing methods can help identify outbreaks of bacteria within the healthcare or community settings and enable the appropriate control measures to be put in place to reduce the spread of the bacteria such as improved hygiene or isolation of patients [42]. This is particularly important for MDR Gram-negative bacteria, which currently pose a threat to public health.

1.1.4 Concern regarding antibiotic resistance in Gram-negative bacteria

Antibiotic resistance in Gram-negative bacteria is a huge concern due to their ability to accumulate resistance genes for multiple classes of antibiotics via horizontal gene transfer and the paucity of new antibiotics being developed against them [56]. According to the WHO priority pathogens list, new antimicrobials are most urgently needed to target carbapenemase-producing *A. baumannii*, *P. aeruginosa* and Enterobacterales [57]. These species are commonly MDR, particularly to the three main classes of antibiotics used to treat Gram-negative infections, which are aminoglycosides, β-lactams and fluoroquinolones [58,59].

1.2 Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics used to treat both Gram-negative and Gram-positive bacterial infections including bloodstream infections, respiratory tract infections and urinary tract infections [60,61]. Aminoglycosides have been classified by the WHO as critically important antibiotics for human medicine as aminoglycosides are one of the limited available antibiotic classes to treat serious infections in the healthcare setting, including those that may be antibiotic resistant or where there is evidence of transmission of resistant bacteria or genes from non-human sources [62]. They are only effective against aerobic bacteria such as Enterobacterales, enterococci, mycobacteria, *P. aeruginosa*, *S. aureus*, some streptococci and some intestinal protozoa. [60,63]. They are rarely used as first-line agents in treatment of disease due to issues with toxicity in patients [64] but their use is increasing due to the emergence of MDR bacterial infections [65,66].

1.2.1 Discovery of aminoglycosides

Streptomycin was the first aminoglycoside to be discovered by Waksman *et al.* [67]. It was isolated from *Streptomyces griseus* in 1944 [67] and was the first antibiotic successfully used to treat *Mycobacterium tuberculosis* [68]. Since 1944, around 200 kinds of aminoglycosides have been isolated from actinomycetes species such as *Streptomyces* spp. and *Micromonospora* spp. [69]. Aminoglycosides produced by *Streptomyces* spp. are known as “mycin” aminoglycosides (e.g. kanamycin) and those produced by *Micromonospora* spp, are “micin” aminoglycosides (e.g. gentamicin) [65]. Semi-synthesised antibiotics have also been introduced, such as amikacin (derived from kanamycin [70]), which have increased antibacterial activity, the ability to evade known resistance mechanisms and reduced toxicity [7] (Table 1.1).

Table 1.1: The year of identification of some of the most clinically-relevant aminoglycosides and the species they were isolated from.

Naturally-produced aminoglycosides				
Aminoglycoside	**Year identified**	**Species**	**Clinical/veterinary use**	**Reference**
streptomycin	1944	*S. griseus*	Clinical	[67]
neomycin	1949	*Streptomyces fradiae*	Clinical	[71]
hygromycin B	1953	*Streptomyces hygroscopicus*	Clinical	[72]
kanamycin	1957	*Streptomyces kanamyceticus*	Clinical	[73]
paromomycin	1959	*Streptomyces rimosus*	Clinical	[74]
spectinomycin	1961	*Streptomyces spectabilis*	Clinical	[75]
gentamicin	1963	*Micromonospora purpurea*	Clinical	[76]
tobramycin	1967	*Streptomyces tenebrarius*	Clinical	[77]
apramycin	1968	*S. tenebrarius*	Veterinary	[78]
ribostamycin	1970	*Streptomyces ribosidificus*	Clinical	[79]
sisomycin	1970	*Micromonospora inyoensis*	Clinical	[80]
lividomycin	1971	*Streptomyces lividus*	Clinical	[81]
fortimicin A	1977	*Micromonospora olivasterospora*	Clinical	[82]
Synthetic aminoglycosides				
Aminoglycoside	**Year identified**	**Derivative of**	**Clinical/veterinary use**	**Reference**
amikacin	1972	kanamycin	Clinical	[70]
arbekacin	1973	kanamycin	Clinical	[83]
netilmicin	1973	sisomicin	Clinical	[84]
dibekacin	1975	kanamycin	Clinical	[85]
isepamicin	1975	gentamicin	Clinical	[86]
plazomicin	2009	sisomicin	Clinical	[87]

1.2.2 Chemical structure

Aminoglycosides consist of an aminocyclitol ring, with both amine and hydroxyl substitutions, which is connected to amino sugars through glycosidic linkages [88]. In clinically-relevant aminoglycosides the aminocyclitol ring can either be 2-deoxystreptamine (DOS) (e.g. amikacin, gentamicin and kanamycin), streptamine (e.g. spectinomycin) or streptidine (e.g. streptomycin) [89] (Figure 1.5). Based on the positions of the glycosidic linkages the aminoglycosides with a DOS ring can be classified into three different groups: monosubstituted (e.g. neamine, which has an amino sugar connected to position 4 of the DOS ring), 4,5-disubstituted DOS and 4,6-disubstituted DOS aminoglycosides (which have amino sugars bound at positions 4, and 5 or 6) [7,61,89]. Those lacking the DOS ring are classified as 'others' (e.g. streptomycin and streptamine) [89]. The amino sugar at position 4 is classed as 'ring I', also known as the primed ring, the aminocyclitol ring is classed as 'ring II' and the amino sugar bound at either position 5 or 6 is classed as 'ring III' (also known as the doubly primed ring). Any additional rings bound to ring III are known as 'ring IV' (or the triply primed ring) [7].

1.2.3 Advantages and disadvantages of aminoglycoside use

There are many advantageous characteristics of aminoglycosides that make them useful antibiotics such as having a broad-spectrum of bactericidal activity as they can be used against a range of Gram-negative and Gram-positive bacteria [60,61]. Aminoglycosides have rapid bactericidal action [61,64], especially as activity is mainly concentration-dependent [60,63], therefore, higher concentrations of the drug can kill pathogens at a faster rate [60]. Furthermore, they exhibit a post-antibiotic effect as they can suppress the growth of bacteria for up to 7.5 hours following clearance of the drug [60]. Aminoglycosides also work synergistically with other antibiotic classes such as β-lactams [63]. β-lactams inhibit cell wall synthesis, which promotes the uptake of the aminoglycoside due to increased cell permeability. Additional benefits of aminoglycosides include chemical stability [61,64]; lack of allergenicity [61] and being inexpensive to produce [61,64].

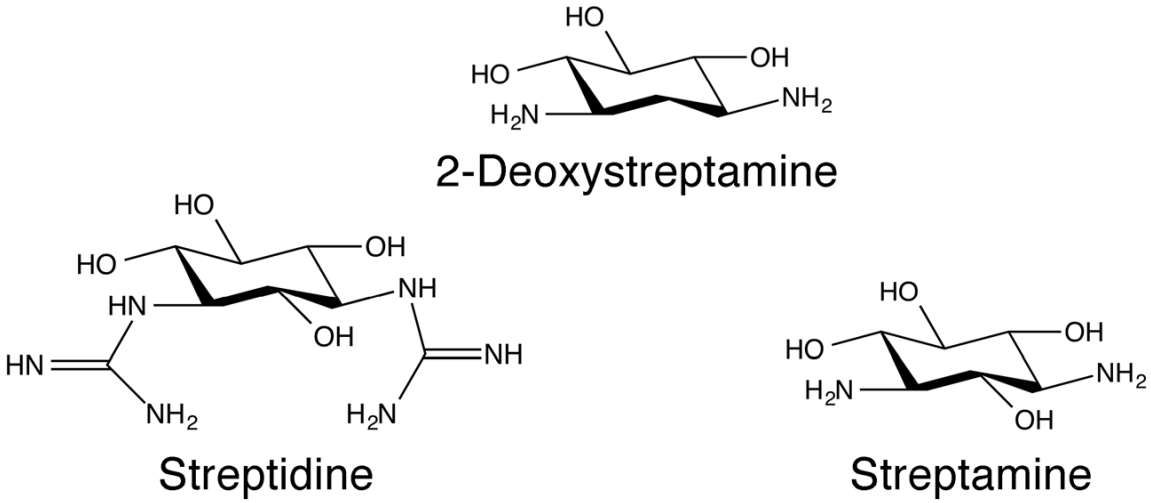


Figure 1.5: Chemical structures of aminocyclitol rings that are found in aminoglycosides. 2-Deoxystreptamine rings are found in 4,5-disubstituted DOS and 4,6-disubstituted DOS aminoglycosides (such as gentamicin and kanamycin); streptidine is found in the aminoglycoside streptomycin and streptamine is found in the aminoglycoside spectinomycin. This figure was obtained from Ramirez *et al.* [90] and is licenced under the Creative Commons Attribution 4.0 International Licence. To view a copy of this licence, visit https://creativecommons.org/licenses/by/4.0/.

However, there are several disadvantages to use, such as lack of activity against anaerobic bacterial infections [61] and the inability to be administered orally due to being absorbed poorly in the gut [7]. Additionally, due to their polycationic nature, aminoglycosides penetrate biological membranes including the blood-brain barrier poorly; therefore, their concentration is low inside human cells, and in tissues, apart from the proximal renal tube, where aminoglycosides are concentrated [7]. As stated earlier, the bactericidal activity of aminoglycosides is concentration-dependent and aminoglycosides have a high post-antibiotic effect meaning that there is a risk of toxicity if the administered dosage is too high (see Section 1.2.4), therefore, aminoglycosides are administered once daily to prevent toxicity in patients, and levels are monitored [91].

1.2.4 Toxicity

The most commonly known toxicities caused by aminoglycosides are nephrotoxicity (renal impairment) and ototoxicity (hearing impairment), but neuromuscular blockade (which causes respiratory depression) can also occur rarely [60,64,65]. Nephrotoxicity is caused by the build-up of the antibiotic in the kidneys; around 5% of the antibiotic is retained in the kidneys, which causes morphological damage to epithelial cells lining the proximal tubules and leads to renal malfunction [92]. Fortunately, the damage caused by nephrotoxicity can be reversible [92]. However, damage caused by ototoxicity is irreversible as aminoglycosides prevent cellular respiration in the mitochondria of the inner ear cells and induce the production of reactive oxygen species, which damage the inner-ear hair cells resulting in hearing loss [93]. Therefore, dosages need to be calculated for individual patients and serum levels need to be monitored to prevent these toxicities in patients [64].

1.2.5 The use of aminoglycosides in the UK

The consumption of aminoglycosides in the UK is relatively low as prescriptions of aminoglycosides accounted for 0.6% (0.13/21.0) Daily Defined Doses (DDDs) per 1,000 inhabitants in 2017 [94]. Consumption was highest in the hospital setting as it accounted for 92.3% (0.12/0.13) DDDs per 1,000 inhabitants in 2017 [94]. The remainder were consumed

by the general practice (GP) setting, which accounted for 7.7% (0.01/0.13) DDDs per 1,000 inhabitants [94].

1.2.6 Uptake and mechanism of action

As aminoglycosides are positively charged, they have an affinity for negatively-charged molecules such as lipopolysaccharides, phospholipids, DNA and RNA [7,61]. This contributes to their mechanism of action, which is to disrupt protein synthesis (specifically the process called translation) through the binding of 16S rRNA in the bacterial ribosome [95].

Bacterial 70S ribosomes are composed of two subunits: the 30S subunit and the 50S subunit [96-98]. The former consists of a single 16S rRNA as well as 21 ribosomal proteins and the latter consists of two rRNAs, (5S rRNA and 23S rRNA) and 33 ribosomal proteins [96]. The ribosome is comprised of three sites where translation, the second stage of protein synthesis following transcription, occurs: the aminoacyl (A) site, the peptidyl (P) site and the exit (E) site [96-98].

During translation, transfer rRNA (tRNA) molecules carrying amino acids (herein called aminoacyl-tRNA), enter the ribosome and the decoding centre (found within the A-site) checks that only cognate aminoacyl-tRNAs (those with nucleotides in their anticodon matching nucleotides in the codon found in the mRNA) are bound to the mRNA [97,98]. This decoding centre comprises of helix 44 of the 16S rRNA as well as two adenine nucleotides known as A1492 and A1493 [97]. Once confirmed, a peptide bond is formed between the amino acid of the cognate aminoacyl-tRNA and the aminoacyl-tRNA stored in the P-site; the two aminoacyl-tRNAs translocate from the A-site and P-site to the P-site and E-site, respectively, so the growing polypeptide chain and the empty tRNA can exit through the E-site [97,98].

1.2.6.1 Initial uptake

Initial uptake of aminoglycosides is self-promoted through the electrostatic binding of positively-charged aminoglycosides to the negatively-charged lipopolysaccharides, phospholipids and other membrane proteins in Gram-negative bacteria and to phospholipids

as well as teichoic acids in Gram-positive bacteria [63] (Figure 1.6). This leads to the disruption of Mg^{2+} bridges between adjacent lipopolysaccharide molecules, which damages the cell membrane and increases its permeability, thus promoting the uptake of aminoglycosides into the cell [99].

1.2.6.2 Energy-dependent phases I and II

Next, energy-dependent phase I occurs [100] where aminoglycosides cross the cytoplasmic membrane in an oxygen-dependent process using metabolic energy from the electron transport system [61], which explains why anaerobic bacteria are intrinsically resistant to aminoglycosides [63]. Following entry into the cytoplasm, energy-dependent phase II occurs [100], where aminoglycosides bind to the 30S subunits of the negatively-charged bacterial ribosomes and induce mistranslation of proteins, which become incorporated into the bacterial cell membrane further increasing cell permeability (Figure 1.6). This allows the entry of additional aminoglycosides into the cell and, eventually, induces cell death [63,101].

Mistranslation of proteins is caused by the binding of aminoglycosides to the ribosomal A-site. The A-site is involved in the identification of cognate and noncognate aminoacyl-tRNA during translation. During this process two universally conserved adenine residues, A1492 and A1493, within the decoding centre flip outwards when the mRNA and aminoacyl-tRNA complex is formed [63]. The two adenine residues form hydrogen bonds between the N1 of these adenine residues [63] and the ribose 2'-OH groups found on the two first bases of the codon as well as the anticodon to determine if the correct bases are matching to form base pairs [7]. The flipping out of these two adenine residues is known as the 'on' state, which promotes a conformational change of the 30S ribosome from an 'open' state, meaning empty, to a 'closed' state, where the aminoacyl-tRNA is bound [102]. The binding of aminoglycosides to the A-site induces the conformational change to the 'on' state, preventing the ribosome from distinguishing between cognate and non-cognate aminoacyl-tRNAs, therefore allowing the incorporation of incorrect amino acids into the polypeptide chain, creating mistranslated proteins [7,63,102,103].

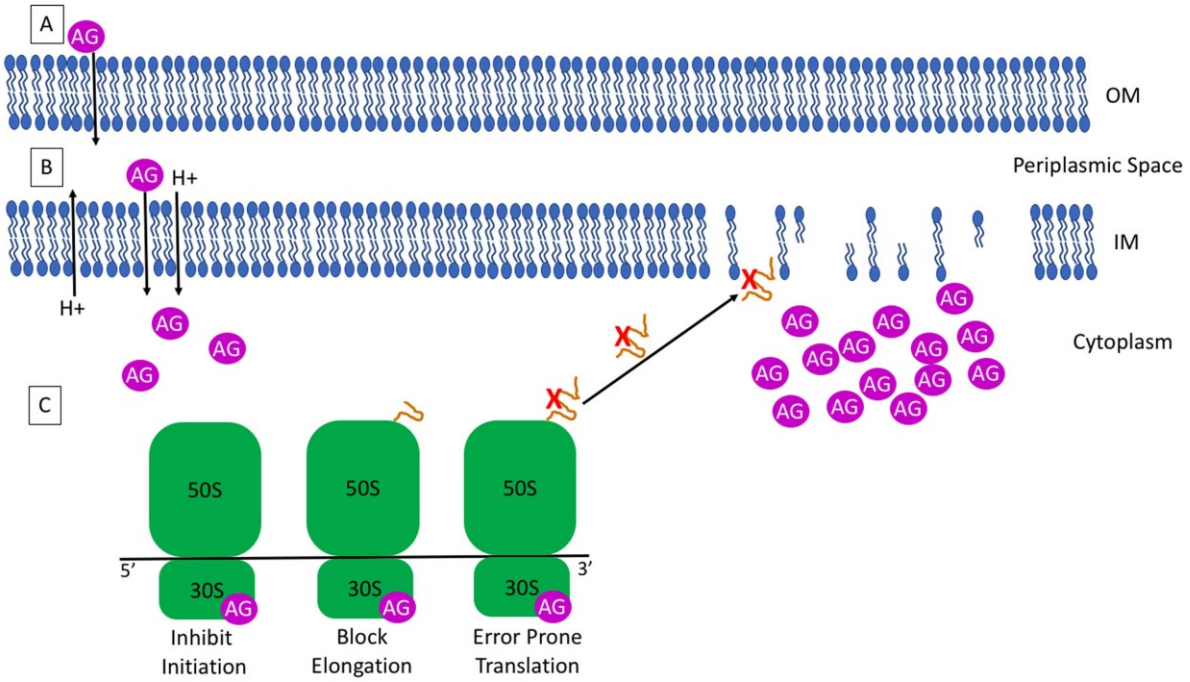


Figure 1.6: The mechanism of action of aminoglycosides. A: A positively-charged aminoglycoside binds to the negatively-charged outer membrane of the bacterial cell, where the aminoglycoside binds to lipopolysaccharides, phospholipids and other membrane proteins in Gram-negative bacteria and to phospholipids as well as teichoic acids in Gram-positive bacteria. Following electrostatic binding the aminoglycoside passes through to the periplasmic space. B: The aminoglycoside passes through the inner membrane into the cytoplasm using metabolic energy from the electron transport system in energy-dependent phase I. C: Following entry into the cytoplasm, the aminoglycoside binds to the negatively-charged 16S rRNA found within the 30S subunit of the bacterial ribosome, where it disrupts translation and leads to the generation of faulty proteins in energy-dependent phase II. These faulty proteins are incorporated into the cell membrane, which leads to the formation of pores and the entry of more aminoglycosides, eventually resulting in cell death. Reproduced from Serio *et al.* [65] with permission from the ASM Press.

1.2.7 Rates of aminoglycoside resistance in the UK

According to the European Antimicrobial Resistance Surveillance Network (EARS-Net), amikacin, gentamicin and tobramycin resistance in the UK in 2017 was identified in 10% *E. coli*, 7.9% *K. pneumoniae*, 4.6% *A. baumannii* and 3.9% *P. aeruginosa* isolates collected from blood and cerebrospinal fluid (Figure 1.7) [104]. Rates of aminoglycoside resistance in the UK were higher than rates found in Denmark, Finland, Norway and Sweden, which ranged from 0%-6.0%, 0%-5.0%, 0%-7.2% and 0%-6.5% in these species, respectively [104]. However, UK rates of resistance were lower than Bulgaria (which had the highest levels of aminoglycoside resistance in *E. coli*, *K. pneumoniae* and *Acinetobacter* spp.), where resistance was found in 36.2% *E. coli*, 63.1% *K. pneumoniae*, 89.1% *A. baumannii* and 28.2% *P. aeruginosa* isolates [104].

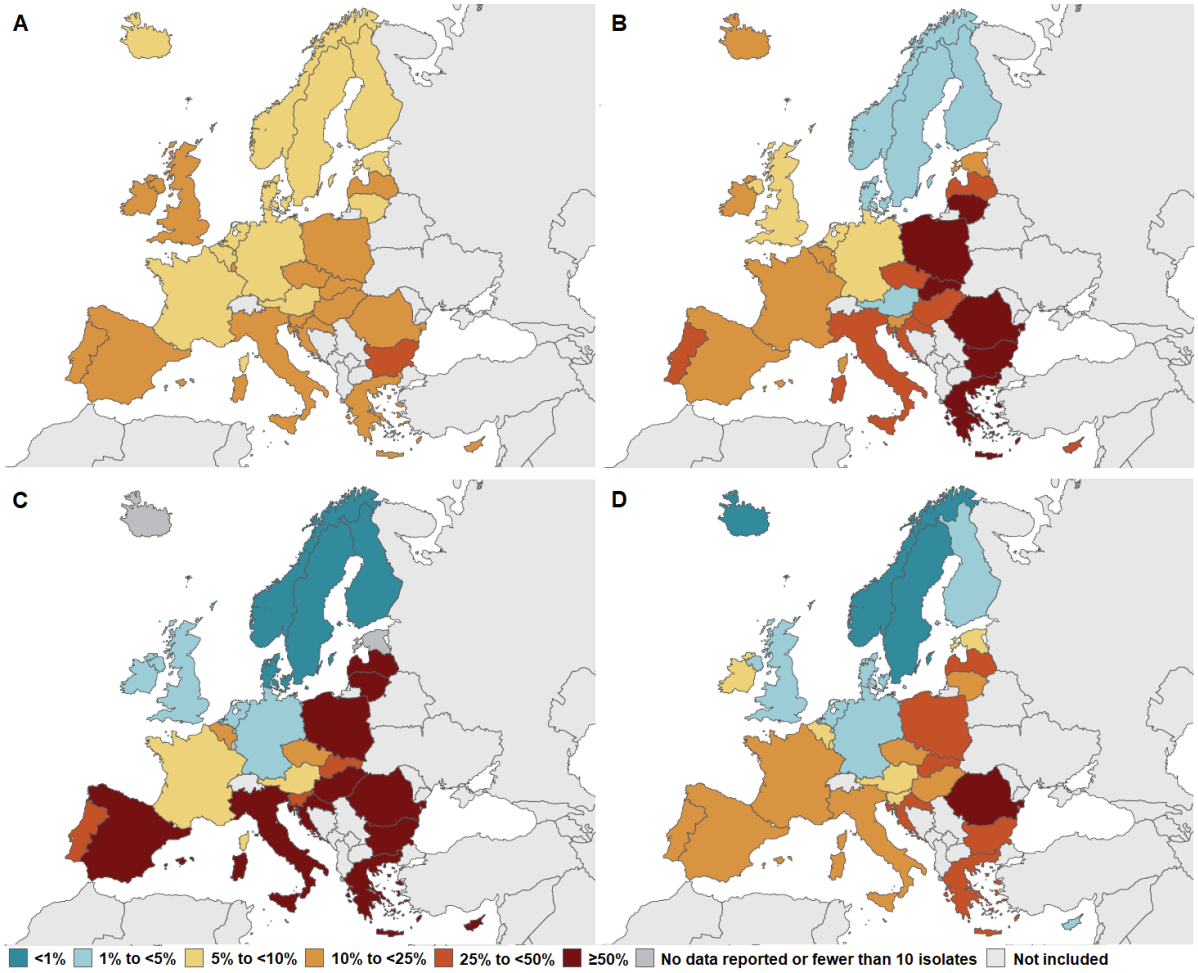


Figure 1.7: Proportion of *E. coli* (A), *K. pneumoniae* (B), *Acinetobacter* spp. (C) and *P. aeruginosa* (D) isolates from blood and cerebrospinal fluid with resistance to amikacin, gentamicin and tobramycin in 2017 according to EARS-Net. These figures were obtained from The European Centre for Disease Prevention and Control (ECDC) [104].

1.2.8 Mechanisms of aminoglycoside resistance

There are several aminoglycoside resistance mechanisms, which include either intrinsic resistance (such as being anaerobic or through mutations in the respiratory chain or adenosine triphosphate (ATP) synthases that result in a low level of transmembrane potential [7,63]); reducing the cellular concentration of aminoglycosides (via efflux pumps or impermeability); chemically modifying the aminoglycoside (via aminoglycoside-modifying enzymes [AMEs]); or by modifying the ribosome (through chromosomal mutation or the use of 16S rRNA methyltransferases [16S RMTases]) [7,63,65,103]. More than one of these resistance mechanisms can exist in a bacterial cell at the same time.

1.2.8.1 Impermeability

The outer membrane of the bacterial cell is the first line of defence against foreign molecules such as antibiotics and has been implicated in aminoglycoside resistance through impermeability, which is caused by either modifying the outer membrane or by porin loss [65,103]. As aminoglycosides are polycationic compounds they have an affinity for lipopolysaccharides, which are negatively charged and are present on the outer membrane. However, this affinity can be reduced by modification of the outer membrane such as by the addition of the positively-charged sugar 4-amino-4-deoxy-L-arabinose to the lipopolysaccharide, therefore inducing resistance by discouraging entry of the aminoglycosides into the cell [105]. The loss of porins, which are water-filled channels that allow the transport of small hydrophilic molecules into the cell (Figure 1.8), has also been indicated to play a role in aminoglycoside resistance but has yet to be confirmed clinically [65,103]. The loss of OmpF porins through mutation in *E. coli* resulted in aminoglycoside resistance [106], but OmpF loss has been indicated to reduce fitness in *E. coli* [107,108], which may indicate why porin loss has rarely been reported as a resistance mechanism for aminoglycosides due to factors such as the potential loss of nutrients [65,103].

1.2.8.2 Efflux pumps

Efflux pumps belonging to the resistance-nodulation cell-division (RND) superfamily have been shown to cause aminoglycoside resistance including AcrD in *E. coli* [109], AdeABC in *A. baumannii* [110] and MexXY-OprM in *P. aeruginosa* [111]. These efflux pumps consist of three components: a transporter protein in the inner membrane, a membrane fusion protein in the periplasm and the third in the outer membrane, which acts as the outer membrane component of the pump [7,65,103] (Figure 1.8). These components create a continuous, tripartite channel that exports substrates directly out of the cell [7]. They use membrane proton force as an energy source and cause bacteria to be intrinsically resistant to low levels of aminoglycosides [7,65,103]. However, overexpression of efflux pumps through the mutation of regulator genes, such as *mexZ* for the efflux pump MexXY-OprM [111], can result in high-level aminoglycoside resistance [103] as well as resistance to other antibiotic classes due to many also being substrates for this efflux pump, such as carbapenems, fluoroquinolones, macrolides and tetracyclines [111].

1.2.8.3 AMEs

Enzyme modification is the main aminoglycoside resistance mechanism used by Gram-negative and Gram-positive bacteria and is carried out by AMEs [63]. AMEs catalyse the modification at $-NH_2$ or -OH groups found on the DOS ring or its sugar moieties [112], which reduces the ability of aminoglycosides to bind to ribosomes and prevents them carrying out energy-dependent phase II, which causes resistance [63]. There are three groups of AMEs called acetyltransferases (AACs), nucleotidyltransferases (ANTs) and phosphotransferases (APHs), which modify aminoglycosides by acetylation, adenylylation and phosphorylation, respectively [112] (Figure 1.9).

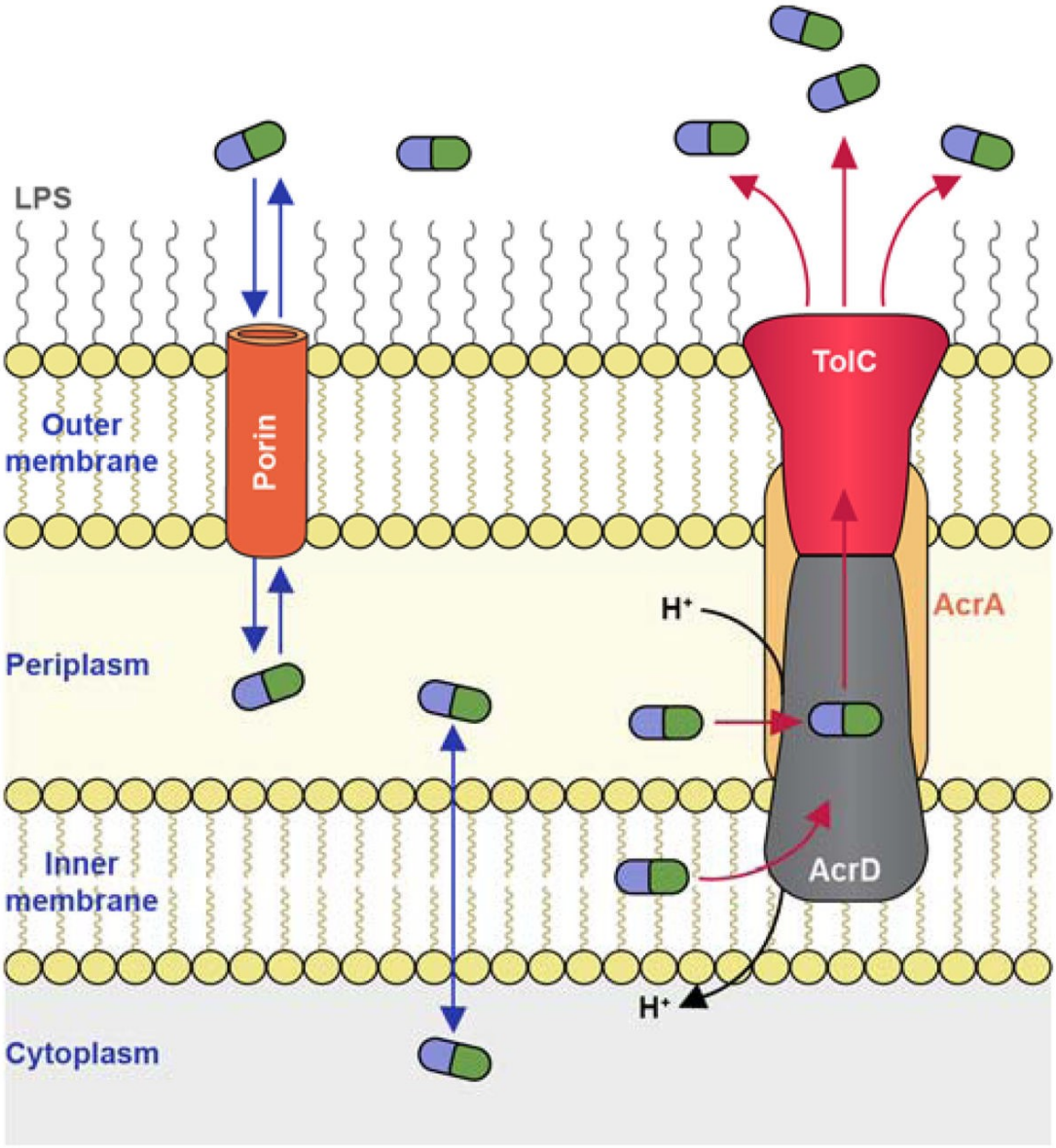


Figure 1.8: Transport of aminoglycosides across the bacterial membrane including influx and efflux by a porin channel and efflux by the RND-type efflux pump AcrAD-TolC. Aminoglycosides are represented by the green and blue lozenges and efflux and influx are represented by the arrows. Efflux pumps require the use of protons (represented as H^+) as an energy source for exportation of the aminoglycoside and other substrates. Reproduced from Garneau-Tsodikova *et al.* [103] with permission from the Royal Society of Chemistry.

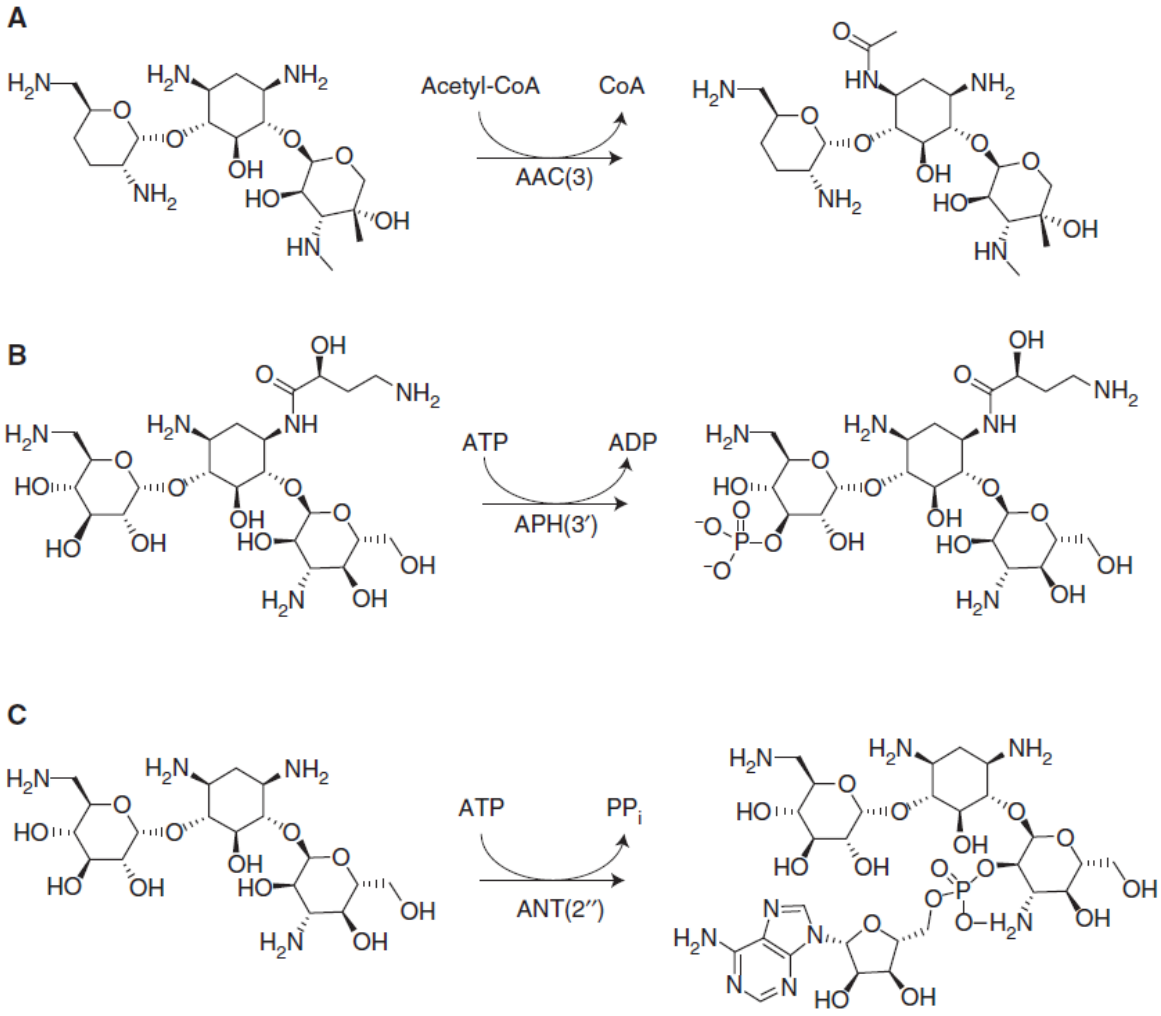


Figure 1.9: Chemical modification of aminoglycosides by AMEs. A: modification of gentamicin catalyzed by the acetyltransferase AAC(3). B: modification of amikacin catalyzed by the phosphotransferase APH(3′). C: modification of kanamycin catalyzed by the nucleotidyltransferase ANT(2″). Reproduced from Krause *et al*. [66] with permission from Cold Spring Harbor Press.

1.2.8.3.1 Nomenclature

Confusingly, there are two different schemes for nomenclature of AMEs: one proposed by Novick *et al.* [113] and the other proposed by Shaw *et al*. [114]. Under the scheme proposed by Novick *et al.* [113], acetyltransferases, nucleotidyltransferases and phosphotransferases are labelled *aac*, *ant* and *aph*, respectively; this is followed by a letter indicating the position of modification where *aacA* stands for a 6′-*N*-acetyltransferase, while *aacB* and *aacC* stand for a 2′-*N*-acetyltransferase and a 3-*N*-acetyltransferase, respectively. This designation can be followed by a number to indicate a subdivision [115].

Shaw *et al.* [114] proposed *aac*, *ant* and *aph* are used to distinguish the enzyme type followed by the position where the aminoglycoside is modified in brackets e.g. (2'), which modifies position 2' of the aminoglycoside; roman numerals are used to distinguish the resistance profile and then a lowercase letter to distinguish unique proteins e.g. *aac(6')-Ia* and *aac(6')-Ib* are unique genes encoding two proteins with the same resistance profile.

1.2.8.3.2 AACs

AACs catalyse the acetylation of -NH_2 groups found on aminoglycosides, using acetyl coenzyme A as the donor substrate, where AACs belonging to groups AAC(1), AAC(2'), AAC(3) and AAC(6') catalyse the modification of -NH_2 groups by the addition of an acetyl group at positions 1, 2', 3 and 6', respectively (Table 1.2) [63,112]. The most prevalent AAC enzyme is AAC(6')-Ib (including its variant AAC(6')-Ib-cr), which confers resistance to amikacin, kanamycin and tobramycin but not gentamicin [90], and has been identified extensively in Gram-negative bacteria including Enterobacterales and the non-fermenters *A. baumannii* and *P. aeruginosa* [63,65,103]. What is worrying is that *aac(6')-Ib-cr* also confers resistance to certain fluoroquinolones such as ciprofloxacin [116] and has been found associated with other resistance genes on plasmids as well as transposons [117]. AAC(6') enzymes have also been found to be part of bifunctional enzymes with other AMEs (such as AAC(6')-Ie-APH(2")-Ia [118], which generates resistance to a broader range of aminoglycosides but this bifunctional enzyme has not yet been identified in Enterobacterales.

Table 1.2: AAC genes according to nomenclature schemes by Novick *et al*. [113] and Shaw *et al*. [114] as well as the resistance profiles and species the genes are associated with.

AAC subclass	Genes according to nomenclature by Shaw *et al*. [114] (nomenclature by Novick *et al*. [113])	Resistance profile	Species
AAC(1)	*aac(1)*	APR, LVD, PRM and RSM	*Enterobacterales*
AAC(2')	*aac(2')-Ia*	DBK, GEN, NET, PLA and TOB	*Providencia stuartii*
	aac(2')-Ib	DBK, GEN, NET and TOB	*A. baumannii* and *Mycobacterium* spp.
	aac(2')-Ic		*Mycobacterium* spp.
	aac(2')-Id		*Mycobacterium smegmatis*
	aac(2')-Ie		*Mycobacterium leprae*
AAC(3)	*aac(3)-Ia* (*aacC1*)	FOR, GEN and SIS	*A. baumannii*, *Enterobacterales* and *P. aeruginosa*
	aac(3)-Ib (*aacCA2*)		*Enterobacterales* and *Pseudomonas* spp.
	aac(3)-Ic (*aacCA3*)		*Enterobacterales* and *Pseudomonas* spp.
	aac(3)-Id (*aacCA5*)		*Enterobacterales*, *P. aeruginosa* and *Vibrio fluvialis*
	aac(3)-Ie (*aacCA5*)		*Enterobacterales*, *P. aeruginosa* and *V. fluvialis*
	aac(3)-IIa (*aacC2*/*aacC3*/*aacC5*)	DBK, GEN, NET, SIS and TOB	*Enterobacterales* and *P. aeruginosa*
	aac(3)-IIb		*Enterobacterales*
	aac(3)-IIc (*aacC2*)		*E. coli* and *P. aeruginosa*
	aac(3)-IIIa (*aacC3*)	DBK, GEN, KAN, NEO, LVD, PRM, SIS and TOB	*P. aeruginosa*
	aac(3)-IIIb		*Pseudomonas* spp.
	aac(3)-IIIc		*P. aeruginosa*
	aac(3)-IVa (*aacC4*)	APR, DBK, GEN, NET, SIS and TOB	*A. baumannii, Enterobacterales* and *P. aeruginosa*
	aac(3)-VIa	GEN, NET and SIS	*Enterobacterales*
	aac(3)-VIIa (*aacC7*)	PRM	*S. rimosus*
	aac(3)-VIIIa (*aacC8*)	NEO	*S. fradiae*
	aac(3)-IXa (*aacC9*)	NEO	*Micromonospora chalcea*
	aac(3)-Xa	DBK, GEN, KAN, NEO and PRM	*S. griseus*

Table 1.2 continued

AAC subclass	Genes according to nomenclature by Shaw *et al*. [114] (nomenclature by Novick *et al*. [113])	Resistance profile	Species
AAC(6')	*aac(6')-Ia* (*aacA1*)	AMK, KAN and TOB	*Enterobacterales* and *P. aeruginosa*
	aac(6')-Ib (*aacA4*)		*Enterobacterales*, *P. aeruginosa* and *Vibrio cholerae*
	aac(6')-Ic/*aac(6')-III*		*Serratia marcescens*
	aac(6')-Ib-cr	AMK, CIP, KAN and TOB	*Enterobacterales* and *P. aeruginosa*
	aac(6')-IIa	DBK, GEN, NET, SIS and TOB	*Enterobacterales* and *Pseudomonas* spp.
	aac(6')-IIb		*Pseudomonas fluorescens*
	aac(6')-IIc	NET	*Enterobacterales* and *Pseudomonas* spp.

AMK = amikacin; APR = apramycin; CIP = ciprofloxacin; DBK = dibekacin; GEN = gentamicin; GEN(B) = gentamicin B; KAN = kanamycin; FOR = fortimicin A; LVD = lividomycin; NEO = neomycin; NET = netilmicin; PLA = plazomicin; PRM = paromomycin; RSM = ribostamycin; SIS = sisomicin and TOB = tobramycin. This table was adapted from Shaw *et al*. [114].

1.2.8.3.3 ANTs

ANTs catalyse the adenylylation of aminoglycosides by using the donor substrate ATP, where they transfer an adenosine monophosphate (AMP) group to -OH groups at positions 2", 3", 4', 6 and 9 of the aminoglycoside via enzyme groups ANT(2"), ANT(3"), ANT(4'), ANT(6) and ANT(9), respectively (Table 1.3) [112]. All enzyme groups are predominately produced by Gram-positive bacteria apart from ANT(2") enzymes, where ANT(2)-Ia is the most prevalent ANT produced by Gram-negative bacteria [65]. This enzyme is produced by Enterobacterales as well as the non-fermenters *A. baumannii* and *P. aeruginosa* [65,112,119]. It confers resistance to dibekacin, gentamicin, kanamycin, sisomicin and tobramycin and the gene *ant(2)-Ia* has been identified on plasmids and MGEs such as transposons [112].

1.2.8.3.4 APHs

APHs catalyse the phosphorylation of -OH groups of aminoglycosides, using ATP as the donor substrate, at positions 2", 3', 3", 4, 6, 7" and 9, using the enzyme groups APH(2"), APH(3'), APH(3"), APH(4), APH(6), APH(7") and APH(9), respectively (Table 1.4) [26,103]. The largest number of APHs belong to the group APH(3') [63,120], where APH(3') genes have been found on broad-host range plasmids and transposons [63] and confer resistance to a range of aminoglycosides such as amikacin, kanamycin, neomycin, paramomycin and ribostamycin in both Gram-negative and Gram-positive bacteria [112,119].

1.2.8.4 Ribosomal modifications

Aminoglycoside resistance can be caused by ribosomal mutations but this is only clinically relevant for streptomycin as mutations in the *rrs* gene, which encodes the 16S rRNA operon, are only effective in *Mycobacterium* spp. due to this species only having one copy of the *rrs* gene (compared with other bacterial species, which have multiple copies) [7,89]. Therefore, a single mutation in the *rrs* gene in *Mycobacterium* spp. can prevent the binding of streptomycin to bacterial ribosomes through the mutation of two highly conserved regions: the 530 loop and the region surrounding nucleotide 912 (based on *E. coli* numbering) in the 16S rRNA, which are part of the A-site [121-124].

Table 1.3: ANT genes according to nomenclature schemes by Novick *et al*. [113] and Shaw *et al*. [114] as well as the resistance profiles and species the genes are associated with.

ANT subclass	Genes according to nomenclature by Shaw *et al*. [114] (nomenclature by Novick *et al*. [113])	Resistance profile	Species
ANT(2")	*ant(2")-Ia* (*aadB*)	DBK, GEN, KAN, SIS and TOB	*A. baumannii*, *Enterobacterales*, *P. aeruginosa* and *V. cholerae*
ANT(3")	*ant(3")-Ia* (*aadA*)	SM and SPM	*Enterobacterales*, *P. aeruginosa* and *V. cholerae*
ANT(4')	*ant(4')-Ia* (*aadD*/*aadD2*)	AMK, DBK, GEN, ISE and TOB	*Bacillus* spp., *Enterococcus* spp. and *Staphylococcus* spp.
	ant(4')-Ib		*Enterococcus* spp. and *Staphylococcus* spp.
	ant(4')-IIa	AMK, ISE and TOB	*Enterobacterales*, *P. aeruginosa* and *V. cholerae*
	ant(4')-IIb		*P. aeruginosa*
ANT(6)	*ant(6)-Ia* (*aadE*)	SM	*C. jejuni*, *Enterococcus* spp., *Staphylococcus* spp. and *Streptococcus* spp.
	ant(6)-Ib		*Bacillus subtilis*, *Campylobacter fetus* and *Clostridioides* spp.
ANT(9)	*ant(9)-Ia* (*aad9*)	SPM	*Enterococcus* spp., *Streptococcus pneumoniae* and *Staphylococcus* spp.

AMK = amikacin; DBK = dibekacin; GEN = gentamicin; ISE = isepamicin; KAN = kanamycin; SIS = sisomicin; SM = streptomycin; SPM = spectinomycin and TOB = tobramycin. This table was adapted from Shaw *et al*. [114].

Table 1.4: APH genes according to nomenclature schemes by Novick *et al*. [113] and Shaw *et al*. [114] as well as the resistance profiles and species the genes are associated with.

APH subclass	Genes according to nomenclature by Shaw *et al*. [114] (nomenclature by Novick *et al*. [113])	Resistance profile	Species
APH(2")	*aph(2")-Ia*	GEN	*Enterococcus* spp. and *Staphylococcus* spp.
	aph(2")-Ib/*aph(2")-IIa*		*E. coli* and *Enterococcus faecium*
	aph(2")-Ic/*aph(2")-IIIa*		*Enterococcus gallinarum*
	aph(2")-Id/aph(2")-IVa	GEN and PLA	*Enterococcus casseliflavus*
	aph(2")-Ie	GEN	*Enterococcus* spp.
APH(3')	*aph(3')-Ia* (*aphA1*/*aphA7*)	GEN(B), KAN, LVD, NEO, PRM and RSM	*Acinetobacter* spp., *Enterobacterales* and *Pseudomonas* spp.
	aph(3')-Ib (*aphA-like*)		*Enterobacterales* and *P. aeruginosa*
	aph(3')-IIa (*aphA2*)	GEN(B), KAN, NEO, PRM and RSM	*Clostridioides botulinum*, *Enterobacterales*, *Listeria monocytogenes*, *Pseudomonas* spp. and *Staphylococcus aureus*
	aph(3')-IIb		*P. aeruginosa*
	aph(3')-IIc		*Stenotrophomonas maltophilia*
	aph(3')-IIIa (*aphA3*)	AMK, GEN(B), ISE, KAN, LVD, NEO, PRM and RSM	*Enterobacterales*, *Enterococcus* spp., *Helicobacter pylori, Staphylococcus* spp. and *Streptococcus* spp.
	aph(3')-IVa (*aphA4*)	KAN, NEO, PRM and RSM	*A. baumannii*, *Bacillus circulans*, *Lactobacillus* spp. and *Pseudomonas* spp.
	aph(3')-Va (*aphA5*)	NEO, PRM and RSM	*Streptomyces* spp. and *Micromonospora* spp.
	aph(3')-Vb (*aphA5a*)		*S. fradiae*
	aph(3')-Vc (*aphA5c*)		*Micromonaspora chalcea*
	aph(3')-VIa (*aphA6*)	AMK, GEN(B), ISE, KAN, NEO, PRM and RSM	*Acinetobacter* spp., *Enterobacterales* and *Pseudomonas* spp.
	aph(3')-VIb		*Enterobacterales*
	aph(3')-VIIa (*aphA7*)	KAN and NEO	*Campylobacter* spp.
APH(3")	*aph(3")-Ia (aphD2*/*aphE)*	SM	*A. baumannii* and S. griseus
	aph(3")-Ib/*strA*		*Enterobacterales* and *Pseudomonas* spp.
	aph(3")-Ic		*Mycolicibacterium fortuitum*

Table 1.4 continued

APH subclass	Genes according to nomenclature by Shaw *et al*. [114] (nomenclature by Novick *et al*. [113])	Resistance profile	Species
APH(4)	*aph(4)-Ia*	HYR	*A. baumannii*, *Enterobacterales*,
			P. fluoroscens and
			S. hygroscopicus
	aph(4)-Ib		*Pseudomonas pseudomallei*
APH(6)	*aph(6)-Ia* (*aphD*)	SM	*Streptomyces* spp. and *Micromonospora* spp.
	aph(6)-Ib		*Streptomyces glaucescens*
	aph(6)-Ic		*Enterobacterales* and
			P. aeruginosa
	aph(6)-Id/*strB*		*Aeromonas bestiarum*, *Edwardsiella tarda*, *Enterobacterales*, *Pasteurella multocida*, *Pseudomonas* spp. and *V. cholerae*
APH(7")	*aph(7")-Ia* (*aph7"*)	HYR	*S. hygroscopicus*
APH(9)	*aph(9)-Ia*	SPM	*Legionella pneumophila*
	aph(9)-Ib	SPM	*S. flavopersicus*

AMK = amikacin; GEN(B) = gentamicin B; HYR = hygromycin B; ISE = isepamicin; KAN = kanamycin; LVD = lividomycin; NEO = neomycin; PLA = plazomicin; PRM = paromomycin; RSM = ribostamycin; SM = streptomycin and SPM = spectinomycin. This table was adapted from Shaw *et al*. [114].

Additionally, mutations in the gene *rspL*, which encodes the ribosomal protein S12, can also cause streptomycin resistance in *Mycobacterium tuberculosis* by disrupting the stability of the 530 loop (which is stabilised by S12) [121].

1.3 16S RMTases

1.3.1 16S RMTases

An emerging mechanism of aminoglycoside resistance, and the focus of this PhD thesis, is modification of the bacterial ribosome by 16S RMTases. These enzymes confer high-level resistance (MICs >256 mg/L) to all clinically-relevant aminoglycosides [125] by adding a methyl group to the N-1 position of residue A1408 or the N-7 position of residue G1405 in the 16S rRNA within the A-site, using S-adenosyl-$_L$-methionine (SAM) as a cofactor [126]. This changes the overall charge of the 16S rRNA from negative to positive, therefore preventing the binding of aminoglycosides to the A-site. Modification of A1408 confers resistance to both 4,5- and 4,6-disubstituted DOS aminoglycosides, such as gentamicin, kanamycin and neomycin, as well as the 4-monosubstituted DOS aminoglycoside apramycin, but modification of the G1405 residue confers resistance only to the 4,6-disubstituted DOS aminoglycosides such as amikacin, gentamicin and tobramycin [127,128]. The difference in resistance profile conferred by N1-A1408 and N7-G1405 16S RMTases is due to the position of ring III of the 4,5- and 4,6-disubstituted DOS aminoglycosides. A hydrogen bond can only form between the N7-G1405 position with a 4,6-disubstituted DOS aminoglycoside due to ring III of the latter being in closer proximity than ring III of a 4,5-disubstituted DOS aminoglycoside (Figure 1.10) [127]. However, the N1-A1408 position interacts with ring I of both 4,5- and 4,6-disubstituted DOS aminoglycosides (Figure 1.10) [127]. Therefore, methylation of positions N1-A1408 and N7-G1405 prevents the formation of these hydrogen bonds and confers resistance. 16S RMTases do not confer resistance to aminoglycosides lacking the DOS ring, such as streptomycin [127].

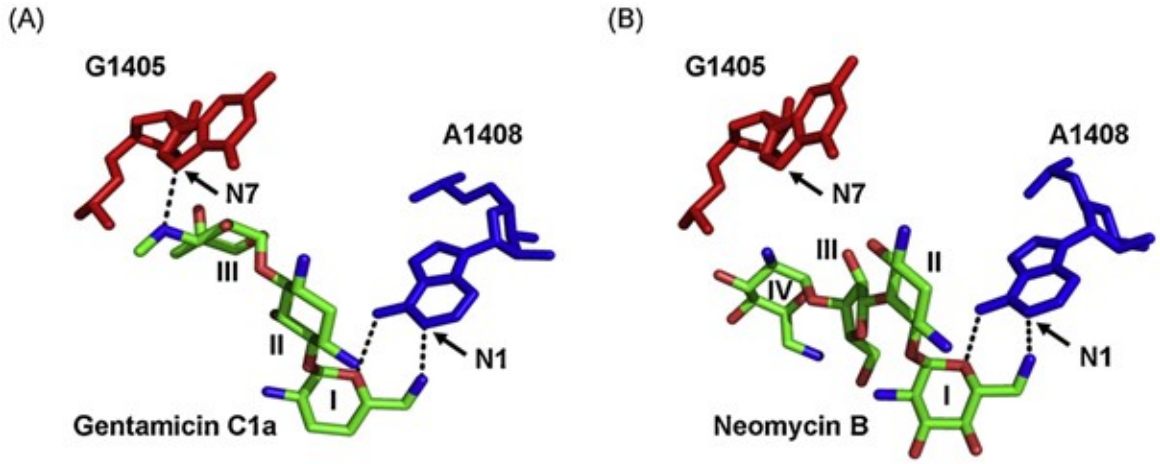


Figure 1.10: The interaction of 4,5- and 4,6- disubstituted DOS aminoglycosides with positions N1-A1408 and N7-G1405 in the 16S rRNA within the ribosomal A-site. A: 4,6- disubstituted DOS aminoglycoside gentamicin C1a; B: the 4,5- disubstituted DOS aminoglycoside neomycin B. Reproduced from Wachino *et al.* [127] with permission from Elsevier.

1.3.2 The origin of 16S RMTases

Aminoglycosides are produced by the actinomycetes, which are spore-forming Gram-positive bacteria [128]. In order to protect their ribosomes from the aminoglycosides that they produce, they utilise 16S RMTases to modify their 16S rRNA within the ribosomal A-site so aminoglycosides are unable bind to the ribosomes [128]. These enzymes were first discovered in actinomycetes in the 1980s, where enzymes modifying the residues A1408 and G1405 were identified, such as KamA from *Streptomyces tenjimariensis* and KgmA from *M. purpurea*, respectively [128].

1.3.3 Discovery of 16S RMTase genes in Gram-negative bacteria

In 1996, the first 16S RMTase gene, *armA*, was found on the plasmid pCTX-M3 (accession number: AF550415), which was isolated from a *Citrobacter freundii* clinical isolate in Poland [129]. However, the *armA* gene remained uncharacterised as it was only identified to be a putative 16S RMTase gene. In 2002 Galimand *et al*. [130] discovered *armA* in a urinary *K. pneumoniae* clinical isolate (strain BM4536) in France and later confirmed the presence of *armA* in pCTX-M3. In 2006, the enzymatic function of ArmA was characterised by Liou *et al*. [126] and it was identified to be a N7-G1405 16S RMTase.

In 2003, another 16S RMTase called *rmtA* was discovered in *P. aeruginosa* strain AR2, which had been isolated in 1997 from sputum in Japan [131]. The *rmtA* gene was found to have a G+C content of 55%, compared with 30% in *armA* and was found to have 30% amino acid identity with ArmA, which led to the belief that RmtA was also a N7-G1405 16S RMTase [127].

To date, ten 16S RMTase genes have been identified, including *armA*, *rmtA-rmtH* and *npmA* as well as several subvariants (Table 1.5). All genes are believed to encode N7-G1405 16S RMTases apart from *npmA*, which encodes the sole N1-A1408 16S RMTase identified in Gram-negative bacteria. Analysis of the G+C content of 16S RMTase genes in Gram-negative bacteria, which is between 30-55%, indicates that they may not have originated from

actinomycetes as the latter have a G+C content around 60% or above; therefore, their origin is currently unknown [127] (Figure 1.11).

1.3.4 Discovery of 16S RMTase genes in Gram-positive bacteria

The expression of the 16S RMTase genes *armA*, *rmtA*-*rmtH* and *npmA* appeared to be limited to Gram-negative bacteria as, until recently, there had been no reports of 16S RMTase genes in Gram-positive bacteria [127,132]. However, *npmA*, and the variant *npmA2*, were discovered in *Clostridioides difficile* clinical isolates from the USA in 2018 [133]. Before this discovery, it was shown by Wachino *et al.* [132] that the expression of *rmtC* in Gram-positive bacteria is possible, but is dependent on the use of optimal promoters that are compatible with Gram-positive bacteria for gene expression. The expression of 16S RMTase genes in Gram-positive bacteria especially *C. difficile,* which is known for causing nosocomial outbreaks [134], being highly infectious and able to produce spores that can live for months outside of the human body [134], means that it has the possibility of spreading 16S RMTase genes to other species of bacteria in patients. This spread could be facilitated via horizontal transfer as recombinases were found associated with *npmA* in *C. difficile* strain CD7814 [133].

Table 1.5: First isolation of 16S RMTase genes *armA*, *rmtA-rmtH* and *npmA*.

Gene	Year isolated	Species	Clinical specimen or animal	Specimen type	Country	GenBank number	Publication
armA	1996	*C. freundii*	Clinical	Unknown	Poland	AF550415	[129]
armA2	Unknown	*A. baumannii*	Clinical	Blood	Myanmar	LC433766	Tada *et al*. (unpublished)
rmtA	1997	*P. aeruginosa*	Clinical	Sputum	Japan	AB083212	[131]
rmtB	2002	*S. marcescens*	Clinical	Sputum	Japan	AB103506	[135]
rmtB2	2006	*Enterobacter cloacae* complex	Clinical	Blood	Mexico	JN968578	Deshpande *et al.* (unpublished)
rmtB3	2005	*E. coli*	Clinical	Blood	USA	JN968579	Deshpande *et al.* (unpublished)
rmtB4	2013	*Proteus mirabilis*	Clinical	Urine	Unknown	KM999534	[136]
rmtC	2003	*P. mirabilis*	Clinical	Throat swab	Japan	AB194779	[137]
rmtD	2005	*P. aeruginosa*	Clinical	Urine	Brazil	DQ914960	[138]
rmtD2	2007	*Klebsiella aerogenes*	Clinical	Unknown	Argentina	HQ401565	[139]
rmtD3	2014	*P. aeruginosa*	Clinical	Sputum	Myanmar	LC229698	[140]
rmtE	Unknown	*E. coli*	Animal	Cattle	USA	GU201947	[141]
rmtE2	Unknown	*E. coli*	Animal	Swine	China	KT428293	[142]
rmtF	2011	*K. pneumoniae*	Clinical	Unknown	La Réunion Island	JQ808129	[143]
rmtF2	Unknown	*P. aeruginosa*	Clinical	Unknown	Nepal	LC050387	[144]
rmtG	2010	*K. pneumoniae*	Clinical	Bone	Brazil	JX486113	[145]
rmtH	2009	*K. pneumoniae*	Clinical	Wound	Iraq	KC544262	[146]
npmA	2003	*E. coli*	Clinical	Urine	Japan	AB261016	[147]
npmA2	Unknown	*C. difficile*	Clinical	Unknown	USA	-	[133]

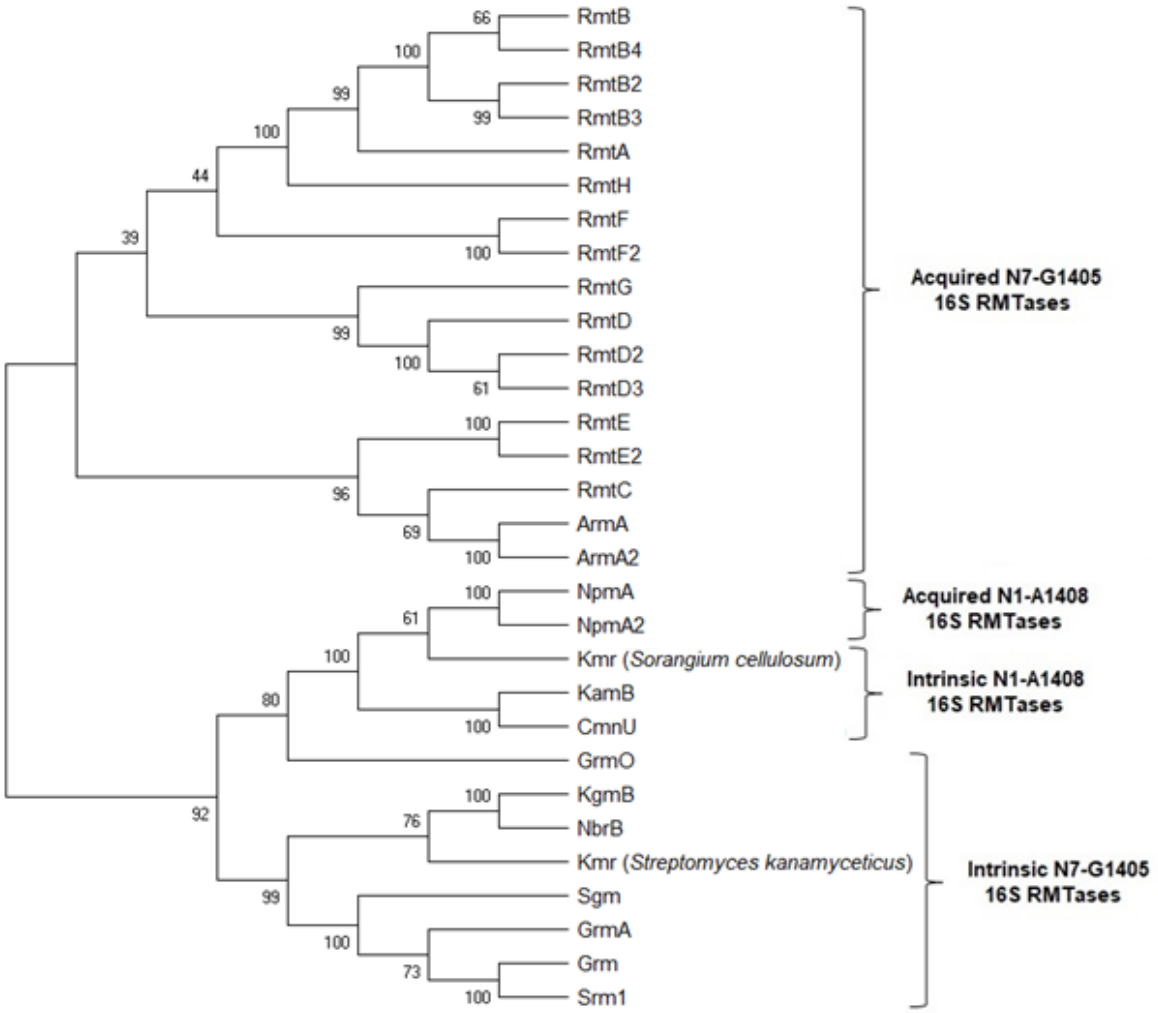


Figure 1.11: Neighbour-joining tree of both acquired and intrinsic 16S RMTases constructed using MEGA X version 10.0.05 [148] using protein sequences acquired from GenBank. This includes: ArmA (accession number: ABG33841.1); ArmA2 (accession number: BBH42896.1); CmnU (accession number: ABR67761); Grm (accession number: AAA25338); GrmA (accession number: AAR98546); GrmO (accession number: AAR98541); KgmB (accession number: AAB20100); KamB (accession number: 3MQ2_A); Kmr from *S. cellulosum* (accession number: ACB88605); Kmr from *S. kanamyceticus* (accession number: BAD20767); NbrB (accession number: AAB95477); Sgm (accession number: 3LCU_A); Srm1 (accession number: AAV28394); RmtA (accession number: BAD12551); RmtB (accession number: BAC81971); RmtB2 (accession number: WP_063854854.1); RmtB3: (accession number: WP_048266647.1); RmtB4: (accession number: BAR72202.1); RmtC (accession number: BAE48305); RmtD (accession number: ABJ53409); RmtD2 (accession number: ADW66545); RmtD3 (accession number: BAX24564.1); RmtE (accession number: ADA63498); RmtE2 (accession number: WP_063866490.1); RmtF (accession number: AGC82133.1); RmtF2 (accession number: BAR64666.1); RmtG: (accession number: AGE00988.1); RmtH: (accession number: AGH19769.1); NpmA (accession number: BAF80809) and NpmA2 (accession number: WP_070112004.1). Branch values represent results of a bootstrap analysis with 1000 iterations.

1.3.5 Worldwide distribution of 16S RMTases

1.3.5.1 *armA*

armA is one of the most widely distributed 16S RMTase genes around the world (Figure 1.12) as it has been found in Europe, Africa, Asia, North America, South America, the Middle East and Oceania [127,149]. *armA* has been identified in a wide range of Gram-negative bacteria including *A. baumannii* [125] (particularly isolates belonging to International Clone II) [150,151], Enterobacterales [125,130,152-156], and *P. aeruginosa* [157]. It has also been identified in species such as *Salmonella* spp. [158-164] and *Shigella flexneri* [165], which are implicated in food-borne diseases. Moreover, *armA* has been identified in bacteria isolated from animals, particularly in China where it has been found in *E. coli* isolates collected from poultry [166-168] and swine [169,170]. Additionally, *armA* has been identified in *E. coli* from farm animals in South Korea [171], *K. pneumoniae* in companion animals from China [172] as well as *Salmonella enterica* serovar California and *S. enterica* serovar Indiana isolates from poultry in China [173]. The variant *armA2* was identified in an *A. baumannii* isolate from Myanmar by Tada *et al*. (accession number: LC433766).

1.3.5.2 *rmtA*

rmtA was initially thought to be restricted to Asia [127,149] as it was mostly identified in *P. aeruginosa* in Japan [131,174-176] as well as *E. coli* [177], *K. pneumoniae* [177] and *P. aeruginosa* [178] in South Korea. However, it was later identified in India in *A. baumannii* [179] and *E. coli* isolates [180] as well as *P. aeruginosa* in Iraq [181] and *K. pneumoniae* in Switzerland [182].

Symbol	16S RMTase
▼	ArmA
▼	ArmA2
▼	RmtA
▼	RmtB
▼	RmtB2
▼	RmtB3
▼	RmtB4
▼	RmtC
▼	RmtD
▼	RmtD2
▼	RmtD3
▼	RmtE
▼	RmtE2
▼	RmtF
▼	RmtF2
▼	RmtG
▼	RmtH
▼	NpmA
▼	NpmA2

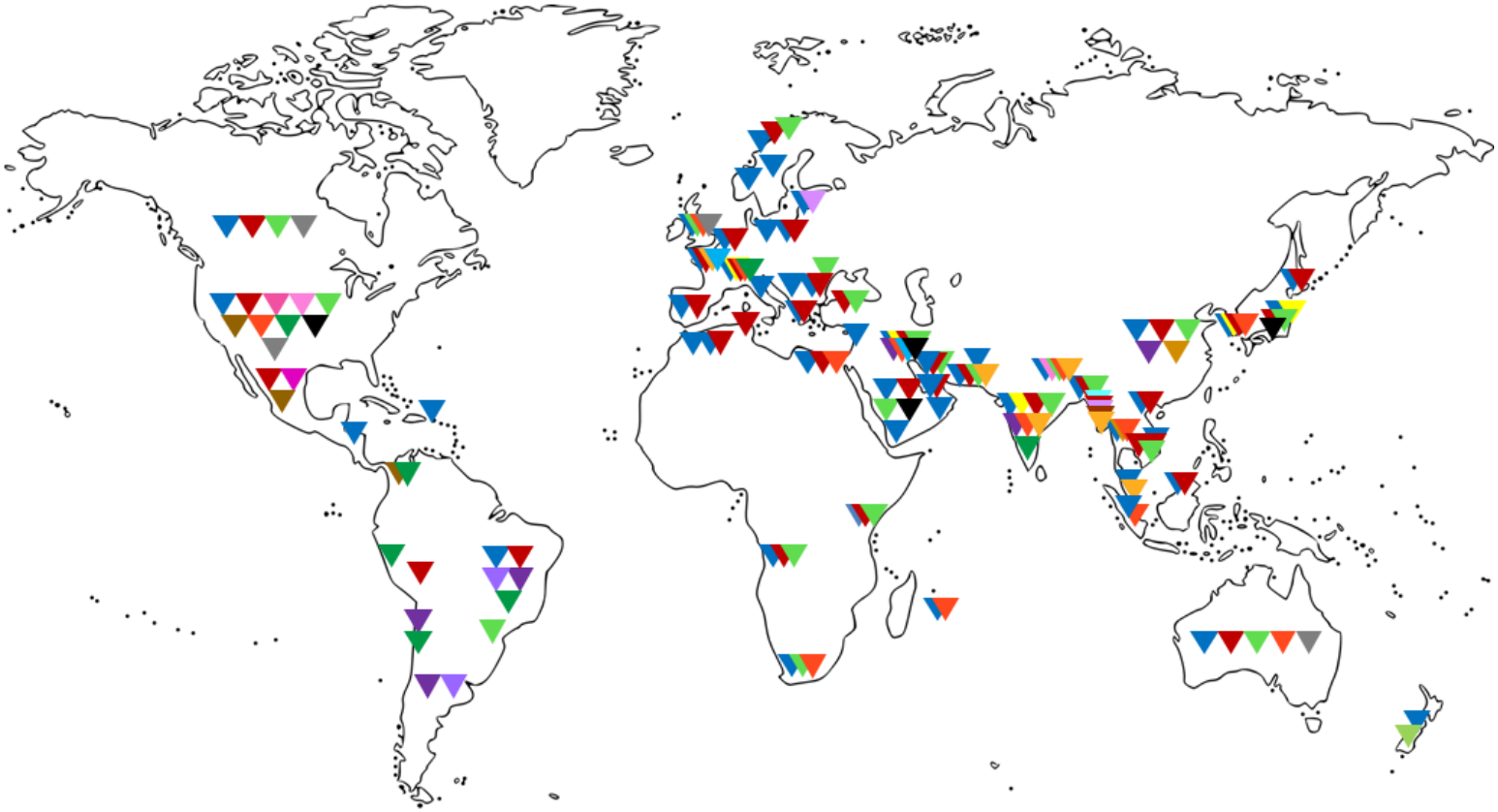


Figure 1.12: The worldwide distribution of 16S RMTases. The legend signifies the different 16S RMTases via coloured triangles. This figure uses data from references 119, 129-131, 133, 135, 137-147, 152-155, 159, 163, 164, 173, 174, 177, 179, 181-296.

1.3.5.3 *rmtB*

Like *armA, rmtB* is one of the most widely distributed 16S RMTase genes worldwide as it has also been found in Europe, Africa, Asia, North America, South America, the Middle East and Oceania [127,149]. *rmtB* has also been identified in a range of Gram-negative bacteria including *A. baumannii* [183], Enterobacterales [154,184-187,266,268] as well as *P. aeruginosa* [269] but appears to be more closely associated with *E. coli* compared with other species, particularly when isolated from animals [149,270,271]. Castanheira *et al*. [188] reported variants *rmtB2* and *rmtB3*, where two *rmtB2*-positive *E. cloacae* complex isolates were isolated from Mexico and one *E. coli* isolate from the USA as well as one *E. cloacae* complex and one *K. pneumoniae* isolate from Mexico harboured *rmtB3*. The variant *rmtB4* has been found in two *P. aeruginosa* isolates from Nepal [144] as well as single *Enterobacter hormaechei* (accession number: CP030347.1) and *P. mirabilis* (accession number: KM999534.1) isolates from the USA.

1.3.5.4 *rmtC*

Like *armA* and *rmtB*, *rmtC* has been identified in Europe, Africa, Asia, North America, South America, the Middle East and Oceania [185,189-194,272,273]. *rmtC* has mainly been identified in Enterobacterales, particularly *K. pneumoniae* clinical isolates [180,184,185,189,190,195-197,273-276] but has also been identified in foodborne pathogens such as *Salmonella* spp. [163,173,200,201]. Additionally, *rmtC* has been identified in *A. baumannii* from India [202] and Uruguay [203] as well as *P. aeruginosa* from India [204,277] and Romania [278].

1.3.5.5 *rmtD*

rmtD has mainly been identified in bacterial isolates from South America, where it has mostly been identified in *K. pneumoniae* [139,145,154,279,280] and *P. aeruginosa* [138,281-285] as well as *Aeromonas hydrophila* [287], *C. freundii* [154], *E. cloacae* complex [154] and *E. coli* [288]. Additionally, *rmtD* has been identified in *A. baumannii* [179] and *E. coli* [180] in India as well as *P. aeruginosa* in Iraq [181]. The subvariant *rmtD2* has been reported less frequently

and has been identified in various Enterobacterales isolates in Argentina [139] as well as *K. pneumoniae* in Brazil [279] and *rmtD3* was initially identified in *P. aeruginosa* in Myanmar [140] but has also been identified in a single *P. aeruginosa* isolate from Poland [275].

1.3.5.6 *rmtE*

Unlike other 16S RMTase genes, *rmtE* has been reported sporadically. It was first identified in multiple *E. coli* isolates in the USA [141,289,290] and was later identified in *A. baumannii* from Mexico (accession number: NZ_ULHD01000074.1), *E. hormaechei* from Thailand (accession number: NZ_NPZP01000120.1) and *K. pneumoniae* from Colombia (accession number: NZ_NCOO01000022.1). It has also been found in combination with *rmtB* in multiple *P. aeruginosa* isolates from Myanmar [296]. The subvariant *rmtE2* has only been reported in two *E. coli* isolates in China [142].

1.3.5.7 *rmtF*

rmtF has mainly been identified in *K. pneumoniae* from countries such as Australia [185], Egypt [205], India [185,206,207], Iraq [196], La Réunion Island [143], Nepal [197], Singapore [208], South Korea [209], Switzerland [210], UK [206] and the USA [211]. *rmtF* has also been identified in other Enterobacterales including *E. cloacae* complex from Australia [185]; *C. freundii* [206,207], *Citrobacter koseri* [207], *E. cloacae* complex [206], *E. coli* [206,291] and *P. mirabilis* [207] from India as well as *E. cloacae* complex from South Africa [212]. Additionally, *rmtF* has been found in *P. aeruginosa* from India [204,277] as well as the subvariant *rmtF2*, which was identified in *P. aeruginosa* isolates from France (accession number: SISM01000089.1), India (accession number: JTZO01000031.1), Nepal [144] and Pakistan (accession number: RHSZ01000084.1). *rmtF2* has also been identified in a *K. pneumoniae* isolate from Singapore [208].

1.3.5.8 *rmtG*

Like *rmtD*, *rmtG* has mainly been reported in South America particularly in *K. pneumoniae* [145,292,293] but has also been identified in *E. hormaechei* (accession number: MH325468.1) and *P. aeruginosa* [294,295]. Additionally, *rmtG* has been identified in *E. coli* and *K.*

pneumoniae isolates from India [207], as well as single *K. pneumoniae* isolates from Switzerland [210] and the USA [213].

1.3.5.9 *rmtH*

rmtH is apparently the rarest of the 16S RMTase genes as it has only been identified in single *K. pneumoniae* isolates from Iraq [146] and Lebanon [214].

1.3.5.10 *npmA*

npmA was first identified in a single *E. coli* isolate from Japan in 2003 [147] but was not reported again until after 2014, where *npmA* has been found in multiple *Enterobacter* spp. and *K. pneumoniae* isolates from Saudi Arabia [189], multiple *P. aeruginosa* isolates from Iraq [181], a single *C. difficile* isolate from the USA [133] and multiple *E. coli* isolates from Iran, where *npmA* was found alone or in combination with *armA* [254]. *npmA2* was discovered in *C. difficile* clinical and veterinary isolates from Australia, Canada, the UK and the USA [133].

1.3.6 Prevalence rates of 16S RMTases

Several studies have been conducted around the world to calculate the prevalence of 16S RMTases in their respective countries. 16S RMTases appear to be found at higher rates in the Far East and Middle East compared with Europe; rates of 2.6% (2/78) were found in extended-spectrum β-lactamase (ESBL)-producing Enterobacterales from Japan [297] compared with 1.3% (5/373) of ESBL-producing Enterobacterales from France [215]. Comparison of nonduplicate Enterobacterales clinical isolates found 16S RMTase genes in 46.3% (57/123), 6.8% (21/307), 1.5% (20/1,310) and 1.1% (20/1,770) of isolates from India [180], Iran [298], Bulgaria [299] and Poland [300], respectively. Finally, rates of 16S RMTases in MDR *A. baumannii* isolates were found to be 88.9% (48/54) in India [202], 76.2% (32/42) in South Korea [301] and 32.9% (23/70) in Bulgaria [216]. Prior to the start of this project there had been no surveillance conducted in the UK so the prevalence rate of 16S RMTases in the UK was unknown.

As stated earlier, AMEs are the most common mechanism of aminoglycoside resistance and studies have proven that they occur more frequently than 16S RMTases. Castanheira *et al*. [302] identified that 18.5% (782/4,217) Enterobacterales collected from European and surrounding countries harboured AMEs compared with 1.4% (60/4,217) isolates producing 16S RMTases. Costello *et al*. [136] identified 72.0% (144/200) Gram-negative isolates from North American, Latin American and European hospitals participating in the SENTRY Antimicrobial Surveillance Program produced AMEs compared with 10.5% (21/200) isolates producing 16S RMTases. Finally, Galani *et al*. [303] identified 85.3% (256/300) carbapenemase-producing *K. pneumoniae* from Greek hospitals produced AMEs compared with 7.7% (23/300) isolates producing 16S RMTases. This shows that the prevalence of 16S RMTase genes is relatively low in comparison to AMEs and that they do not currently pose a significant threat to the use of aminoglycosides but there are several factors that make their current emergence worrying.

1.3.7 The threat of 16S RMTase genes to public health

The emergence of 16S RMTases is concerning as they confer high-level pan-aminoglycoside resistance (MIC >256 mg/L) with only a single gene compared with other aminoglycoside resistance mechanisms that need several genes to achieve the same level of resistance [127,304]. Additionally, they appear to be associated with other antibiotic resistance genes (see Section 1.3.7.1); are found on MGEs (see Section 1.3.7.2); appear to have several reservoirs that could contribute to their spread in the community setting and across the globe (see Sections 1.3.7.3 to 1.3.7.4) and can cause resistance to the newly developed aminoglycoside called plazomicin (see Section 1.3.7.5).

1.3.7.1 Association with other antibiotic resistance genes

Over the years 16S RMTase genes have been found associated with other antibiotic resistance genes in MDR bacteria. Carbapenemase genes (which encode enzymes that cause resistance to carbapenems and the majority of cephalosporins and penicillins [305]) appear to be highly associated with 16S RMTase genes, particularly bla_{NDM}, which has been

found associated with *armA* [217,306,307], *rmtA* [182], *rmtB* [144,218,308], *rmtC* [194,218,277] and *rmtF* [206,277], whereas *rmtD* appears to be associated with bla_{SPM-1} (specifically in *P. aeruginosa* ST277 isolates) [138,206,282,309] but also has been reported in bla_{KPC-2}-positive isolates (as well as *rmtG*) [145].

16S RMTase genes have also been found associated with ESBL genes, which encode enzymes that confer resistance to β-lactams including penicillins, cephalosporins and monobactams but not carbapenems or cephamycins [310]. The most common gene they are found associated with is $bla_{CTX-M-15}$, which has been identified with *armA*, *rmtB*, *rmtC*, *rmtD*, *rmtF*, *rmtG*, *rmtH* [145,205,219,220,277,311] but has not yet been found associated with *rmtE* or *npmA*.

16S RMTase genes, particularly *armA* and *rmtB*, have also been found associated with plasmid-mediated quinolone resistance (PMQR) genes such as *qnrB*, *qnrS* as well as *aac(6')-Ib-cr* [156,162,170,312-319], and *rmtB* has also been found closely associated with the quinolone efflux pump gene *qepA* [169,312,314,320-322].

The association of 16S RMTase genes with carbapenemase, ESBL and PMQR genes means that treatment options for Gram-negative infections will be limited as aminoglycosides, β-lactams and fluoroquinolones are three of the main antibiotic classes used to treat Gram-negative bacterial infections [58,59].

1.3.7.2 Association with MGEs

One troubling characteristic of 16S RMTase genes is that they are associated with MGEs, which consist of transposable elements and plasmids, as these facilitate their spread to other bacteria through horizontal transfer.

1.3.7.2.1 Transposable elements

Transposable elements are genetic structures that can move within and between genomes through a process known as transposition [323,324]. During this process, a transposase encoded by the transposable element cleaves phosphodiester bonds within the DNA, which

releases the transposable element so it can insert itself into DNA elsewhere [324-326]. The 3'-OH groups on the ends of the released transposable element then serve as nucleophiles, which attack 5'-phosphate groups in the target DNA to enable insertion [324,326]. Gaps adjacent to the transposable element are then filled by DNA repair systems, generating the repeats that flank the transposable element and enable transposition [324].

There are several types of transposable elements including insertion sequence (IS) elements, transposons, integrons and IS*CR* elements. All have been found associated with 16S RMTase genes, enabling their spread between plasmids and chromosomes as well as between bacteria.

1.3.7.2.1.1 IS elements

IS elements are the smallest and simplest transposable elements. They range from 0.5-2 kb in size and only encode genes involved in transposition [324,327,328]. IS elements only have a single gene encoding a transposase as well as short-terminal inverted repeats (~15-40 bp), which act as binding sites for the transposase [324,326,328].

1.3.7.2.1.2 Transposons

Transposons differ from IS elements as they encode genes for transposition as well as for other functions such as antibiotic resistance [324,329]. There are two types of transposons: composite transposons and complex transposons.

Composite transposons have a modular structure, where identical IS elements flank a region containing genes encoding a function such as antibiotic resistance but lack genes involved in transposition [324,325,330]. Instead, genes involved in transposition are provided by the IS elements, which also act as recognition sites for the transposase [324]. The 16S RMTase gene *npmA* has previously been identified in a composite transposon as it was flanked by two IS*26* elements (Figure 1.13) [147].

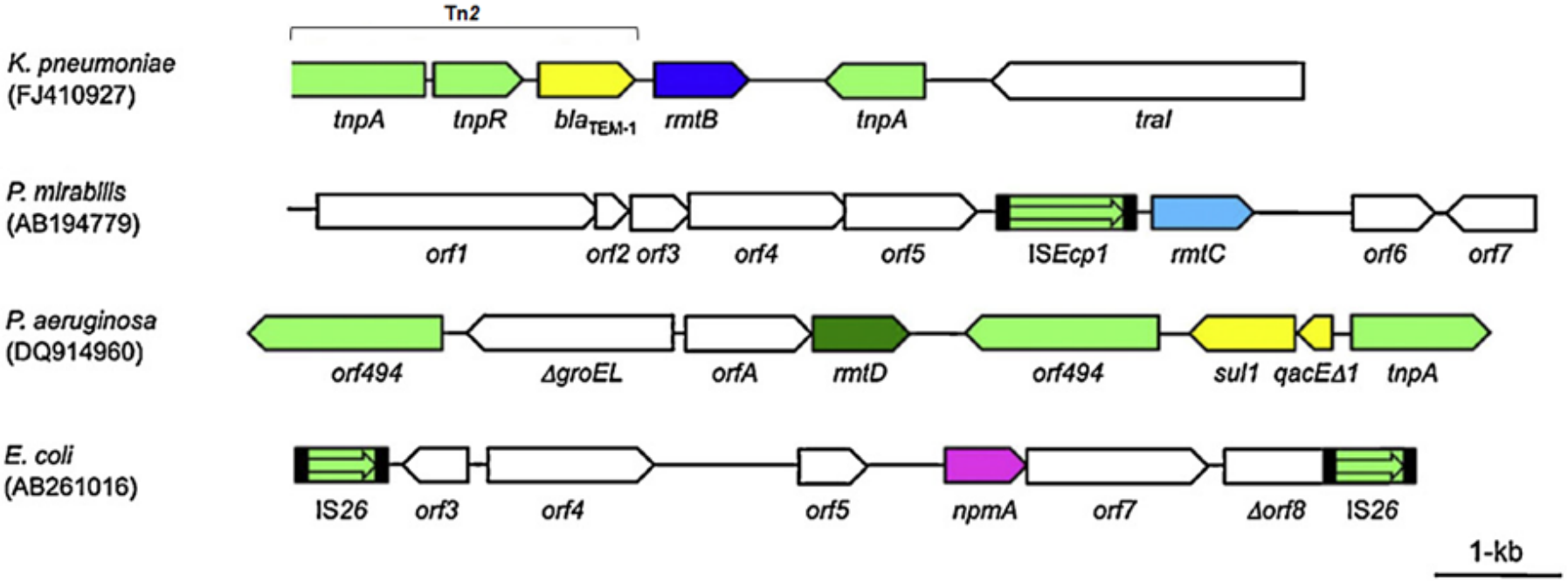


Figure 1.13: Association of Tn*2* with *rmtB*, IS*Ecp1* with *rmtC*, IS*CR14* (orf494) with *rmtD* and IS*26* with *npmA*. Light green arrows represent MGEs; yellow arrows represent antibiotic resistance genes other than 16S RMTase genes; the dark blue arrow represents *rmtB*; the light blue arrow represents *rmtC*; the dark green arrow represents *rmtD* and the pink arrow represents *npmA*. Reproduced from Wachino *et al.* [127] with permission from Elsevier.

Complex transposons contain genes that are involved in both transposition and function (such as antibiotic resistance) but are flanked by short inverted-terminal repeats (~15-40 bp) [324,325]. Tn*3* is one of most common complex transposons identified in Gram-negative bacteria, particularly Enterobacterales and *P. aeruginosa* [324,331], and is frequently associated with the 16S RMTase gene *rmtB* (Figure 1.13) [135,169,221,315,332]. However, Tn*3* associated with *rmtB* was previously misclassified and has now been reclassified to Tn*2* [333].

1.3.7.2.1.3 Integrons

Bacterial integrons can capture short DNA sequences (~1 kb), known as gene cassettes using an integrase, encoded by the gene *int* [324,330,334]. The integrase inserts the gene cassettes, which can encode antibiotic resistance genes, into an insertion site known as *attI* [324,330]. Gene cassettes are inserted into the *attI* site by the integrase to form a continuous array of genes, where the most recent gene cassette is nearest to the *int* gene [324,330,334].

Class 1 integrons are integrons with a specific structure and are commonly associated with antibiotic resistance genes. They consist of two conserved segments (CS), known as 3'-CS and 5'-CS, which flank the region containing the gene cassettes [324,335,336]. The 5'-CS consists of the *int* gene as well as the insertion site *attI* and provides a promotor for the expression of the gene cassettes [324,336]. The 3'-CS consists of the truncated gene *qacEΔ1*, which confers resistance to quaternary ammonium compounds, and *sul1*, which confers sulphonamide resistance along with two other genes known as *orf5* and *orf6* [324,336].

Complex class 1 integrons encompass an IS*CR* element at the 3'-CS, such as IS*CR1*, which is associated with another variable region encoding antibiotic resistance genes that are separate from the region containing the gene cassettes [324]. The 16S RMTase gene *armA* as well as the macrolide resistance genes *mphE* and *msrE* are found within this variable region in the complex class 1 integron found in Tn*1548* [153,162,163,165] (Figure 1.14).

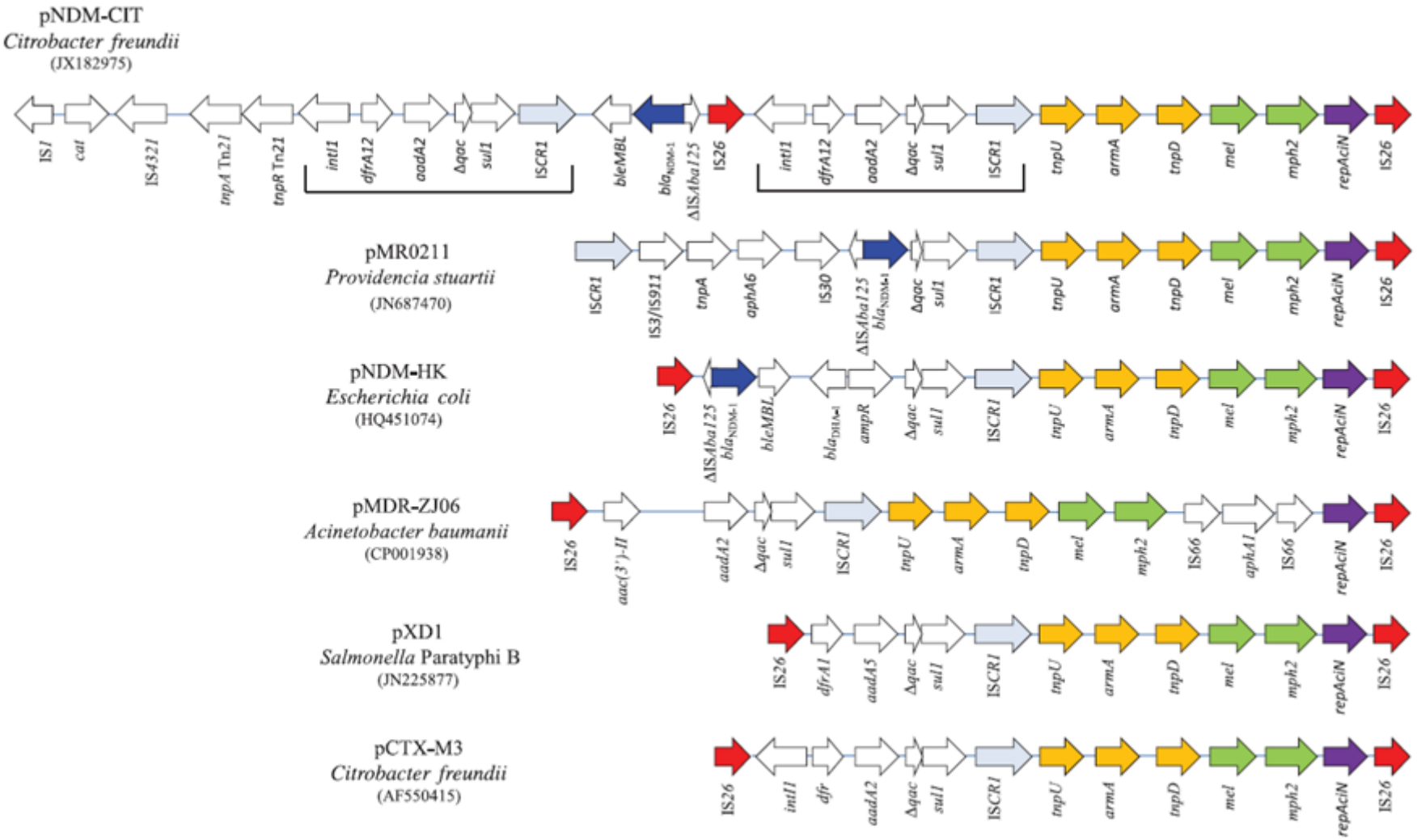


Figure 1.14: Comparison of Tn*1548* associated with *armA* in plasmids pNDM-CIT, pMR0211, pNDM-HK, pMDR-ZJ06, pXD1 and pCTX-M-3. Tn*1548* is represented by orange arrows; bla_{NDM-1} is designated by blue arrows; *mel* (also known as *msrE*) and *mph2* (also known as *mphE*) are designated by green arrows; IS*CR1* and IS*26* are designated by grey and red arrows, respectively and the purple arrows represent the *repAciN* gene. Reproduced from Dolejska *et al.* [306] by permission of Oxford University Press.

1.3.7.2.1.4 IS*CR* elements

Unlike other transposable elements, IS*CR* elements transpose by a process called rolling-circle replication. They have distinct terminal repeats known as *ori*IS and *ter*IS, where rolling-circle replication is initiated at *ori*IS and terminated at *ter*IS [324,330,336]. During rolling-circle replication a single strand of the IS*CR* element is nicked at the 5'-end near *ori*IS and becomes covalently bonded to the transposase [337]. A new copy of the IS*CR* element is generated using the intact DNA strand as a template by a replication complex, which displaces the nicked strand while replication takes place, forming a single-stranded loop of DNA [337]. Once *ter*IS is reached the single-stranded loop is displaced, with the transposase still bound at the 5'-end. The free 3'-OH group at the 3'-end of the single strand can be used to as a nucleophile to attack a target DNA site for insertion [337]. This generates a new 3'-OH group in the target DNA, which attacks the 5'-end of the IS*CR* element attached to the transposase, leading to insertion of the IS*CR* element [337].

However, around 1.0% to 10.0% of the time, *ter*IS is not recognised so replication continues on downstream of *ter*IS until it is terminated at another *ter*IS [324,330,334]. This means that genes such as antibiotic resistance genes that are adjacent to *ter*IS can be mobilised as part of the IS*CR* element due to the misreading of *ter*IS and is thought to be the reason behind the generation of complex class 1 integrons [324,334].

Several 16S RMTase genes have been found associated with IS*CR* elements including IS*CR14* (*rmtD*), which was originally named *orf494* (Figure 1.13) [338] and IS*CR2* (*rmtH*) (Figure 1.15) [214]. Additionally, *rmtC* has been found associated with IS*Ecp1* (Figure 1.13) [201,311,339], which is similar to IS*CR* elements due to transposing by rolling-circle replication and mobilising adjacent genes through failed termination [330]. However, IS*Ecp1* has less mobilising ability than IS*CR* elements as the size of DNA that can be mobilised by IS*Ecp1* appears to be limited [334].

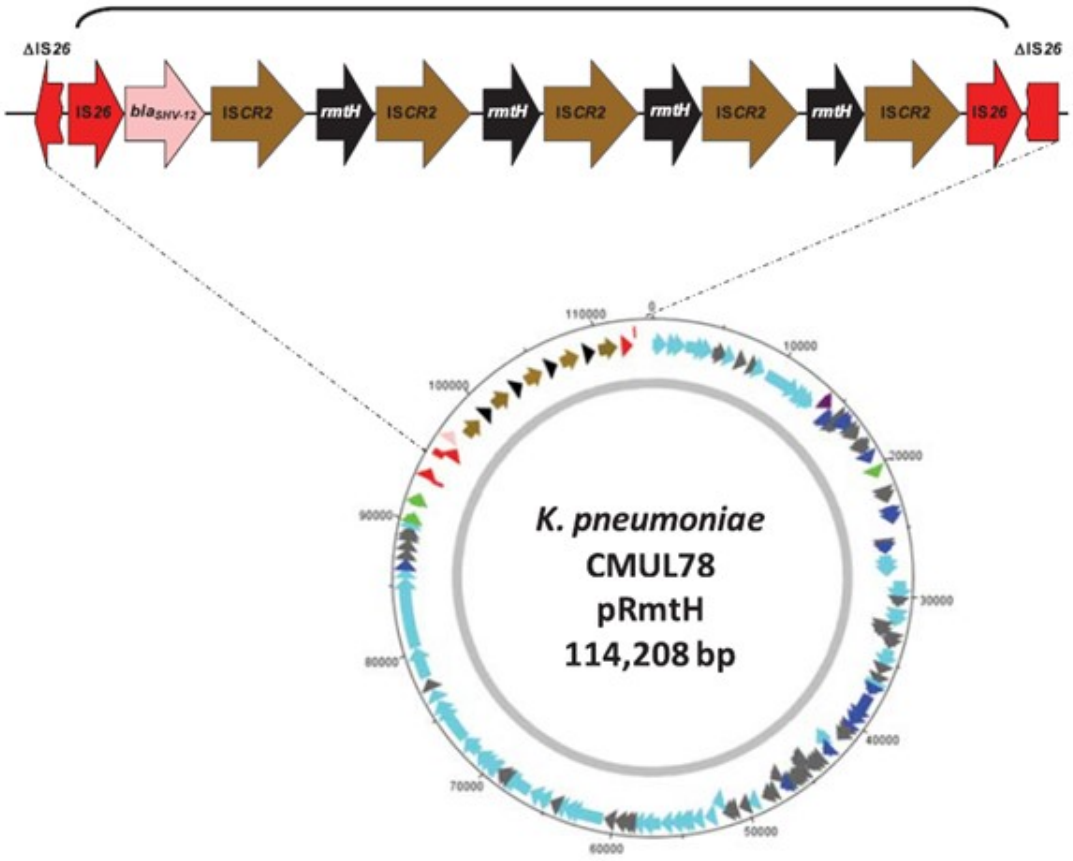


Figure 1.15: Association of *rmtH* with IS*CR2* found in Tn*6329* found on plasmid pRmtH. Reproduced from Beyrouthy *et al.* [214] by permission of Oxford University Press.

1.3.7.2.1.5 Association of MGEs with 16S RMTase genes in the UK

In the UK, there have only been three studies that have investigated the genetic environment of 16S RMTase genes. Hopkins *et al*. [200] identified IS*Ecp1* was associated with *rmtC* in *S. enterica* serovar Virchow but the 5' end of IS*Ecp1* could not be amplified indicating that a partially deleted IS*Ecp1* element or a variant of the IS*Ecp1* element was associated with *rmtC*. Hidalgo *et al*. [164] found that *armA* in *S*. enterica serovar Thompson isolate and *S. enterica* serovar Worthington isolates (which were from Public Health England's (PHE) Laboratory of Gastrointestinal Pathogens culture collection) was associated with truncated Tn*1548* transposons. The Tn*1548* transposon from *S*. enterica serovar Thompson was missing the variable region downstream of IS*CR1* containing *mphE* and *msrE* genes and the other (from *S. enterica* serovar Worthington) was missing *qacE*Δ*1* and the gene cassettes as part of the class 1 integron. Finally, Hidalgo *et al*. [206] found IS*91* was located downsteam of *rmtF* in *K. pneumoniae*. Further investigation into the association of MGEs with 16S RMTase genes in the UK needs to be performed to prove that these MGEs are commonly identified with these 16S RMTases genes and to see if they are involved in the emergence of 16S RMTase genes within the UK.

1.3.7.2.2 Plasmids

1.3.7.2.2.1 Plasmids

Plasmids are small, circularised segments of double-stranded DNA that are separate from bacterial chromosomes and can replicate independently [341,342]. Plasmids can contain virulence factors and/or antibiotic resistance genes, which can improve the ability of the bacteria to survive. They can spread between bacteria through horizontal gene transfer, which occurs through a process called conjugation. During conjugation, an extracellular appendage, known as a sex pilus, connects a donor bacterial cell (which contains the plasmid) to a recipient bacterial cell (which lacks the plasmid) so a copy of the plasmid can be transferred into the recipient [341]. However, some plasmids known as 'mobilizable' plasmids are not

conjugative as they lack the genes necessary for conjugation but are able to be transferred alongside conjugative plasmids into bacterial cells [343].

The stability of plasmids within the recipient cells following conjugation has been used as a basis for the classification of plasmids known as plasmid incompatibility [344]. Plasmid incompatibility is based on the discovery by Datta and Hedges [345], where they discovered that plasmids sharing the same replication controls are unable to be stably maintained within a bacterial cell. Therefore, those sharing the same controls are 'incompatible' and those with different controls are 'compatible', which led to plasmids being designated incompatibility (Inc) groups such as IncF [342]. Plasmids are also classified on their ability to replicate in a range of bacterial species, which is known as the plasmid host range [346]. Plasmids are classified as having either a broad-host range or narrow-host range. Broad-host range plasmids were originally classified by Datta and Hedges [347] based on their ability to be transferred among Enterobacterales and *Pseudomonas* spp. Now broad-host-range plasmids are based on their ability to spread and be maintained in bacteria from various phylogenetic subgroups, whereas narrow-host-range plasmids are restricted to certain species [346,348]. Plasmids belonging to Inc groups such as IncP and IncQ have been classed as having a broad-host range and those belonging to IncF and IncX (which occur in Enterobacterales) have been classed as having a narrow-host range [341,346].

16S RMTase genes have been identified on broad-host range plasmids such as IncA/C [213,349], IncL/M [130,153,161,222] and IncN [145,223,266,277,350] plasmids as well as narrow-host range plasmids such as IncF plasmids [153,162,169,214,224,266,351]. Most of these plasmids carry other antibiotic resistance genes meaning their spread will promote multidrug resistance and will lead to the co-selection of 16S RMTase genes based on the use of antibiotics other than aminoglycosides. Worryingly, *armA* was found on an IncP plasmid [159], which has a broad-host range including Gram-positive bacteria meaning that 16S RMTase genes could spread to more species of Gram-positive bacteria in the future, limiting treatment options.

1.3.7.2.2.2 Plasmids associated with 16S RMTase genes in the UK

There have been few studies in the UK that have investigated plasmids harbouring 16S RMTase genes. Hornsey *et al*. [352] identified an IncF plasmid that harboured *rmtB*, *bla*$_{NDM-5}$, *aadA5* (conferring spectinomycin resistance) and *dfrA17* (conferring trimethoprim resistance) in an *E. coli* isolate recovered from a patient transferred to the UK from India. Hidalgo *et al*. [164] identified two plasmids that harboured *armA* called pB1015 and pB1016, which were isolated from *S. enterica* serovar Thompson and *S. enterica* serovar Worthington isolates, respectively. pB1015 belonged to IncHI2 and pB1016 belonged to IncA/C but the latter was also found to harbour the ESBL *bla*$_{VEB-5}$ and *bla*$_{CMY-2}$ (conferring cephalosporin resistance). Additionally, Hidalgo *et al*. [206] identified untypeable plasmids harbouring *rmtF* and *bla*$_{NDM}$ from *K. pneumoniae* isolates from the UK. Finally, Turton *et al*. [353] identified the plasmid KpvST147L (accession number: CM007852.1), which was isolated from a *K. pneumoniae* isolate. This plasmid harboured *armA* as well as *dfrA5* (conferring trimethoprim resistance), *mphA* (conferring macrolide resistance), *aph(3')-VIb* (conferring aminoglycoside resistance), *sul1, sul2* (conferring sulphonamide resistance) and genes encoding a range of virulence proteins. The association of *armA* with a plasmid encoding multiple antibiotic resistance genes and virulence genes is concerning as MDR bacteria harbouring this plasmid would have increased virulence and the possibility of being untreatable. Additional studies need to be conducted to determine how widespread these plasmids are as well as what other plasmids are circulating within the UK that may be contributing to the spread of 16S RMTase genes.

1.3.7.3 Identification in animals and the food chain

16S RMTase genes have also been identified in animals (including companion animals and livestock) and in bacteria from supermarket meat. The 16S RMTase genes *armA* and *rmtB* (but mostly *rmtB*) have been identified in farm animals such as poultry [162,166-168,173,354-359], swine [169,266,315,332] and cattle [360,361], particularly in China where the majority of such animal studies have been conducted [162,166-170,172,173,266,270,332,351,354-361].

The presence of 16S RMTase genes in bacteria from livestock poses a threat not just to the food chain but a threat to those in close proximity to farm animals, for example an 11-month old child became infected with an *armA*-producing *Salmonella enterica* serotype Stanley isolate after contact with farm animals in India [163] and *rmtB*-containing plasmids were found to have disseminated between farm workers and swine from a farm in China [169]. There have been several incidences of 16S RMTase genes identified in bacteria isolated from supermarket meat [159,187,200], particularly poultry [159,187], indicating that meat may be a potential source of 16S RMTase-positive bacteria in the community. Additionally, the identification of 16S RMTase genes in companion animals [172,351,358] means that they may act as a reservoir in the community setting too. However, it is unclear whether meat, livestock or companion animals acquired 16S RMTase genes independently or from humans. Therefore, continued surveillance needs to be carried out in animals (both companion and livestock) to help limit the spread of 16S RMTase genes to the human population and support the concept of 'One Health'. 'One Health' is a collaborative approach between government officials as well as healthcare professionals, such as clinicians, scientists and veterinarians, to achieve better public health outcomes in humans as well as animals, plants and the environment through research or legislation [362].

1.3.7.4 Identification in the environment

In addition to clinical samples and animals, 16S RMTase genes have also been found in the environment. Meta-genomic analysis of toilet wastewater from planes arriving in an international airport in Copenhagen, Denmark, identified the presence of *npmA* [363]. Additionally, analysis of wastewater from hospitals as well as treatment plant influent and effluent from sewage sites in Basel, Switzerland, identified *armA* and *rmtB* genes in *E. coli* isolates [225]. The presence of 16S RMTase genes in wastewater, particularly water that has been treated, is concerning as it indicates that wastewater is a potential reservoir for bacteria harbouring 16S RMTase genes (as well as other antibiotic resistance genes) and that additional treatment to sewage is needed to ensure the eradication of antibiotic-resistant

bacteria and to prevent the release of antibiotic-resistant bacteria to aquatic environments. *rmtD* was identified in a *P. aeruginosa* isolate collected from Tietê River in Brazil [282] indicating that aquatic environments already serve as reservoirs for 16S RMTase genes, and other antibiotic resistance genes, and have the potential to disseminate these genes throughout the community setting.

1.3.7.5 Resistance to plazomicin

Plazomicin is a new FDA-approved aminoglycoside, under the name Zemdri, to be used for the treatment of complicated urinary tract infections caused by Enterobacterales in the USA [65] but is currently (August 2019) unavailable in Europe as it has been filed for approval. It was developed by the pharmaceutical company Achaogen and was initially designed to be used against carbapenem-resistant Enterobacterales with the ability to resist modification by clinically-relevant AMEs including AAC(3), AAC(6′), ANT(2″) and APH(2″) [65,127,149]. However, it has minimal activity against *A. baumannii* and *P. aeruginosa* [364] due to upregulation of efflux pumps, changes in membrane permeability or porin loss in these species [365,366]. Therefore, it is not useful in treating carbapenem-resistant *A. baumannii* and *P. aeruginosa*, which are on WHO's priority list of antibiotic-resistant bacteria.

A study by Castanheira *et al*. [302] screened plazomicin against 4,217 Enterobacterales isolates collected from 45 hospitals between 2014 and 2015 from Europe as well as surrounding countries and found it inhibited 95.8% (4,040/4,217) isolates at the recommended breakpoint of ≤2 mg/L. The inhibition by plazomicin was higher than amikacin (94.1%), gentamicin (84.3%) and tobramycin (77.9%) when the EUCAST clinical breakpoints were applied. A total of 227 carbapenem-resistant Enterobacterales were identified, of which 84.6% (192/227) were inhibited by plazomicin at ≤2 mg/L. Additionally, in another study Castanheira *et al*. [367] found that plazomicin at ≤2 mg/L was able to inhibit 96.4% (4,207/4,362) Enterobacterales from 70 hospitals from the USA during 2014 to 2015. This included 97 carbapenem-resistant Enterobacterales, where plazomicin inhibited 99.0% (96/97) isolates.

However, Livermore *et al*. [368] showed that plazomicin is ineffective against Enterobacterales harbouring 16S RMTase genes, as 93.8% (15/16) NDM-producing Enterobacterales with MICs of ≥64 mg/L for plazomicin were identified to harbour 16S RMTase genes. As 16S RMTase genes appear to be closely associated with metallo-β-lactamase genes this means that the use of this drug will be restricted to bacteria harbouring ESBLs (e.g. CTX-M-15), serine carbapenemases (e.g. KPC), oxacillinases (e.g. OXA-48) or metallo-β-lactamase producers lacking 16S RMTase genes [369]. This was confirmed by Castanheira *et al*. [302], where out of 37 metallo-β-lactamase-producing Enterobacterales from European hospitals, plazomicin was only able to inhibit 40.5% (15/37) of the isolates as 95.5% (21/22) of the resistant isolates carried 16S RMTase genes. Therefore, a future generation of aminoglycosides needs to be produced that are not susceptible to 16S RMTases in order to help combat resistance.

1.3.8 Development of aminoglycosides resistant to 16S RMTases

As most 16S RMTases are unable to inhibit 4-5-disubstituted DOS aminoglycosides, researchers are modifying 4-5-disubtituted DOS aminoglycosides in order to improve their selectivity for the bacterial ribosome over the eukaryotic ribosome to reduce toxicity without affecting antibacterial potency. Examples include the 4′-O-ethyl modification of paramomycin as well as the 4′-O-ethyl modification and N6'-hydroxyethyl modification of neomycin [370,371].

Another promising candidate is a derivative of apramycin called TS3112, which is resistant to all 16S RMTases but NpmA [65,69]. It was developed by the Institute of Microbial Chemistry and was shown to have high efficacy against 16S RMTase-producing *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* meaning that it could be used in the future to target 16S RMTase-producing Gram-negative bacteria that are MDR [65,69].

An alternative solution to combating aminoglycoside resistance generated by 16S RMTases is the design of 16S RMTase inhibitors. Vinal *et al*. [372] discovered three regions within the methyltransferase core fold of NpmA that were important for binding to the bacterial 30S

ribosomal unit or the substrate SAM: the β2/β3 linker, the β5/β6 linker and the β6/β7 linker. The β2/β3 linker was found to be important in the ability of NpmA to bind to the 16S rRNA within the 30S ribosomal subunit through the use of four positively-charged lysine residues as their removal would lead to a complete loss of binding affinity and resistance. The β5/β6 linker and the β6/β7 linker were found to be involved in substrate recognition as their removal would result in loss of resistance due to the inability of NpmA to bind to SAM. Therefore, these three regions have the potential to be possible targets for future 16S RMTase inhibitors targeting N1-A1408 16S RMTases. However, their role in N7-G1405 16S RMTases needs to be investigated to see if these regions are involved in ribosome binding and substrate specificity so they can be targeted by future N7-G1405 16S RMTase inhibitors.

1.3.9 Other antibiotics in development that could target 16S RMTase-producing bacteria

Additionally, other new antibiotics are in the pipeline to target Gram-negative bacteria that are currently in phase III clinical trials that could possibly be used against MDR bacteria harbouring 16S RMTases. This includes sulopenem, which is a new carbapenem originally designed by Pfizer Japan that can be used against ESBL-producing Enterobacterales, but is not effective against *P. aeruginosa* [373,374]. Additionally, the siderophore cephalosporin called cefiderocol was developed by Shionogi & Co. Ltd and can be used against Enterobacterales, *A. baumannii* and *P. aeruginosa* isolates that produce ESBLs, and carbapenemases such as KPC, metallo-β-lactamases and OXA enzymes [375-377]. Furthermore, a combination of imipenem (a carbapenem), cilastatin (an inhibitor of the human enzyme renal dehydropeptidase, which degrades imipenem) and relebactam (a β-lactamase inhibitor) has been designed by Merck & Co. Inc. to be used against carbapenem-resistant Enterobacterales, and possibly carbapenem-resistant *P. aeruginosa* [377].

Unfortunately, this highlights that there are few newly developed antibiotics in the pipeline to treat Gram-negative bacteria, particularly carbapenem-resistant Enterobacterales, *A. baumannii* and *P. aeruginosa*, which pose the biggest threat in antibiotic resistance. Therefore,

future research is needed to help develop new antibiotics to target these bacteria and the distribution of currently available as well as newly designed antibiotics need to be controlled in order to prevent the development of further antibiotic resistance and the onset of the 'post-antibiotic' era.

1.4 Conclusion

In conclusion, the emergence of 16S RMTases is concerning as they cause high-level resistance, by gain of a single gene, to the most clinically-relevant aminoglycosides used to treat Gram-negative bacterial infections. Their association with MGEs and other antibiotic resistance genes, particularly carbapenemase genes, means their emergence will contribute to the generation of MDR bacteria with severely limited treatment options available. As the prevalence rate of 16S RMTase genes was unknown in the UK, this PhD project was set up to determine this as well as the reason behind their emergence.

1.5 Hypothesis

16S RMTases are emerging in the UK primarily due to an association with plasmids containing carbapenemase genes or 'high-risk' bacterial clones known to harbour carbapenemase genes. They remain uncommon in bacteria that lack carbapenemase genes.

1.6 Aims

The aims of this study were to: i) determine the prevalence of 16S RMTase genes in Gram-negative bacteria within the UK and, ii) determine if they are associated with particular plasmids that carry carbapenemase genes or 'high-risk' bacterial clones known to harbour carbapenemase genes.

Chapter 2: General materials and methods

2.1 Introduction

This chapter describes the bacterial isolates used throughout the study as well as the standard methods used to grow bacterial isolates; extract bacterial DNA; identify antibiotic resistance genes; type bacterial isolates and perform WGS. Additional methods used for specific studies are described separately in the respective chapters.

2.2 Bacterial isolates, growth conditions and storage

2.2.1 Bacterial isolates

Bacterial isolates used throughout this study were Gram-negative bacteria belonging to the family Enterobacterales or non-fermenting species *A. baumannii* or *P. aeruginosa*. Bacterial isolates used in Chapter 4 were from the Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, which is the national reference laboratory for the investigation of unusual antibiotic resistance in, and characterisation of, healthcare-associated infections. Most isolates are referred to AMRHAI by UK diagnostic laboratories for investigation of resistance to last-line antibiotics (primarily carbapenems). Isolates, from 2003-2015, were selected for inclusion in this study due to pan-aminoglycoside resistance (MICs amikacin >64 mg/L, gentamicin >32 mg/L and tobramycin >32 mg/L).

In Chapter 5, the WGS data used to analyse the presence of 16S RMTase genes was obtained from MDR bacteria collected for the Bacteraemia Programme of the British Society for Antimicrobial Chemotherapy (BSAC) Resistance Surveillance Project from 2001-2013. These isolates were consecutively sent from designated laboratories throughout the UK on a systematic annual basis to screen for antibiotic resistance. As multidrug resistance (classified as resistance to a minimum of three antibiotic classes) was the only selection criteria, this isolate collection was not biased towards carbapenem resistance unlike the isolate collection from the AMRHAI Reference Unit.

Bacterial isolates from Chapter 6 were collected from UK hospitals participating in the surveillance study of 16S RMTases within the UK, designed as part of this project, from 1st

May to 31st October 2016. These bacterial isolates displayed the highest level of amikacin resistance detectable during antibiotic susceptibility testing by the participating hospital laboratories, which included no zone of inhibition (30 µg amikacin discs) during disk diffusion, an amikacin gradient strip displaying a MIC >256 mg/L or the highest MIC range obtainable when using automated susceptibility testing methods.

Finally, Chapter 7 used WGS data obtained from bacterial isolates from the AMRHAI Reference Unit (see Chapter 4), the Bacteraemia Programme of the BSAC Resistance Surveillance Project (see Chapter 5) and the prospective surveillance study (see Chapter 6) to investigate the genetic environment of 16S RMTase genes and for the analysis of 16S RMTase gene-containing plasmids that were successfully obtained from these bacterial isolates in order to identify what plasmids are circulating in the UK.

2.2.2 Growth conditions

Bacterial isolates were cultured on cystine–lactose–electrolyte-deficient agar (CLED) agar and incubated overnight at 37 °C for 16-20 hours. Liquid cultures, when required, were produced by inoculating 10 ml of Luria-Bertani (LB) broth (Media Services, PHE Microbiology Services Colindale) with one bacterial colony and incubating at 37 °C overnight on a shaker for 16-20 hours.

2.2.3 Bacterial glycerol stocks and storage

Glycerol stocks of bacterial strains were made by adding bacterial growth from an agar plate using a 10 µl loop (Alpha Laboratories Ltd, Eastleigh, England) to 1 ml of 15% (v/v) glycerol in a 2 ml cryotube (Media Services, PHE Microbiology Services Colindale). The bacterial suspension was vortexed until thoroughly mixed and stored at -80 °C.

2.3 MIC determination

Bacterial isolates were screened against AMRHAI's panel of antibiotics for Gram-negative bacteria (Table 2.1) to determine MICs using agar dilution, as previously described [378], and interpreted according to EUCAST clinical breakpoints version 8.1 [379]. A range of inhibitors

were also included to identify specific antibiotic resistance genes including avibactam (to detect β-lactamase production), clavulanic acid (to detect ESBL production), cloxacillin (to detect AmpC-type β-lactamase production), ethylenediaminetetraacetic acid (EDTA) (to detect metallo-β-lactamase production) and tazobactam (to detect β-lactamase production). In addition to testing for β-lactamase production, the inhibitors avibactam and tazobactam were used in combination with the antibiotics ceftazidime and piperacillin, respectively, as these combinations are used to treat patients.

Briefly, suspensions of test isolates, as well as nine bacterial controls with known MIC values (Table 2.2), were prepared to a 0.5 McFarland standard by inoculating 3 ml Iso-Sensitest broth with a maximum of five bacterial colonies and were measured using a densitometer (BioMérieux, Marcy-l'Etoile, France). Fifty-five microlitres of bacterial suspension containing 10^4 colony forming units (CFU) was added to 110 µl Iso-Sensitest broth in each well of a round-bottom 96-well microtitre plate (Thermofisher Scientific, Surrey, UK). The plate was placed in a 96-pin multipoint inoculator (Mast Group Ltd, Merseyside, UK), in order to inoculate agar plates containing doubling dilutions of antibiotics with 1 µl of the suspensions, with plates inoculated from the lowest to the highest antibiotic concentration.

The agar plates containing doubling dilutions of antibiotics were made using ISO-sensitest agar and Mueller-Hinton (MH) agar (Media Services, PHE Microbiology Services Colindale). Control plates for the inhibitors avibactam (4 mg/L), clavulanic acid (2 mg/L and 4 mg/L), cloxacillin (100 mg/L), EDTA (320 mg/L) and tazobactam (4 mg/L) were included where all plates were ISO-sensitest agar except for the EDTA control, which was a MH agar plate.

Additionally, two sets of ISO-sensitest, MH and CHROMagar (Bioconnections, Knypersley, UK) agar plates were inoculated at the beginning and end of plate inoculation: The ISO-sensitest and MH plates were used to confirm bacterial growth. The CHROMagar plates were used to indicate the species of bacterial isolates and likelihood of contamination from splashing during the inoculation process or the initial use of a mixed bacterial culture, evidenced by the presence of multiple colours in one inoculation spot.

Table 2.1: The range in concentration (mg/L) of antibiotics and inhibitors used in the agar dilution method to calculate MICs as well as the type of agar used.

Antibiotic +/- inhibitor	Range tested (mg/L)	Concentration of inhibitor (mg/L)	Agar
Aminoglycosides			
amikacin	0.5-64	-	ISO-sensitest
gentamicin	0.125-32	-	ISO-sensitest
tobramycin	0.125-32	-	ISO-sensitest
Penicillins			
amoxicillin + clavulanic acid	0.125-64	2	ISO-sensitest
ampicillin	0.5-64	-	ISO-sensitest
carbenicillin	16-512	-	ISO-sensitest
piperacillin + tazobactam	1-64	4	ISO-sensitest
temocillin	1-128	-	ISO-sensitest
2nd generation cephalosporins			
cefoxitin	1-64	-	ISO-sensitest
3rd generation cephalosporins			
cefotaxime	0.125-256	-	ISO-sensitest
cefotaxime + cloxacillin	0.125-256	100	ISO-sensitest
cefotaxime + clavulanic acid	0.06-32	2	ISO-sensitest
ceftazidime	0.125-256	-	ISO-sensitest
ceftazidime + avibactam	0.06-32	4	ISO-sensitest
ceftazidime + clavulanic acid	0.06-32	2	ISO-sensitest
4th generation cephalosporins			
cefepime	0.125-64	-	ISO-sensitest
cefepime + clavulanic acid	0.06-32	2	ISO-sensitest
5th generation cephalosporins			
ceftolozane + tazobactam	0.25-16	4	ISO-sensitest
Carbapenems			
ertapenem	0.125-16	-	MH
imipenem	0.06-128	-	MH
imipenem + EDTA	0.03-16	-	MH
meropenem	0.06-32	-	MH
Monobactams			
aztreonam	0.125-64	-	ISO-sensitest
Fluroquinolones			
ciprofloxacin	0.125-8	-	ISO-sensitest
Tetracyclines			
minocycline	0.125-32	-	ISO-sensitest
Glycylcyclines			
tigecycline	0.25-16	-	ISO-sensitest
Polymyxins			
colistin	0.5-32	-	ISO-sensitest

Table 2.2: Bacterial controls used in the agar dilution method.

Species	Strain
E. cloacae complex	NCTC 13406
E. coli	NCTC 10418
E. coli	NCTC 11560
E. coli	NCTC 11954
E. coli	NCTC 12241
K. pneumoniae	NCTC 13368
P. aeruginosa	NCTC 12903
P. aeruginosa	PS 10586
P. aeruginosa	PS2297

Following plate inoculation, test isolates were streaked onto CLED agar to check the purity of the bacterial suspensions by dipping a 1 µl loop into the bacterial suspensions used for plate inoculation and streaking on 1/3 of a CLED agar plate and incubated overnight at 37 ˚C. MIC determination was repeated for any test isolate that appeared to have a contaminated suspension.

The agar plates were incubated at 37 °C for 18-20 hours and bacterial growth was identified using a Sorcerer™ Colony Counter (Perceptive Instruments Ltd., Suffolk, UK), which automatically calculates the MIC as the lowest concentration that inhibited bacterial growth. Results were only valid if the bacterial controls matched expected MICs within a 2-fold difference.

2.4 PCR to identify antibiotic resistance genes

2.4.1 Multiplex PCRs for the detection 16S RMTase genes

2.4.1.1 Positive control bacterial strains

Positive control bacterial strains (Table 2.3) with the genes *armA* and *rmtB-rmtD* were provided by AMRHAI; bacterial strains carrying *rmtA*, *rmtE* and *rmtF* were provided by Professor Bruno González-Zorn at the Complutense University of Madrid, *rmtG* was provided by Dr Laurent Poirel from the University of Fribourg, *rmtH* was provided by Dr Yohei Doi at the University of Pittsburgh School of Medicine and the *npmA* gene was synthesised by Eurofins Genomics (Germany), cloned into a pEX-A2 plasmid and transformed into *E. coli* DH10B (see Section 2.4.1.2).

2.4.1.2 Construction of *npmA* control bacterial strain

The *npmA* gene was synthesised and cloned into a pEX-A2 plasmid at Eurofins Genomics (Figure 2.1). The 2.5 kb plasmid, which was lyophilised, was reconstituted by adding 44 µl sterile distilled water (Media Services, PHE Microbiology Services Colindale) to 2.2 µg plasmid DNA to make a final concentration of 50 ng/µl. One microlitre of the plasmid suspension was added to a 0.5 ml Eppendorf tube (Sigma Aldrich, Dorset, UK) containing 25 µl *E. coli* DH10B

cells (ThermoFisher Scientific) and placed on ice. The bacterial suspension was added to a pre-chilled 0.1 cm Gene Pulser cuvette (Bio-Rad, Hertfordshire, UK) and electroporated using the GenePulser Xcell™ Electroporation System (Bio-Rad) at 1.8 kV. Following electroporation, 0.5 ml Super-Optimal Broth with Catabolite Repression (SOC) broth (Bioline, London, UK) was added to the cuvette and mixed with the cell suspension and transferred to a 15 ml centrifuge tube (ThermoFisher Scientific). The centrifuge tube was placed on an Innova 2100 platform shaker (New Brunswick Scientific, Herts, UK) and incubated for 1 hour at 37 °C. Following incubation, 100 µl of the cell suspension was spread onto a LB agar plate containing 100 mg/L ampillicin to select for bacteria harbouring pEX-A2 (as it encoded the ampicillin resistance gene *ampR*) and was incubated overnight at 37 °C.

Single colonies from the agar plate were selected and plated on an additional LB + 100 mg/L ampicillin agar plate, which was incubated overnight at 37 °C. DNA was extracted from the bacterial growth from each colony as described in Section 2.4.1.3 and tested with a singleplex *npmA* PCR as described in Section 2.4.1.4 but with 1 µM of *npmA* primers only. Gel electrophoresis was performed as described in Section 2.4.1.6.

The *E. coli* DH10B isolate harbouring *npmA* was streaked onto a Muellar-Hinton plate supplemented with 256 µg/ml amikacin to test the ability of *npmA* to confer high-level resistance. As the isolate grew on the 256 µg/ml amikacin plate, this confirmed that the isolate had a MIC of >256 µg/ml.

A single *npmA*-positive colony was sub-cultured overnight onto CLED agar and the resulting growth made into a glycerol stock that was stored at -80 °C for future use (see Section 2.2.3).

Table 2.3: Bacterial controls used for the detection of 16S RMTase genes via two multiplex PCRs.

Gene	Bacterial strain	Clinical or laboratory strain	Provider
armA	*E. coli*	Clinical	AMRHAI
rmtA	*E. coli* TOP10	Laboratory	Professor Bruno González-Zorn
rmtB	*E. coli*	Clinical	AMRHAI
rmtC	*E. coli*	Clinical	AMRHAI
rmtD	*P. aeruginosa*	Clinical	AMRHAI
rmtE	*E. coli* TOP10	Laboratory	Professor Bruno González-Zorn
rmtF	*K. pneumoniae*	Clinical	Professor Bruno González-Zorn
rmtG	*K. pneumoniae*	Laboratory	Dr Laurent Poirel
rmtH	*K. pneumoniae*	Laboratory	Dr Yohei Doi
npmA	*E. coli* DH10B	Laboratory	Eurofins Genomics

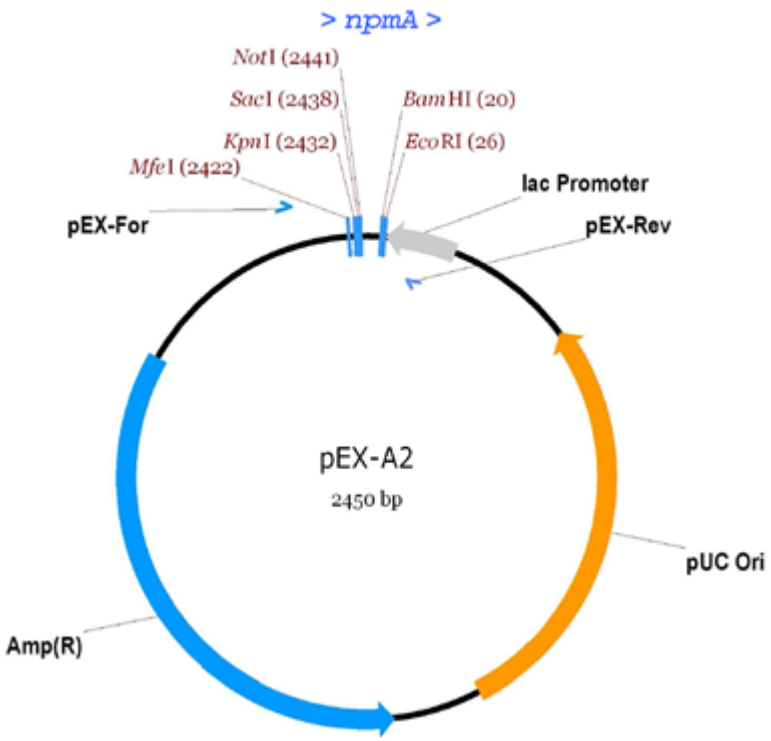


Figure 2.1: Plasmid pEX-A2 containing *npmA*. The figure was provided by Eurofins Genomics.

2.4.1.3 DNA extraction from bacterial isolates

Four to five bacterial colonies were harvested from CLED agar plates using a 1 µl loop and suspended in 100 µl PCR-grade water (Sigma Aldrich) in 0.2 ml MicroAmp® 8-Tube strips (Applied Biosystems, Warrington, UK). The samples were denatured at 95 °C for 5 mins using a 2720 Thermal Cycler (Applied Biosystems) and spun down using a Prism™ mini centrifuge (Labnet International, Rutland, UK).

2.4.1.4 Multiplex PCRs for the detection of 16S RMTase genes

Two multiplex PCRs were used to detect the 16S RMTase genes. Multiplex 1 was based on a published PCR protocol by Doi and Arakawa [304], which detects *armA* and *rmtA-rmtD*, but with the *armA* primers redesigned to allow for better separation of the *armA* and *rmtD* amplicons on an agarose gel. Primers for *armA* and multiplex 2, which detects *rmtE-H* and *npmA,* were designed using the website MPprimer version 1.4 (which was previously accessed with the URL: http://biocompute.bmi.ac.cn/MPprimer/ but is now accessed using: https://archive.is/DowMq) [380], where selected settings included a primer annealing temperature of 55 °C and primer length of ~20 base pairs (bp). Amplicon sizes were set manually by providing a range value ± 50 bp of the desired product size. The specificity of resultant primers (Table 2.4) to the respective genes was evaluated *in silico* using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Lyophilised primers (Sigma Aldrich) were reconstituted with PCR-grade water (Sigma Aldrich), according to the manufacturer's instructions, to make 100 µM primer stock solutions, which were stored at -20 °C until use. For multiplex 1, the 100 µM primer stock solutions were diluted to 1 µM by adding 10 µl of each forward and reverse primer to 900 µl PCR-grade water (Sigma Aldrich) to make a total volume of 1 ml. For multiplex 2, the 100 µM primer stock solutions for were diluted to 0.2 µM (*rmtE*, *rmtG*, *rmtH* and *npmA*) and 0.3 µM (*rmtF*) by adding 2 µl and 3 µl, respectively, of each forward and reverse primer to 978 µl PCR-grade water (Sigma Aldrich) to make a total volume of 1 ml.

Table 2.4: Primer sequences for detection of 16S RMTase genes.

Name	Primer Sequence (5'→3')	Size (bp)	Melting temperature (°C)	GC content (%)	Target	Product size (bp)	Multiplex PCR 1 or 2	Reference
armA–F	AAAGTACAATCAGGGGCAGTT	21	61.4	42.8	*armA*	269	1	This study
armA–R	TCGTCGTCTTTAACTTCCCAA	21	63.4	42.8			1	This study
rmtA–F	CTAGCGTCCATCCTTTCCTC	20	57.48	55.00	*rmtA*	634	1	[304]
rmtA–R	TTGCTTCCATGCCCTTGCC	19	61.29	57.89			1	[304]
rmtB–F	GCTTTCTGCGGGCGATGTAA	20	61.37	55.00	*rmtB*	173	1	[304]
rmtB–R	ATGCAATGCCGCGCTCGTAT	20	63.60	55.00			1	[304]
rmtC–F	CGAAGAAGTAACAGCCAAAG	20	54.66	45.00	*rmtC*	711	1	[304]
rmtC–R	ATCCCAACATCTCTCCCACT	20	57.72	50.00			1	[304]
rmtD–F	CGGCACGCGATTGGGAAGC	19	64.87	55.56	*rmtD*	401	1	[304]
rmtD–R	CGGAAACGATGCGACGAT	18	58.31	68.42			1	[304]
rmtE–F	TTGCGTTGCCATTACTGCAC	20	60.04	50.00	*rmtE*	353	2	This study
rmtE–R	CAATGAGGAAGGCAGGTTTTCT	22	58.84	45.45			2	This study
rmtF–F	TTGCCGCGATACAGAAAACC	20	59.20	50.00	*rmtF*	582	2	This study
rmtF–R	GTGCTTTTCCATGCCCACG	19	60.08	57.89			2	This study
rmtG–F	GGAAATCGATAAAGCGGCGC	20	60.38	55.00	*rmtG*	494	2	This study
rmtG–R	TGGGGAACGAACAGATCAGC	20	60.04	55.00			2	This study
rmtH–F	ATATCAACTGCTGTGCCCGG	20	60.46	55.00	*rmtH*	673	2	This study
rmtH–R	GATAAGGGTGTGGTGCTGCA	20	60.32	55.00			2	This study
npmA–F	GGTCAGTTTGATCGTGTGCA	20	58.77	50.00	*npmA*	195	2	This study
npmA–R	AGCTGCAATAACAAACACCACA	22	59.31	40.91			2	This study

PCR mastermix was prepared using ThermoPrime 2X Reddymix PCR Master Mix (ThermoFisher Scientific), PCR-grade water (Sigma Aldrich) and the primer mix as stated in Table 2.5. A volume of 0.8 µl DNA (or PCR-grade water for the negative control) was added to 9.2 µl of the PCR mastermix in 0.2 ml MicroAmp® 8-Tube strips (Applied Biosystems) and the PCR, according to Professor Arakawa's PCR conditions (Table 2.6), was run using a T100™ Thermal Cycler (Bio-Rad).

2.4.1.5 PCR Controls

During DNA extraction, positive bacterial controls (Table 2.3) for PCR controls A, B, C and D were included where one colony from each bacterial isolate was mixed together in 100 µl PCR-grade water (Sigma Aldrich). Control A contained control strains positive for *armA* and *rmtA*; Control B had *rmtB*, *rmtC* and *rmtD*; Control C had *rmtE* and *rmtF* and Control D had *rmtG*, *rmtH* and *npmA* (Figure 2.2). During PCR set-up, a no-template control (NTC) was included where 0.8 µl PCR-grade water was added to 9.2 µl PCR mastermix in place of DNA template. Failure of a positive control to amplify or DNA amplification in the NTC invalidated the PCR, leading to a repeat run.

2.4.1.6 Gel electrophoresis

Five microlitres of amplified product were run on 2.0% (w/v) agarose gels, which were made with 1.4 g Ultrapure agarose (Invitrogen, Paisley, United Kingdom) and 70 ml 0.5X UltraPure™ TBE buffer (Invitrogen), for one hour at 120 V. Hyperladder™ 100 bp (Bioline) was used as a DNA size standard. Agarose gels were stained for one hour with 200 ml 3X GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), which was made from 60 µl 10,000X GelRed Nucleic Acid Gel Stain (Biotium) and 200 ml 0.1 M NaCl. Results were visualised using UV light and photographed using the InGenius gel documentation system (Syngene, Synoptics Ltd, UK) and Genesnap software version 06.03.01 (Syngene).

2.4.1.7 PCR validation

PCR validation (see Chapter 3) was conducted as described by Bogaerts *et al*. [381].

Table 2.5: 16S RMTase multiplex PCR constituents per PCR reaction.

Reagent	Volume (µl)
ThermoPrime 2X Reddymix PCR Master Mix	5.0
PCR-grade water	1.0
Primer mix	3.2
DNA	0.8
Total	10.0

Table 2.6: PCR cycling conditions for the 16S RMTase multiplex PCRs.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	96	5 min	1
Denaturation	96	30 sec	30
Annealing	55	30 sec	
Extension	72	1 min	
Final extension	72	5 min	1

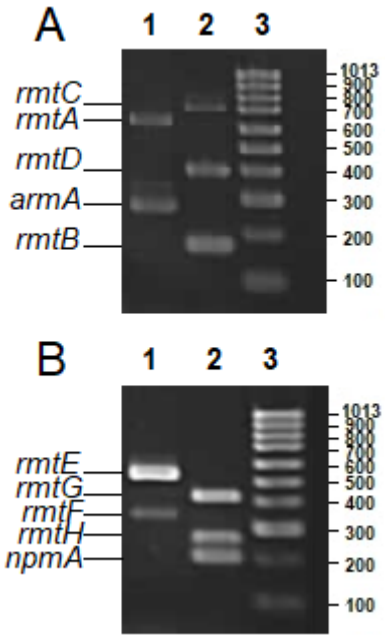


Figure 2.2: Positive controls A, B, C and D for multiplex PCRs 1 and 2 detecting 16S RMTase genes *armA*, *rmtA*-*rmtH* and *npmA*. A: multiplex PCR 1. Lane 1: Control A (*armA* and *rmtA)*; Lane 2: Control B (*rmtB*, *rmtC* and *rmtD)* and Lane 3: 100 bp ladder. B: multiplex PCR 2. Lane 1: Control C (*rmtE* and *rmtF);* Lane 2: Control D (*rmtG*, *rmtH* and *npmA)* and Lane 3: 100 bp ladder.

2.4.2 Real-time PCR (rtPCR) for the detection of KPC, NDM, OXA-48 and VIM carbapenemase genes

Isolates were screened for the carbapenemase genes KPC, NDM, OXA-48-like and VIM using a rtPCR utilised by the AMRHAI Reference Unit as described previously [382].

2.4.2.1 DNA extraction from bacterial isolates

One to two colonies from isolates to be tested, as well as the positive controls for KPC, NDM, OXA-48 and VIM carbapenemase genes (Table 2.7), were harvested from CLED agar plates using a 1 µl loop and suspended in 100 µl *Bacillus thuringiensis* ATCC 29730 suspension (made from suspending colonies of *B. thuringiensis* ATCC 29730 in 100 ml PCR-grade water to 0.5 McFarland standard). *B. thuringiensis* ATCC 29730 acts as internal positive control due to the resistant nature of its spores so amplification of its DNA is indicative of successful DNA extraction. The samples were denatured at 99 °C for 30 mins using a 2720 Thermal Cycler (Applied Biosystems).

2.4.2.2 Detection of KPC, NDM, OXA-48 and VIM carbapenemase genes

For the rtPCR, lyophilised primers (Sigma Aldrich) were reconstituted with PCR-grade water (Sigma Aldrich), according to the manufacturer's instructions, in order to make 100 µM primer stock solutions and were stored at -20 °C until use. The 100 µM primer (Table 2.8) stock solutions were diluted to 30 µM by adding 15 µl of each forward and reverse primer to 70 µl PCR-grade water (Sigma Aldrich) to make a total volume of 100 µl. Lyophilised probes (Eurofins Genomics) were reconstituted with qPCR probe dilution buffer (10 mM Tris-HCl; pH 8; 1 mM EDTA) (Eurofins Genomics), according to the manufacturer's instructions, in order to make 100 µM probe stock solutions and were stored at -20 °C until use. The 100 pM probe (Table 2.9) stock solutions (Eurofins Genomics) were diluted to 10 pM by adding 10 µl of the probe to 90 µl PCR-grade water to make a total volume of 100 µl. The primers and probes were pooled together to make a primer mix and a probe mix as stated in Table 2.10.

Table 2.7: Bacterial controls used in the carbapenemase rtPCR.

Bacterial strain	Carbapenemase
K. pneumoniae NCTC 13438	KPC-3
K. pneumoniae NCTC 13443	NDM-1
K. pneumoniae NCTC 13442	OXA-48
E. coli clinical isolate	VIM-4

Table 2.8: Primer sequences for the carbapenemase rtPCR.

Name	Primer sequence (5'→3')	Size (bp)	Melting temperature (°C)	GC content (%)	Target	Reference
KPC-F	GCAGCGGCAGCAGTTTGTTGATT	23	73.0	52.1	*bla*$_{KPC}$	[382]
KPC-R	GTAGACGGCCAACACAATAGGTGC	24	69.2	54.1		[382]
NDM-F	CCAGCAAATGGAAACTGGCGAC	22	71.6	54.5	*bla*$_{NDM}$	[382]
NDM-R	ATCCAGTTGAGGATCTGGGCG	21	69.6	57.1		[382]
OXA-48-F	GATTATGGTAATGAGGACATTTCGGGC	27	70.1	44.4	*bla*$_{OXA-48}$	[382]
OXA-48-R	CATATCCATATTCATCGCAAAAAACCACAC	30	71.0	36.6		[382]
VIM-F	TTGCTTTTGATTGATACAGCGTGGGG	26	73.4	46.1	*bla*$_{VIM}$	[382]
VIM-R	GTACGTTGCCACCCCAGCC	19	69.9	68.4		[382]
Bt-F	GCAACTATGAGTAGTGGGAGTAATTTAC	28	62.0	39.2	*B. thuringiensis cry1* gene	[382]
Bt-R	TTCATTGCCTGAATTGAAGACATGAG	26	68.8	38.4		[382]

Table 2.9: Probe sequences for the carbapenemase rtPCR.

Name	Primer sequence (5'→3')	Size (bp)	5' label	3' label	Target	Reference
KPC	CAGTCGGAGACAAAACCGGAACCTGC	26	ROX	BHQ-2	*bla*$_{KPC}$	[382]
NDM	ACCGAATGTCTGGCAGCACACTTC	24	JOE	BHQ-1	*bla*$_{NDM}$	[382]
OXA-48	CCATTGGCTTCGGTCAGCATGGCTTGTTT	29	Cy5.5	BBQ-650	*bla*$_{OXA-48}$	[382]
VIM	TCTCGCGGAGATTGAAAAGCAAATTGGACTTCC	33	CY5	BBQ-650	*bla*$_{VIM}$	[382]
Bt	ACGGAGTAGTAGTAAAACCTACAGTCCTAAAGC	33	FAM	BHQ-1	*B. thuringiensis cry1* gene	[382]

Table 2.10: Primer and probe mix constituents per PCR reaction.

Primer	**Working stock concentration (µM)**	**One reaction volume (µl)**
KPC	30	0.25
OXA-48	30	0.5
NDM	30	0.75
VIM	30	0.75
Bt	30	0.75
Total volume	-	3.0
Probe	**Working stock concentration (µM)**	**One reaction volume (µl)**
KPC	10	0.25
OXA-48	10	0.5
NDM	10	0.5
VIM	10	0.25
Bt	10	0.25
Total volume	-	1.75

PCR mastermix was prepared using Platinum™ Quantitative PCR SuperMix-UDG (ThermoFisher Scientific), PCR-grade water (Sigma Aldrich) and the primer and probe mixes as stated in Table 2.11. Five microlitres of DNA (or PCR-grade water for the NTC) was added to 20 µl of the PCR mastermix in 0.2 ml PCR tubes (Qiagen, Crawley, UK) and the PCR was run using a Rotorgene-Q instrument (Qiagen) and Rotor-Gene 6000 Series Software version 1.7 under the PCR cycling conditions stated in Table 2.12. Rotor-Gene Q software version 2.0.3 was used to analyse the results and carbapenemase-positive isolates were identified.

Rotor-Gene Q software version 2.0.3 was used to analyse the results and carbapenemase-positive isolates were identified using a cut-off value (cycle threshold (Ct) ≤ 35). Results were only valid if the internal positive control was positive in all samples tested, the positive controls were positive and the NTC was negative.

2.5 Typing of bacterial isolates

2.5.1 PFGE

2.5.1.1 Preparation of the agarose plugs

Bacterial isolates were streaked onto CLED agar and grown overnight at 37 °C. Bacterial suspensions were made up to 2.3-2.7 McFarland standard by suspending a 1 µl loopful of bacteria into 1 ml sodium chloride/EDTA (SE) buffer (75 mM NaCl, 25 mM EDTA pH 7.5) and were measured using a densitometer (BioMérieux). Four hundred microlitres of the bacterial suspensions were mixed 1:1 with 2% Macrosieve Low Melt Agarose (Flowgen, Nottingham, UK) in SE buffer (which was kept at 50-56 °C in a water bath throughout use) in a sterile 1.5 ml Eppendorf tube and the mixtures were dispensed into PFGE block moulds. The PFGE block moulds were stored at 4 °C until the agarose plugs had set.

Table 2.11: Carbapenemase rtPCR reaction constituents per PCR reaction.

Reagent	Volume (µl)
Platinum™ Quantitative PCR SuperMix-UDG	12.5
PCR-grade water	2.75
Primer mix	3.0
Probe mix	1.75
DNA	5.0
Total	25.0

Table 2.12: PCR cycling conditions for the carbapenemase rtPCR.

Temperature (°C)	Time	Cycles
60	1 min	1
95	10 min	
95	15 sec	40
58	1 min	

Lysis buffer was prepared by adding 500 µg/ml lysozyme (Sigma Aldrich) to the appropriate volume of First Lysis buffer (6 mM Tris, 100 mM EDTA, 1 M NaCl, 0.5% w/v Brij-58, 0.2% w/v sodium deoxycholate, 0.5% N-lauroyl sarcosine, 1 mM $MgCl_2$) and was incubated at 37 °C on an Innova 2100 platform shaker (New Brunswick Scientific) until the lysozyme was dissolved. Once set, the agarose plugs were removed from the block moulds, transferred to sterile bijoux containing 3 ml Lysis buffer and incubated overnight at 37 °C with shaking.

The Lysis buffer was removed and replaced with 3 ml Alkaline Lysis buffer (1% (w/v) N-lauroyl sarcosine, 0.5 M EDTA pH 9.5) and 3.6 µl Proteinase K (Sigma Aldrich) was added to each sample. The samples were incubated at 56 °C overnight with shaking. Following incubation, the agarose plugs were washed with 3 ml TRIS-EDTA (TE) buffer (10 mM Tris, 10 mM EDTA, pH 7.5) three times at 2 hour intervals and were incubated at 4 °C. The final wash was replaced with 2 ml TE buffer and the agarose plugs were stored at 4 °C until use.

When ready to be tested 2 mm pieces of agarose plug per strain were cut using a scalpel and incubated in 100 ml 1X FastDigest buffer (ThermoFisher Scientific) in 0.5 ml Eppendorf tubes for 30 min at 4 °C. After incubation the buffer was removed and replaced with fresh 1X FastDigest buffer and 3.2 µl of the restriction enzyme *Xba*I (for Enterobacterales) or *Apa*I (for *A. baumannii*), and incubated at 37 °C for 30 mins.

2.5.1.2 Gel electrophoresis

The agarose plugs, along with Lambda Ladder PFG Marker (New England Biolabs, Hertfordshire, UK) to size the fragments, were loaded into the wells of a 1.2% agarose gel comprising of 2.04 g Certified™ Molecular Biology Agarose (Bio-Rad) and 170 ml 0.5X UltraPure™ Tris-Borate EDTA (TBE) buffer (Invitrogen). The agarose gel was run on a CHEF DRII apparatus (Bio-Rad) at 6 V for 30 hours at 12 °C with an initial pulse time of 5 sec and a final pulse time of 35 sec. Following the run, the agarose gel was stained with 200 ml 3X GelRed™ Nucleic Acid Gel Stain (Biotium) for one hour and the results were visualised using UV light and photographed using the InGenius gel documentation system (Syngene) and Genesnap software 06.03.01 (Syngene) and analysed using Bionumerics Software version

6.1 (Applied Maths, Sint-Martens-Latem, Belgium), where cluster analysis was performed to create dendrograms using the unweighted pair group method with arithmetic mean (UPGMA) algorithm based on the Dice similarity coefficient with 0.5% optimisation and 1.5% tolerance.

2.5.2 VNTR analysis for the typing of *K. pneumoniae*

VNTR analysis was carried out as previously described by Turton *et al*. [383] to amplify 11 VNTR loci (A, D, E, H, J, K, N1, N2, 52, 45 and 60). In 2016 the loci 52, 45 and 60 were added to the VNTR scheme.

2.5.2.1 Preparation of the PCR mastermixes

Separate PCR mastermixes were prepared for VNTR loci A, E, H, J, K and N1 using 2X MyTaq HS Red Mix (Qiagen), PCR-grade water (Sigma Aldrich) and non-fluorescent primers (Table 2.13). For loci 45, 52, 60, D and N2 one mastermix was prepared using Platinum® Taq DNA Polymerase (Invitrogen), 10X CoralLoad PCR buffer (Qiagen), deoxynucleotide triphosphates (dNTPs) (Qiagen), PCR-grade water (Sigma Aldrich) and fluorescently-labelled primers for each locus (Table 2.13).

All forward and reverse primers were made into 10 µM primer mixes by adding 20 µl of each 100 µM stock primer solution to 160 µl of water. Two microlitres of DNA extract for loci A, E, H, J, K and N1 was added to 18 µl PCR mastermix (Table 2.14) and 2 µl DNA extract for loci 45, 52, 60, D and N2 was added to 23 µl PCR mastermix (Table 2.15) in a 96-well plate (ThermoFisher Scientific). The 96-well plate was covered with foil (ThermoFisher Scientific) and sealed using a heat sealer (Eppendorf). The PCR for loci A, E, H, J, K and N1 was run using a PTC-225 DNA Engine Tetrad thermal cycler (MJ Research, San Francisco, CA) and the PCR for loci 45, 52, 60, D and N2 was run on a T100™ Thermal Cycler (Bio-Rad) according to the PCR conditions in Tables 2.16 and 2.17.

Table 2.13: Primer and probe sequences for VNTR analysis of *K. pneumoniae*.

Locus	Name	Primer sequence (5'→3')	Repeat unit size	5' reporter dye
A	A-F	AGCGTATCTGCCATTGCC	140	-
	A-R	CAGCATGGCCAGTTTGTC		-
E	E-F	CCAAATCCGGGTATTTATCG	118	-
	E-R	TTCGATACCCATCCGGAAG		-
H	H-F	ATGACCAAGGAAGAACCCG	124	-
	H-R	CTTTACCTGGCATGCGAACG		-
J	J-F	ACCGGATTAAGCGCTATTCC	124	-
	J-R	TTCCTCGCCCACGGATAG		-
K	K-F	GAGCTGGCGGCTGGAATA	118	-
	K-R	GCAATCTGCCCGGAAATA		-
N1	N1-F	CATCAGGTGCAAGATTCCA	116	-
	N1-R	TGAGCGATTGCTGGCCTA		-
52	52-F	TTTGGCGGCAGCGGTTTCCC	21	6-FAM
	52-R	GCCAGAAAAAGGCGCGCAGC		-
45	45-F	CGCTGACACATTGACGAAAACAGAGA	12	6-FAM
	45-R	ATGAATATTGCCCAGTTTCTGGAACAA		-
60	60-F	CGGTACGAATCTGTTGGATTAAG	7	VIC
	60-R	GGCCTTCTTCCGGGTCTAT		-
D	D-F	GCAGGTCTCGTCTTCATTCC	14	NED
	D-R	TGACCATCGAAGAGGCG		-
N2	N2-F	GATGCGGCAAGCACCAC	57	PET
	N2-R	ACGCCCTGACCATTATGC		-

Table 2.14: PCR reaction constituents for *K. pneumoniae* loci A, E, H, J, K and N1 per PCR reaction.

Reagent	Volume (µl)
2X MyTaq HS Red Mix	10.0
10 µM F/R primer mix	0.8
PCR-grade water	7.2
DNA	2.0
Total	20.0

Table 2.15: PCR reaction constituents for *K. pneumoniae* loci 52, 45, 60, D and N2 per PCR reaction.

Reagent	Volume (µl)
Platinum® Taq DNA Polymerase	0.3
10X CoralLoad PCR buffer	2.5
dNTPs	0.5
52 F/R primer mix	0.4
45 F/R primer mix	0.4
60 F/R primer mix	0.4
D F/R primer mix	0.3
N2 F/R primer mix	0.6
PCR-grade water	17.6
DNA	2.0
Total	25.0

Table 2.16: PCR cycling conditions for *K. pneumoniae* loci A, E, H, J, K and N1.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	1 min	1
Denaturation	95	20 sec	30
Annealing	58	20 sec	
Extension	72	45 sec	
Denaturation	94	30 sec	5
Annealing	58	30 sec	
Extension	72	45 sec	
Final extension	72	10 min	1

Table 2.17: PCR cycling conditions for *K. pneumoniae* loci 52, 45, 60, D and N2.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	3 min	1
Denaturation	94	30 sec	30
Annealing	58	45 sec	
Extension	72	1 min	
Final extension	68	30 sec	1

2.5.2.2 Gel Electrophoresis

Five microlitres of amplified product for loci A, E, H, J, K and N1 were run on 1.5% (w/v) agarose gels, which were made with 2.7 g Certified™ Molecular Biology Agarose (Bio-Rad) and 180 ml 0.5X UltraPure™ TBE buffer (Invitrogen), at 150 V for 100 min. Hyperladder™ 50 bp (Bioline) was used as a DNA size standard. Agarose gels were stained and results were visualised according to Section 2.4.1.6. Based on the size of the amplicons the number of repeats per locus could be determined using a table of expected sizes as seen in Table 2.18.

2.5.2.3 Fragment analysis

As loci 45, 52, 60, D and N2 have small repeat units the size of the PCR products was determined using an ABI 3730XL Genetic Analyser (Applied Biosystems) at the Genomic Services Unit (PHE, Colindale).

The amplified products for loci 45, 52, 60, D and N2 were diluted 1:40 by adding 5 µl of each PCR product to 125 µl water to give optimum peak intensity. Ten microlitres Hi-Di formamide (Applied Biosystems) was added to 0.5 µl Genescan LIZ-600 Size Standard (Applied Biosystems) per reaction and 10.5 µl of this mixture was added to each well of a MicroAmp optical semi-skirted sequencing plate (Applied Biosystems) followed by 1 µl diluted PCR product. The 96-well plate was covered in adhesive foil (ThermoFisher Scientific), sealed and submitted to the Genomic Services Unit for fragment size analysis.

Results were analysed using the software Peak Scanner version 2.0 (Applied Biosystems) to determine the size of the peaks per sample, which were used in conjunction with the tables of expected sizes to calculate the number of repeats per loci (Table 2.19).

Table 2.18: Number of repeats per *K. pneumoniae* locus (A, E, H, J, K and N1) based on size of the amplicons.

	A	E	H	J	K	N1
Repeat Number	**Size (bp)**	**Size (bp)**	**Size (bp)**	**Size (bp)**	**Size (bp)**	**Size (bp)**
0	129	64	171	155	186	122
1	241	176	295	279	304	238
2	381	316	419	403	422	354
3	521	456	543	527	540	470
4	661	596	669	641	658	586
5	801	736	791	775	776	702
6	941	876	915	897	894	818
7	1,081	1,016	1,039	1,021	1,012	934
8	1,221	-	1,163	1,145	1,130	1,050
9	1,361	-	-	1,269	-	-
10	1,501	-	-	1,393	-	-
11	1,641	-	-	1,517	-	-
12	1,781	-	-	1,641	-	-
13	1,921	-	-	-	-	-
14	2,061	-	-	-	-	-

Table 2.19: Number of repeats per *K. pneumoniae* locus (45, 52, 60, D and N2) based on size of the amplicons.

	52	45	60	D	N2
Repeat Number	**Size (bp)**	**Size (bp)**	**Size (bp)**	**Size (bp)**	**Size (bp)**
0	130	254	-	-	124
1	151	266	321	131	181
2	172	278	328	145	238
3	193	290	335	-	295
4	214	302	342	-	352
5	235	314	349	-	409
6	258	326	356	-	466
7	279	338	363	-	523
8	-	-	370	-	580
9	-	-	377	-	637
10	-	-	384	-	694
11	-	-	391	-	751
12	-	-	-	-	808
13	-	-	-	-	865
14	-	-	-	-	922

2.5.2.4 VNTR analysis

Following data analysis VNTR profiles were inferred in the order A, E, H, J, K, D, N1, N2, 52, 45, 60 and a dendrogram was created using Bionumerics Software version 6.1 (Applied Maths), where cluster analysis was performed using the categorical parameter with a tolerance of 0%. STs were inferred from VNTR profiles from AMRHAI's ST/VNTR library, which was built based on results for isolates for which both STs and VNTR profiles had been determined.

2.5.3 VNTR analysis for the typing of *P. aeruginosa*

VNTR analysis was carried out as previously described by Turton *et al*. [384] to amplify nine VNTR loci (61, 172, 207, 209, 211, 213, 214, 217 and 222).

2.5.3.1 Preparation of the PCR mastermixes

Separate PCR mastermixes were prepared for each *P. aeruginosa* locus using HotStarTaq Plus DNA Polymerase (Qiagen), 10X CoralLoad Concentrate (Qiagen), PCR-grade water (Sigma Aldrich), fluorescently-labelled primers (for loci 61, 207 and 209) and non-fluorescent primers (for loci 172, 211, 213, 214, 217 and 222) as in Table 2.20. Each pair of non-fluorescent primers was made into a 10 µM primer mix by adding 20 µl of each 100 µM stock primer solution to 160 µl of water. Forward and reverse fluorescently-labelled primers were diluted to 10 µM (by adding 20 µl of 100 µM stock primer solution to 180 µl of water) and added separately to their respective PCR mastermix (Table 2.21). Two microlitres of the DNA extract was added to 18 µl PCR mastermix in 96-well plates (ThermoFisher Scientific). The 96-well plate was covered with foil (ThermoFisher Scientific) and sealed using a heat sealer (Eppendorf). The PCR was run using a T100™ Thermal Cycler (Bio-Rad) according to the PCR conditions in Table 2.22.

Table 2.20: Primer and probe sequences for VNTR analysis of *P. aeruginosa*.

Locus name	Primer name	Primer sequence (5'→3')	Repeat unit size (bp)	5' reporter dye	Reference
61	61-F	CTTGCCGTGCTACCGATCC	6	VIC	[385]
	61-R	CCCCCATGCCAGTTGC		-	[385]
172	172-L	GGATTCTCTCGCACGAGGT	54	6-FAM	[385]
	172-R	TACGTGACCTGACGTTGGTG		-	[386]
207	207-L	ACGGCGAACAGCACCAGCA	6	NED	[386]
	207-R	CTCTTGAGCCTCGGTCACT		-	[386]
209	209-L	CAGCCAGGAACTGCGGAGT	6	-	[386]
	209-R	CTTCTCGCAACTGAGCTGGT		-	[386]
211	211-L	ACAAGCGCCAGCCGAACCTGT	101	-	[386]
	211-R	CTTCGAACAGGTGCTGACCGC		-	[386]
213	213-L	CTGGGCAAGTGTTGGTGGATC	103	-	[386]
	213-R	TGGCGTACTCCGAGCTGATG		-	[386]
214	214L	AAACGCTGTTCGCCAACCTCTA	115	-	[386]
	214-R	CCATCATCCTCCTACTGGGTT		-	[386]
217	217-L	TTCTGGCTGTCGCGACTGAT	109	-	[386]
	217-R	GAACAGCGTCTTTTCCTCGC		-	[386]
222	222-L	AGAGGTGCTTAACGACGGAT	101	-	[386]
	222-R	TGCAGTTCTGCGAGGAAGGCG		-	[386]

Table 2.21: PCR reaction constituents for *P. aeruginosa* loci 172, 211, 213, 214, 217 and 222 as well as 61, 207 and 209 per PCR reaction.

Loci 172, 211, 213, 214, 217 and 222	
Reagent	**Volume (µl)**
2X Qiagen Master Mix	10.0
10X CoralLoad Concentrate	2.0
PCR-grade water	5.2
Primer mix (10 µM)	0.8
DNA	2.0
Total	20.0
Loci 61, 207 and 209	
Reagent	**Volume (µl)**
2X Qiagen Master Mix	10.0
10X CoralLoad Concentrate	2.0
PCR-grade water	5.2
Forward primer (10 µM)	0.4
Reverse primer (10 µM)	0.4
DNA	2.0
Total	20.0

Table 2.22: PCR cycling conditions for *P. aeruginosa* loci 61, 172, 207, 209, 211, 213, 214, 217 and 222.

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 min	1
Denaturation	94	1 min	35
Annealing	60	1 min	
Extension	72	1 min	
Final Extension	72	10 min	1

2.5.3.2 Gel Electrophoresis

Five microlitres of amplified product for loci 172, 211, 213, 214, 217 and 222 were run on 1.5% (w/v) agarose gels as described in Section 2.5.2.2. Agarose gels were stained and results were visualised according to Section 2.4.1.6. Based on the size of the amplicons the number of repeats per locus could be determined using a table of expected sizes as seen in Table 2.23.

2.5.3.3 Fragment analysis

As loci 61, 207 and 209 have small repeat units the size of the PCR products was determined using an ABI 3730XL Genetic Analyser (Applied Biosystems) at the Genomic Services Unit (PHE, Colindale).

The amplified products for loci 61, 207 and 209 were diluted 1:40 by adding 5 µl of each PCR product to 185 µl PCR-grade water to give optimum peak intensity. Ten microlitres Hi-Di formamide (Applied Biosystems) was added to 0.5 µl Genescan LIZ-600 Size Standard (Applied Biosystems) per reaction and 10.5 µl of this mixture was added to each well of a MicroAmp optical semi-skirted sequencing plate (Applied Biosystems) followed by 1 µl diluted PCR product. The 96-well plate was covered in adhesive foil (ThermoFisher Scientific), sealed and submitted to the Genomic Services Unit for fragment size analysis.

Once the results were obtained they were analysed using the software Peak Scanner version 2.0 (Applied Biosystems) to determine the size of the peaks per sample, which were used in conjunction with the tables of expected sizes to calculate the number of repeats per locus (Table 2.24).

2.5.3.4 VNTR analysis

Following data analysis VNTR profiles were inferred in the order 172, 211, 213, 214, 217, 222, 207, 209, 61 and a dendrogram was created using Bionumerics Software version 6.1 (Applied Maths), where cluster analysis was performed using the categorical parameter with a tolerance of 0%. STs were inferred from the VNTR profiles according to Wright *et al*. [387].

Table 2.23: Number of repeats per *P. aeruginosa* locus (172, 211, 213, 214, 217 and 222) based on size of the amplicons.

	172	**211**	**213**	**214**	**217**	**222**
Repeat Number	**Size (bp)**	**Size (bp)**	**Size (bp)**	**Size (bp)**	**Size (bp)**	**Size (bp)**
1	195	259	221	196	497	188
2	249	360	324	311	606	289
3	303	461	427	426	715	390
4	357	562	530	541	824	491
5	411	663	640	656	933	592
6	465	764	743	771	1,042	693
7	519	865	846	886	-	794
8	573	966	949	1,001	-	895
9	627	-	1,052	-	-	996
10	681	-	1,155	-	-	-
11	735	-	1,258	-	-	-
12	789	-	-	-	-	-
13	843	-	-	-	-	-
14	897	-	-	-	-	-

Table 2.24: Number of repeats per *P. aeruginosa* locus (207, 209 and 61) based on size of the amplicons.

	207	**209**	**61**
Repeat Number	**Size (bp)**	**Size (bp)**	**Size (bp)**
1	-	119	-
2	-	125	-
3	124	131	-
4	130	137	78
5	136	143	83
6	142	149	89
7	149	154	96
8	155	160	103
9	161	-	109
10	167	-	115
11	173	-	121
12	180	-	127
13	186	-	133
14	192	-	139
15	198	-	145
16	-	-	151
17	-	-	157
18	-	-	163
19	-	-	169
20	-	-	175
21	-	-	181

2.6 WGS

2.6.1 DNA extraction

Bacterial isolates were plated onto CLED agar plates and incubated overnight at 37 °C. Following incubation, a 1 µl loopful of each bacterial isolate was transferred to wells of a 96-deep well block plate (Qiagen) containing 250 µl lysis buffer comprising of ATL buffer (Qiagen), RNase A (Qiagen) and Proteinase K (Qiagen) (Table 2.25). The plate was sealed using adhesive foil (Sigma Aldrich), was vortexed for 30 sec using the Vortex Mixer Wizard X (VELP Scientifica, Italy) and was incubated at 59 °C for 45 min using the Eppendorf ThermoMixer FP (Eppendorf) to lyse the bacterial cells. The plate was centrifuged for 1 min at 3000 rpm using the Heraeus™ Multifuge™ X1R centrifuge (ThermoFisher Scientific) and genomic DNA was extracted using the QIAsymphony extraction robot (Qiagen) and the QiaSymphony DSP DNA mini kit (Qiagen) according to manufacturer's instructions, and eluted into a FrameStar® 96 Well Semi-Skirted PCR Plate (4titude Ltd, Dorking, UK).

2.6.2 WGS

WGS was conducted by the Wellcome Trust Sanger Institute using the Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) with 150-bp paired-end reads.

2.7 Plasmid analysis

2.7.1 Extraction of plasmid DNA

Bacterial isolates were streaked onto CLED agar and grown overnight at 37 °C. A single colony was used to inoculate 10 ml LB broth (Media Services, PHE Microbiology Services Colindale) and was incubated overnight at 37 °C on an Innova 2100 platform shaker (New Brunswick Scientific) at 225 rpm. Three millilitres of the bacterial suspension were used for plasmid extraction, which was carried out using the PureYield™ Plasmid Miniprep System (Promega, Southampton, UK) according to the manufacturer's instructions. The plasmid DNA was stored at -20 °C until use.

2.7.2 Electroporation of plasmid DNA into *E. coli* TOP10 cells

Plasmid DNA was electroporated into TOP10 *E. coli* cells (Invitrogen) as described in Section 2.4.1.2. Following incubation in SOC broth, the 0.5 ml cell suspension was inoculated into 10 ml LB broth with 50 mg/L amikacin (Sigma Aldrich, Dorset, UK) and was incubated overnight at 37 °C on an Innova 2100 platform shaker (New Brunswick Scientific) at 225 rpm. Following incubation, 10 µl of the cell suspension was plated onto LB agar containing 50 mg/L amikacin and incubated overnight at 37 °C. Any resulting colonies were plated onto LB agar with 256 mg/L amikacin and incubated overnight at 37 °C to select for those with 16S RMTase genes. Colonies that exhibited growth underwent DNA extraction and were screened with the two multiplex PCRs to confirm the presence of 16S RMTase genes, as described in Section 2.1.4.3 to Section 2.4.1.6.

Table 2.25: Constituents of lysis buffer for per sample.

Reagent	Volume (µl)
ATL buffer	226.0
RNase A	4.0
Proteinase K	20.0
Total	250.0

Chapter 3: Validation of two multiplex PCRs to detect 16S RMTase genes

3.1 Introduction

Antibiotic susceptibility testing is important for the identification of antibiotic-resistant bacteria in the clinical setting in order for antibiotics to be correctly administered to patients and is carried out using a variety of techniques, including agar dilution, microbroth dilution, disk diffusion, gradient strips and automated systems such as the VITEK 2 [28,33]. However, these methods can be laborious and are very time-consuming due to each technique requiring pure bacterial cultures and having several incubation periods [28,33], resulting in delays in the diagnosis of antibiotic-resistant infections and in ensuring the patient is administered an effective treatment. Therefore, molecular methods are increasingly being used in diagnostic laboratories to identify the antibiotic resistance mechanisms present in bacterial isolates in order to aid the diagnosis and treatment of patients, for outbreak investigation and surveillance of antibiotic resistance [388].

The relatively low cost of PCR and the ability to detect more than one gene in a single PCR assay through the use of multiplexing has led to the use of multiple PCRs to detect antibiotic resistance genes in clinical isolates [35,389-391]. As resistance mechanisms can be identified sooner using PCR than by the use of phenotypic susceptibility testing alone (<4 hours for PCR *vs.* 48-72 hours for phenotypic susceptibility testing), patients infected or colonised with certain antibiotic resistant bacteria (e.g. patients carrying carbapenemase-producing bacteria [392]) can be identified faster and infection prevention and controls measures can be implemented if necessary in order to prevent spread to other patients [388].

As 16S RMTase genes are known to cause high-level resistance (MICs >256 mg/L) to either 4,6-disubstituted DOS aminoglycosides such as amikacin, gentamicin and tobramycin (*armA*, *rmtA*-*rmtH* and *npmA*) or 4,5-disubstituted DOS aminoglycosides such as neomycin (*npmA*) and are mainly associated with plasmids [149], the use of PCR to detect 16S RMTase genes could benefit patient management as it would alert clinicians that aminoglycosides would be ineffective as well as the risk to other patients due to transmission of host pathogens or horizontal gene transfer of 16S RMTase gene-encoding plasmids. In the last few years,

several multiplex PCRs have been designed to detect 16S RMTase genes but many are now outdated due to the identification of new 16S RMTase gene families and/or novel gene variants. In 2007, Doi and Arakawa [304] published details of two multiplex PCRs to detect *armA, rmtB* and *rmtC* in Enterobacterales and *rmtA* and *rmtD* in *P. aeruginosa.* Over the years, additional multiplex PCRs have been designed that detect 16S RMTase genes via conventional (Berçot *et al.* [393] and Corrêa *et al*. [394]) or rtPCR (Guo *et al*. [274]), or detect 16S RMTase genes alongside other resistance genes such as AMEs (Hu *et al*. [395]). As no published PCR was able to identify all of the 16S RMTase gene families and variants known at the start of this project, a new multiplex PCR was designed and validated to detect *rmtE*, *rmtF*, *rmtG*, *rmtH* and *npmA* (and known variants). Doi and Arakawa's PCR was also validated to check its ability to detect all known variants of *armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD*. Unlike the PCR by Corrêa *et al*. [394], where all ten 16S RMTase gene families are detected in a single reaction, two multiplex PCRs were used to allow easy differentiation between amplicons, which becomes especially important if isolates have more than one 16S RMTase gene.

3.2 Aims

The aims of this study were to: i) validate two multiplex PCRs for the detection of 16S RMTase genes according to Bogaerts *et al*. [381], and, ii) calculate the analytical sensitivity, specificity and reproducibility.

3.3 Methods

3.3.1 Bacterial isolates used for the PCR Validation

A blinded panel of 65 bacterial isolates was provided by PHE's AMRHAI Reference Unit. The panel comprised of 65 Gram-negative bacteria including 60 Enterobacterales and five non-fermenting bacteria whose 16S RMTase gene content had been previously determined by WGS. The bacterial isolates in the panel were *K. pneumoniae* (27.7%, 18/65), *E. coli* (15.4%, 10/65), *C. freundii* (10.8%, 7/65), *E. cloacae* complex (10.8%, 7/65), *K. aerogenes* (4.6%, 3/65), *P. aeruginosa* (4.6%, 3/65), *Klebsiella* spp. (3.1%, 2/65), *Klebsiella oxytoca* (3.1%,

2/65), *P. stuartii* (3.1%, 2/65), *A. baumannii* (1.5%, 1/65), *Acinetobacter calcoaceticus* (1.5%, 1/65), *Morganella morganii* (1.5%, 1/65), *P. mirabilis* (1.5%, 1/65). *Providencia rettgeri* (1.5%, 1/65) and *Citrobacter* sp. (1.5%, 1/65). Additionally, the panel of isolates included *E. coli* TOP10 (3.1%, 2/65) and *E. coli* DH10B (1.5%, 1/65) transformants positive for *rmtA*, *rmtE* and *npmA*, respectively, as well as single *K. pneumoniae* isolates positive for *rmtG*, and *rmtH* as no clinical isolates positive for these genes had been identified within the AMRHAI culture collection.

3.3.2 DNA extraction

DNA was extracted from the bacterial isolates according to Section 2.4.1.3.

3.3.3 Multiplex PCRs for the detection of 16S RMTase genes

Two multiplex PCRs were used to detect the 16S RMTase genes *armA*/*armA2*, *rmtA*, *rmtB/rmtB2/rmtB3/rmtB4*, *rmtC*, *rmtD/rmtD2*/*rmtD3*, *rmtE/rmtE2*, *rmtF/rmtF2*, *rmtG*, *rmtH* and *npmA* as described in Section 2.4.1.4 and results were visualised using gel electrophoresis as described in Section 2.4.1.6.

3.3.4 PCR validation procedure

PCR validation was performed according to the recommendations of Bogaerts *et al*. [381]. In order to calculate sensitivity, a minimum of ten isolates positive for each 16S RMTase gene were tested once. In instances where the minimum of ten isolates per gene could not be met, the available isolate(s) were extracted again until the target of ten DNA extracts per gene was achieved. This was done for isolates positive for *rmtA*, *rmtD*, *rmtE*, *rmtG*, *rmtH* and *npmA* as only one clinical isolate was available for *rmtD* and the others were either transformants (*rmtA*, *rmtE* and *npmA*) or positive controls (*rmtG* and *rmtH*) from external providers.

To calculate specificity, at least 20 isolates negative for 16S RMTase genes (or those negative for genes for one multiplex PCR but positive for genes in the other) were included in the panel and were tested once. Reproducibility was measured by testing one isolate per individual gene three times intra- and inter-run.

The sensitivity and specificity of each multiplex PCR were calculated using the MedCalc diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic_test.php), which used the formulas found in Table 3.1.

3.4 Results

3.4.1 Multiplex PCR 1 and 2 results

Following testing with multiplex PCRs 1 and 2 the blinded panel was found to contain 42 16S RMTase gene-positive isolates and 23 gene-negative isolates. The results of each multiplex PCR can be found in Tables 3.2; the 16S RMTase gene content was correctly identified in all isolates.

3.4.2 Calculation of specificity, sensitivity and reproducibility

The analytical sensitivity of multiplex PCRs 1 and 2, which is the probability that the result will be positive when an 16S RMTase gene(s) is present, was found to be 100% (95% confidence interval [CI] 93.3-100) for both multiplex PCRs. The analytical specificity, which is the probability of the result being negative when an 16S RMTase genes is absent, was 100% (95% CI 94.6-100) for both multiplex PCRs. Both multiplex PCRs also had a reproducibility of 100% (Figures 3.1 and 3.2).

Table 3.1: Formulas for the calculation of sensitivity and specificity.

Statistic	Formula
Sensitivity	$\frac{\text{No. of true positives}}{\text{No. of true positives + No. of false negatives}}$
Specificity	$\frac{\text{No. of true negatives}}{\text{No. of false positives + No. of true negatives}}$

Table 3.2: Results of multiplex PCRs 1 and 2 in the detection of 16S RMTase genes in the blind panel of 16S RMTase-positive and -negative isolates, provided from PHE's AMRHAI Reference Unit. Isolates 13, 33, 34, 40, 41 and 42, positive for *rmtA*, *rmtD*, *rmtE*, *rmtG*, *rmtH* and *npmA*, respectively, were extracted an additional nine times to generate ten samples to test to calculate sensitivity.

Number	Species	16S RMTase gene present as determined by WGS	Multiplex 1 result	Multiplex 2 result
1	*C. freundii*	*armA*	*armA*	Negative
2	*C. freundii*	*armA*	*armA*	Negative
3	*E. cloacae* complex	*armA*	*armA*	Negative
4	*E. cloacae* complex	*armA*	*armA*	Negative
5	*E. coli*	*armA*	*armA*	Negative
6	*K. pneumoniae*	*armA*	*armA*	Negative
7	*K. pneumoniae*	*armA*	*armA*	Negative
8	*K. pneumoniae*	*armA*	*armA*	Negative
9	*Citrobacter* sp.	*armA*	*armA*	Negative
10	*E. coli*	*armA* + *rmtB*	*armA* + *rmtB*	Negative
11	*K. pneumoniae*	*armA* + *rmtF*	*armA*	*rmtF*
12	*K. pneumoniae*	*armA* + *rmtF*	*armA*	*rmtF*
13	*E. coli*	*rmtA*	*rmtA*	Negative
14	*E. cloacae* complex	*rmtB*	*rmtB*	Negative
15	*E. cloacae* complex	*rmtB*	*rmtB*	Negative
16	*E. coli*	*rmtB*	*rmtB*	Negative
17	*K. pneumoniae*	*rmtB*	*rmtB*	Negative
18	*K. pneumoniae*	*rmtB*	*rmtB*	Negative
19	*M. morganii*	*rmtB*	*rmtB*	Negative
20	*P. stuartii*	*rmtB*	*rmtB*	Negative
21	*C. freundii*	*rmtB* + *rmtC*	*rmtB* + *rmtC*	Negative
22	*K. pneumoniae*	*rmtB* + *rmtF*	*rmtB*	*rmtF*
23	*E. coli*	*rmtB* + *rmtF*	*rmtB*	*rmtF*
24	*C. freundii*	*rmtC*	*rmtC*	Negative
25	*C. freundii*	*rmtC*	*rmtC*	Negative
26	*E. cloacae* complex	*rmtC*	*rmtC*	Negative
27	*E. coli*	*rmtC*	*rmtC*	Negative
28	*K. pneumoniae*	*rmtC*	*rmtC*	Negative
29	*K. pneumoniae*	*rmtC*	*rmtC*	Negative
30	*P. rettgeri*	*rmtC*	*rmtC*	Negative
31	*E. coli*	*rmtC* + *rmtF*	*rmtC*	*rmtF*
32	*K. pneumoniae*	*rmtC* + *rmtF*	*rmtC*	*rmtF*
33	*P. aeruginosa*	*rmtD*	*rmtD*	Negative
34	*E. coli*	*rmtE*	Negative	*rmtE*
35	*K. oxytoca*	*rmtF*	Negative	*rmtF*
36	*K. pneumoniae*	*rmtF*	Negative	*rmtF*
37	*K. pneumoniae*	*rmtF*	Negative	*rmtF*

Table 3.2 continued

Number	Species	16S RMTase gene present as determined by WGS	Multiplex 1 result	Multiplex 2 result
38	*K. pneumoniae*	*rmtF*	Negative	*rmtF*
39	*K. pneumoniae*	*rmtF*	Negative	*rmtF*
40	*K. pneumoniae*	*rmtG*	Negative	*rmtG*
41	*K. pneumoniae*	*rmtH*	Negative	*rmtH*
42	*E. coli*	*npmA*	Negative	*npmA*
43	*A. baumannii*	Negative	Negative	Negative
44	*A. calcoaceticus*	Negative	Negative	Negative
45	*C. freundii*	Negative	Negative	Negative
46	*C. freundii*	Negative	Negative	Negative
47	*K. aerogenes*	Negative	Negative	Negative
48	*K. aerogenes*	Negative	Negative	Negative
49	*K. aerogenes*	Negative	Negative	Negative
50	*E. cloacae* complex	Negative	Negative	Negative
51	*E. cloacae* complex	Negative	Negative	Negative
52	*E. coli*	Negative	Negative	Negative
53	*E. coli*	Negative	Negative	Negative
54	*E. coli*	Negative	Negative	Negative
55	*E. coli*	Negative	Negative	Negative
56	*K. oxytoca*	Negative	Negative	Negative
57	*K. pneumoniae*	Negative	Negative	Negative
58	*K. pneumoniae*	Negative	Negative	Negative
59	*K. pneumoniae*	Negative	Negative	Negative
60	*Klebsiella* sp.	Negative	Negative	Negative
61	*Klebsiella* sp.	Negative	Negative	Negative
62	*P. aeruginosa*	Negative	Negative	Negative
63	*P. aeruginosa*	Negative	Negative	Negative
64	*P. mirabilis*	Negative	Negative	Negative
65	*P. stuartii*	Negative	Negative	Negative

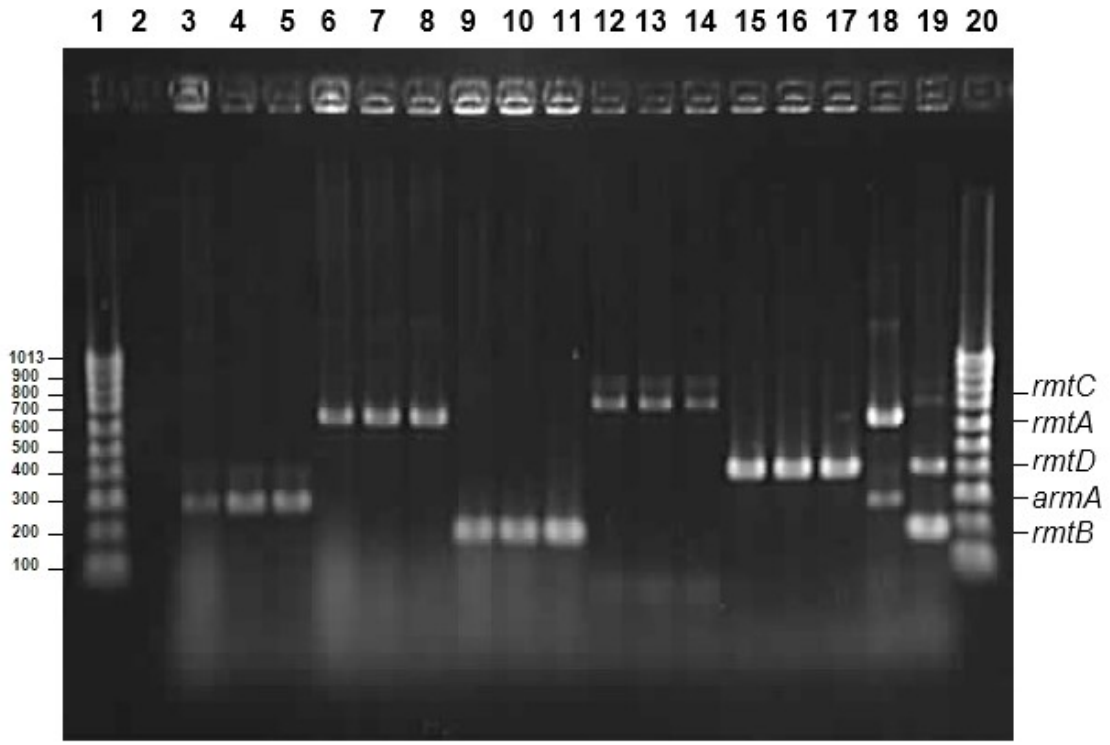


Figure 3.1: Reproducibility results for multiplex 1. Reproducibility was measured by testing one isolate per individual gene three times intra- and inter-run. Lane 1: 100 bp ladder; Lane 2: NTC; Lanes 3-4: *armA*; Lanes 6-8: *rmtA*; Lanes 9-11: *rmtB*; Lanes 12-14: *rmtC*; Lanes 15-17: *rmtD*; 18: Control A (*armA* and *rmtA*); 19: Control B (*rmtB, rmtC* and *rmtD*); Lane 20: 100 bp ladder.

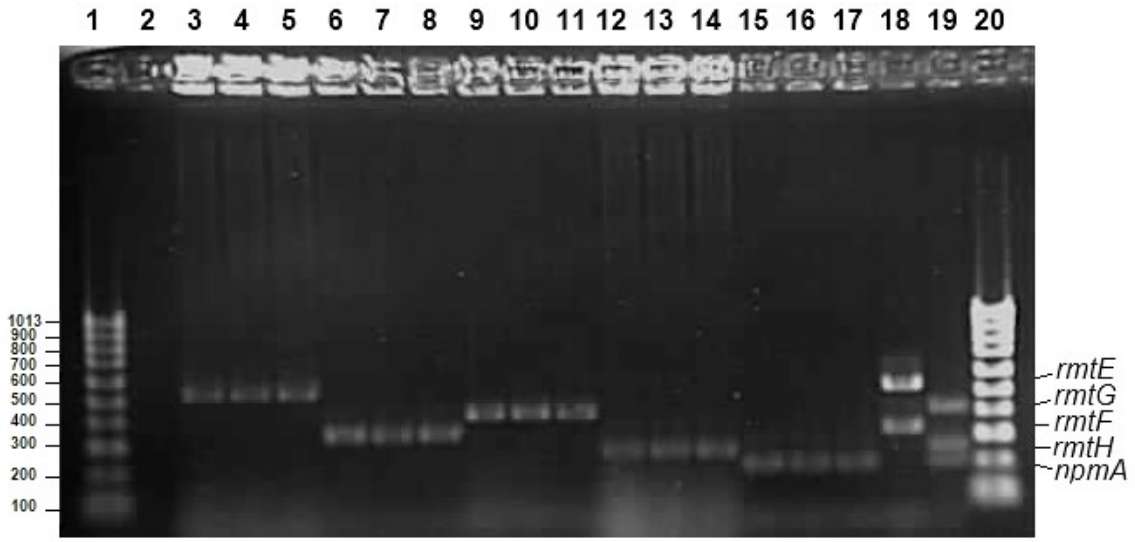


Figure 3.2: Reproducibility results for multiplex 2. Reproducibility was measured by testing one isolate per individual gene three times intra- and inter-run. Lane 1: 100 bp ladder; Lane 2: NTC; Lanes 3-4: *rmtE*; Lanes 6-8: *rmtF*; Lanes 9-11: *rmtG*; Lanes 12-14: *rmtH*; Lanes 15-17: *npmA*; 18: Control C (*rmtE* and *rmtF*); 19: Control D (*rmtG*, *rmtH* and *npmA*); Lane 20: 100 bp ladder.

3.5 Discussion

Over the years, several multiplex PCRs have been developed to detect 16S RMTase genes, but many do not identify all ten 16S RMTase gene families and the only published PCR to do so (Corrêa *et al.* [394]) was not able to identify the variant *rmtE2*. Therefore, a multiplex PCR detecting genes *rmtE/rmtE2, rmtF, rmtG, rmtH* and *npmA*, was designed to be used alongside the previously published PCR by Doi and Arakawa [304], detecting *armA* and *rmtA-rmtD,* under identical PCR conditions. Both PCRs were validated and found to have analytical sensitivity and specificity of 100%, as well as 100% reproducibility demonstrating that both multiplex PCRs can be used to identify all 16S RMTase genes and variants known to date.

Since carrying out this work, additional variants of 16S RMTase genes have been identified including *armA2*, *rmtB4*, *rmtD3* and *rmtF2* [140,396]. Analysis of the specificity of the primers used in the two multiplex PCRs to each of the variant genes showed that there were no nucleotide differences in the primer-binding sites of these newly identified variants, from which it may be inferred that the two multiplex PCRs described here could also be used to detect these novel variants.

Alternative testing methods are becoming available for the detection of 16S RMTases, including a loop-mediated isothermal amplification assay [174] for the rapid detection (<1 hour) of genes *armA*, *rmtA* and *rmtB*. Additionally, an immunochromatographic assay that uses novel monoclonal antibodies to detect ArmA within 15 minutes, was developed by Oshiro *et al.* [397] and a rapid (<4 hour) colorimetric test based on the detection of bacterial growth via glucose metabolism in the presence of a set concentration of amikacin and gentamicin was produced by Nordmann *et al*. [398]. These tests allow diagnostic laboratories that may not have access to PCR equipment to detect 16S RMTases, but the limitations of these assays include their inability to identify the specific resistance genes for outbreak or surveillance purposes (in the case of the colorimetric test); the lack of detection of all 16S RMTases (in the case of the loop-mediated isothermal amplification assay and the immunochromatographic assay), or the lack of specificity to detect 16S RMTases alone as the colorimetric test may

detect other resistance mechanisms causing aminoglycoside resistance such as AMEs. Also, the colorimetric test cannot be used for non-fermenting bacteria such as *A. baumannii* and *P. aeruginosa*, which are known to harbour 16S RMTase genes. Lastly, Nordmann *et al*. [399] have designed a culture medium containing 30 mg/L amikacin and gentamicin to detect 16S RMTases as well as AMEs that exhibit both amikacin and gentamicin resistance in pan-aminoglycoside-resistant Gram-negative bacteria. However, as the medium is not selective for bacteria only carrying 16S RMTase genes, additional methods such as PCR will need to be undertaken to confirm their presence.

One limitation of PCR is that it will not be able to detect novel 16S RMTase genes if the novel genes have significant sequence diversity compared to known 16S RMTase genes, whereas other detection methods such as the colorimetric test by Nordmann *et al*. [398] and the culture medium by Nordmann *et al*. [399] could. Additionally, PCR may detect 16S RMTase genes that are not being expressed, which may have detrimental effects on patient care if aminoglycosides are wrongly taken out of a patient's treatment regime, however, this has rarely been reported [153,400,401].

In conclusion, a multiplex PCR assay with a sensitivity, specificity and reproducibility of 100% was designed for the identification of 16S RMTase genes *rmtE/rmtE2, rmtF/rmtF2, rmtG, rmtH* and *npmA,* which can be used in conjunction with the multiplex PCR proposed by Doi and Arakawa [304] for the detection of all currently known 16S RMTase gene families and variants simultaneously for either diagnostic or surveillance purposes.

Chapter 4: A retrospective study to investigate the occurrence of 16S RMTase genes in pan-aminoglycoside-resistant Gram-negative bacteria referred to PHE's AMRHAI Reference Unit.

4.1 Introduction

Prior to the beginning of this study there were few data available on the occurrence of 16S RMTases in the UK. Hopkins *et al*. [200] identified *rmtC* in 23.2% (13/56) high-level amikacin-resistant (MIC >500 mg/L) *Salmonella enterica* isolated between January 2004 and December 2008. Mushtaq *et al*. [272] identified 16S RMTase genes in 100% (10/10) bla_{NDM}-producing *E. coli* isolates from ten UK hospitals, specifically *rmtC* (70.0%, 7/10) and *armA* (30.0%, 3/10). *rmtB* was identified in a bla_{NDM-5}-producing *E. coli* isolate from a patient who was previously hospitalized in India by Hornsey *et al.* [352], and Hidalgo *et al*. [164] identified *armA* in four high-level amikacin-resistant (MIC >500 mg/L) isolates belonging to *S. enterica* serovar Thompson (n=1) and *S. enterica* serovar Worthington (n=3). In a further study, Hidalgo *et al*. [206] screened 132 bla_{NDM}-producing Enterobacterales referred to PHE's AMRHAI Reference Unit between 2009 to 2011 for *armA* and *rmtA*-*rmtF*. In total 88.6% (117/132) of isolates were positive for 16S RMTase genes, with *rmtC* (49.2%, 65/132), followed by *armA* (47.0%, 62/132) the most common 16S RMTase genes identified.

In this study, Gram-negative bacterial isolates displaying pan-aminoglycoside resistance (MICs amikacin >64 mg/L, gentamicin >32 mg/L and tobramycin >32 mg/L), spanning 2003-2015 from the AMRHAI Reference Unit's strain collection were screened for 16S RMTase genes.

4.2 Aims

The aims of this study were to: i) identify the occurrence of 16S RMTase genes amongst pan-aminoglycoside-resistant Gram-negative bacterial isolates (*A. baumannii*, Enterobacterales and *P. aeruginosa*) archived in the AMRHAI strain collection, and ii) to determine if 16S RMTase genes were associated with particular bacterial clones.

4.3 Methods

4.3.1 Identification of pan-aminoglycoside-resistant isolates amongst AMRHAI's strain collection

Bacterial isolates (n=2,185) displaying pan-aminoglycoside resistance (MICs amikacin >64 mg/L, gentamicin >32 mg/L and tobramycin >32 mg/L) spanning from December 2003 to December 2015 were identified in the AMRHAI strain archive using the programme WHONET version 5.6 (WHO, Boston, MA, USA). The bacterial isolates in the AMRHAI strain archive were sent by laboratories from the UK and the Republic of Ireland for the investigation of unusual antibiotic resistance patterns (particularly carbapenem resistance). These isolates included 902 Enterobacterales, 856 *A. baumannii* and 427 *P. aeruginosa*.

WGS data were available for 1,819 carbapenemase-producing Enterobacterales (CPE) referred to AMRHAI between October 2012 and October 2015. These WGS data were generated either as part of AMRHAI's sequencing of the first novel CPE isolate from each new patient or as part of a research project [402].

Isolates were de-duplicated based on patient names, removing those with identical bacterial species, sample collection dates, the same PFGE and/or multi-locus VNTR profiles and, for those with WGS data available, identical antibiotic resistance gene content as well as ST. Additionally, isolates with existing WGS data were removed from the list of pan-aminoglycoside-resistant bacteria to be screened for 16S RMTase genes by PCR. Where MIC data were available, isolates that were not pan-aminoglycoside resistant were removed from the list of isolates with existing WGS data.

Deduplication resulted in a selection of 1,123 pan-aminoglycoside-resistant bacterial isolates, which were screened for 16S RMTase genes using the two multiplex PCRs described in Chapter 2, as well as an additional 453 isolates with WGS data, where antibiotic resistance gene content generated by Genefinder [403] was analysed for the presence of 16S RMTase genes. In total, these consisted of 806 (51.1%) Enterobacterales, 550 (34.9%) *A. baumannii*

and 220 (14.0%) *P. aeruginosa* (Figure 4.1). The remaining 428 isolates were unavailable for testing either because they had been discarded from storage (88.1%, 377/428) or were no longer viable (11.9%, 51/428) (Figure 4.1).

4.3.2 Retrieval of bacterial isolates from AMRHAI archives

Bacterial isolates were retrieved from either stocks frozen at -80 °C or original agar slopes from AMRHAI's archives. The isolates were streaked onto CLED agar plates and grown overnight at 37 °C.

4.3.3 Identification of 16S RMTase genes

DNA from the bacterial isolates was extracted as in Section 2.4.1.3 and was subjected to two multiplex PCRs to detect the genes *armA*, *rmtA*-*rmtH* and *npmA* as described in Sections 2.4.1.4 and 2.4.1.6.

4.3.4 Identification of carbapenemase genes

Carbapenemase genes were previously identified by the AMRHAI Reference Unit with bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$ and bla_{VIM} identified by in-house PCR as previously described [164,272,389,404] or by a rtPCR as described in Section 2.4.2. $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ genes were identified as previously described [405] and $bla_{GES\text{-}5}$, bla_{IMI}, bla_{IMP}, bla_{GIM}, bla_{SIM}, bla_{SME}, and bla_{SPM} were identified as previously described [389,406-408] or via Genefinder, where WGS data were available [403].

4.3.5 Determination of MICs

MICs were previously determined by the AMRHAI Reference Unit using the BSAC agar dilution method and interpreted using EUCAST guidelines (see Section 2.3).

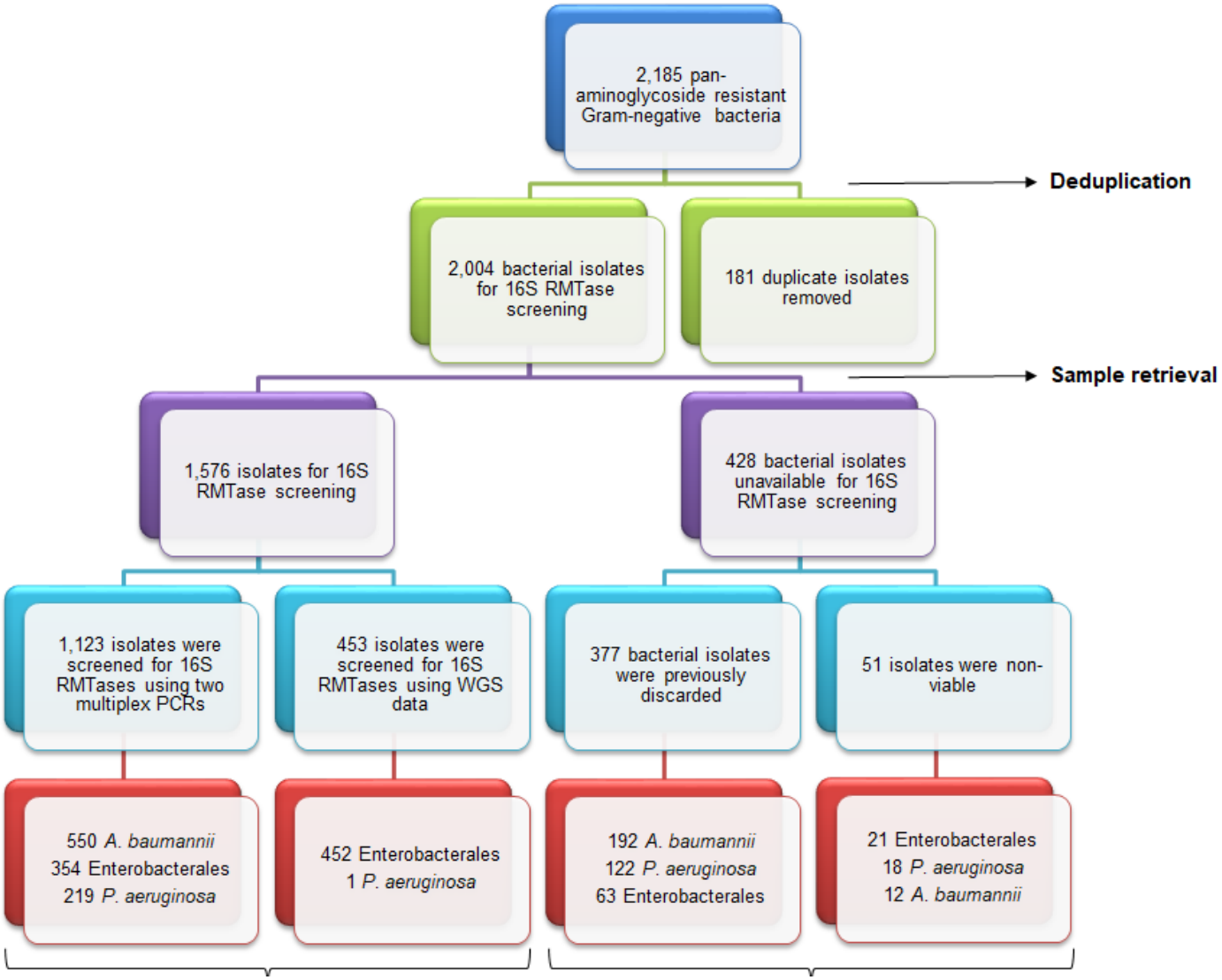


Figure 4.1: The process of deduplication and retrieval of Gram-negative bacterial isolates used throughout this study as well as the number of isolates that were discarded or non-viable.

4.3.6 Bacterial Typing

Bacterial typing was previously carried out by the AMRHAI Reference Unit by either VNTR (for *K. pneumoniae* and *P. aeruginosa*) or PFGE (for *A. baumannii* and Enterobacterales other than *K. pneumoniae*) as described in Section 2.5. Additionally, *A. baumannii* isolates were screened by the AMRHAI Reference Unit using a multiplex PCR targeting the porin gene *ompA*, the pilus assembly system gene *csuE* and the intrinsic carbapenemase gene $bla_{\text{OXA-51-like}}$ to determine if they belonged to International Clone II as previously described [409]. Isolates with WGS data available were typed using MLST data, which was generated using Genefinder [403] and eBURST diagrams were generated using the PHYLOViZ online tool (http://www.phyloviz.net/) [410]. Strain clusters were identified using Bionumerics Software version 6.1 (Applied Maths).

4.3.7 Analysis of 16S RMTase gene-negative isolates

PCR-negative *A. baumannii* and Enterobacterales isolates were streaked onto three sets of MH agar (Media Services, PHE Microbiology Services Colindale) supplemented with 256 mg/L amikacin, gentamicin or tobramycin and grown overnight at 37 °C. Isolates that grew on all three plates were sent for WGS as described in Section 2.6 and analysis of the data was conducted as per Section 4.3.8.

To screen for potentially novel 16S RMTase genes, DNA sequences of contigs were analysed using the 'sequence search' tool from Pfam 31.0 (https://pfam.xfam.org/) [411] to determine whether DNA within the contigs encoded proteins belonging to the same protein families as known 16S RMTases. A six-frame translation was performed by Pfam 31.0 to generate a set of protein sequences, where the presence of the ribosomal RNA methyltransferase family FmrO (Pfam ID: PF07091), which ArmA and RmtA-RmtH belong to, as well as the Methyltransf_4 family (Pfam ID: PF02390), which NpmA belongs to, could be identified. Two searches were carried out using different E-value cut-offs, where the default E-value of 1.0 was used as well as an E-value of 1×10^{-5}, where the latter provided a more stringent search.

4.3.8 WGS and analysis of WGS data

Additional WGS was carried out as described in Section 2.6 on 16S RMTase-producing *P. aeruginosa* isolates and an *rmtE*-positive *A. baumannii* isolate to determine MLST profiles and the genetic environment of the 16S RMTase genes. WGS was also performed on PCR-negative isolates displaying high-level aminoglycoside resistance in order to investigate whether these isolates harboured novel 16S RMTase genes.

Purity of the samples was checked using KmerFinder 3.0 (https://cge.cbs.dtu.dk/services/KmerFinder/) [412-414], antibiotic resistance genes were identified using ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/) [39] and MLST was conducted using MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/) [415]. Additionally, *rmtE3* was aligned with *rmtE1* and *rmtE2* using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) [416].

4.3.9 Analysis of patient demographic information

Anonymised patient demographic information was gathered from sample request forms sent to the AMRHAI Reference Unit from referring laboratories using WHONet version 5.6 (WHO).

4.3.10 Ethical approval

Ethical approval was not required as it was deemed to be an enhanced surveillance study.

4.3.11 Statistical analysis

Statistical analysis was performed using the Chi Square test (Excel 2016). A p value ≤0.05 was deemed to be statistically significant.

4.4 Results

4.4.1 Identification of 16S RMTase genes

Analysis of AMRHAI's WGS data of carbapenemase-producing Gram-negative bacterial isolates and the use of two multiplex PCRs identified one or more 16S RMTase genes in 83.2% (1,312/1,576) isolates, of which 58.1% (762/1,312) were Enterobacterales, 40.5%

(531/1,312) were *A. baumannii*, and 1.4% (19/1,312) were *P. aeruginosa* (Table 4.1). The most common 16S RMTase gene identified was *armA* (66.3%, 870/1,312), followed by *rmtC* (11.2%, 147/1,312), *rmtF* (10.7%, 140/1,312), *rmtB* (7.4%, 97/1,312), *rmtD* (0.4%, 5/1,312), and *rmtE* (0.1%, 1/1,312). Fifty-two (4.0%) isolates harboured various two gene combinations (Table 4.1). No *rmtA*, *rmtG*, *rmtH* or *npmA* genes were identified.

The distribution of 16S RMTase genes in different Gram-negative bacterial species can be found in Figure 4.2. *armA* alone was mainly found in *A. baumannii* (60.9%, 530/870) and accounted for almost all high-level aminoglycoside resistance detected in this species (96.4%, 530/550). *K. pneumoniae* accounted for over a quarter of *armA*-positive (28.9%, 251/870) isolates, and was also the most common species harbouring *rmtC* (47.6%, 70/147), *rmtF* (96.4%, 135/140) and two-gene combinations (73.1%, 38/52). In contrast, *rmtB* was mainly found in *E. coli* (76.3%, 74/97), including 55.6% (5/9) of *rmtB* genes in combination with other 16S RMTase genes. *rmtD* and *rmtE* were only found in 26.3% (5/19) *P. aeruginosa* isolates and 0.2% (1/531) *A. baumannii* isolates, respectively.

WGS of the 16S RMTase-producing *P. aeruginosa* isolates identified the *rmtB* subvariants *rmtB4* (90.0%, 9/10) and *rmtB1* (10.0%, 1/10); the *rmtD* subvariants *rmtD3* (80.0%, 4/5) and *rmtD1* (20.0%, 1/5) and the *rmtF* subvariant *rmtF2* (100%, 3/3).

Additionally, WGS of the *rmtE*-positive *A. baumannii* isolate identified a new subvariant, which had one SNP at nucleotide 20 (T→C), leading to a valine to alanine substitution and another SNP at nucleotide 141 (T→A), which substituted asparagine with lysine. The latter mutation was not found in *rmtE1* or *rmtE2* (Figure 4.3), therefore this subvariant was designated *rmtE3* (accession no: MH572011) according to the nomenclature scheme proposed by Doi *et al.* [417]. No subvariants were identified in the Enterobacterales.

Table 4.1: Total number of 16S RMTase genes found in pan-aminoglycoside-resistant Gram-negative bacteria (n=1,576) from AMRHAI's strain collection, 2003-2015.

Species	16S RMTase gene(s)													Total
	Negative	*armA*	*rmtB*	*rmtC*	*rmtD*	*rmtE*	*rmtF*	*armA* + *rmtB*	*armA* + *rmtC*	*armA* + *rmtF*	*rmtB* + *rmtC*	*rmtB* + *rmtF*	*rmtC* + *rmtF*	
A. baumannii	19	530	-	-	-	1	-	-	-	-	-	-	-	550
K. pneumoniae	19	251	8	70	-	-	135	1	17	15	-	2	3	521
P. aeruginosa	201	-	10	1	5	-	3	-	-	-	-	-	-	220
E. coli	10	33	74	38	-	-	-	1	1	1	1	3	-	162
***E. cloacae* complex**	5	11	3	17	-	-	-	-	2	-	-	-	-	38
***Klebsiella* spp.**	4	20	-	5	-	-	2	-	1	1	-	-	-	33
***Providencia* spp.**	-	6	1	5	-	-	-	-	2	-	-	-	-	14
C. freundii	1	5	-	5	-	-	-	-	-	-	1	-	-	12
***Enterobacter* spp.**	1	2	-	4	-	-	-	-	-	-	-	-	-	7
***Proteus* spp.**	1	5	-	1	-	-	-	-	-	-	-	-	-	7
***Citrobacter* spp.**	1	3	-	1	-	-	-	-	-	-	-	-	-	5
S. marcescens	2	2	-	-	-	-	-	-	-	-	-	-	-	4
M. morganii	-	1	1	-	-	-	-	-	-	-	-	-	-	2
Escherichia hermannii	-	1	-	-	-	-	-	-	-	-	-	-	-	1
Total	264	870	97	147	5	1	140	2	23	17	2	5	3	1,576

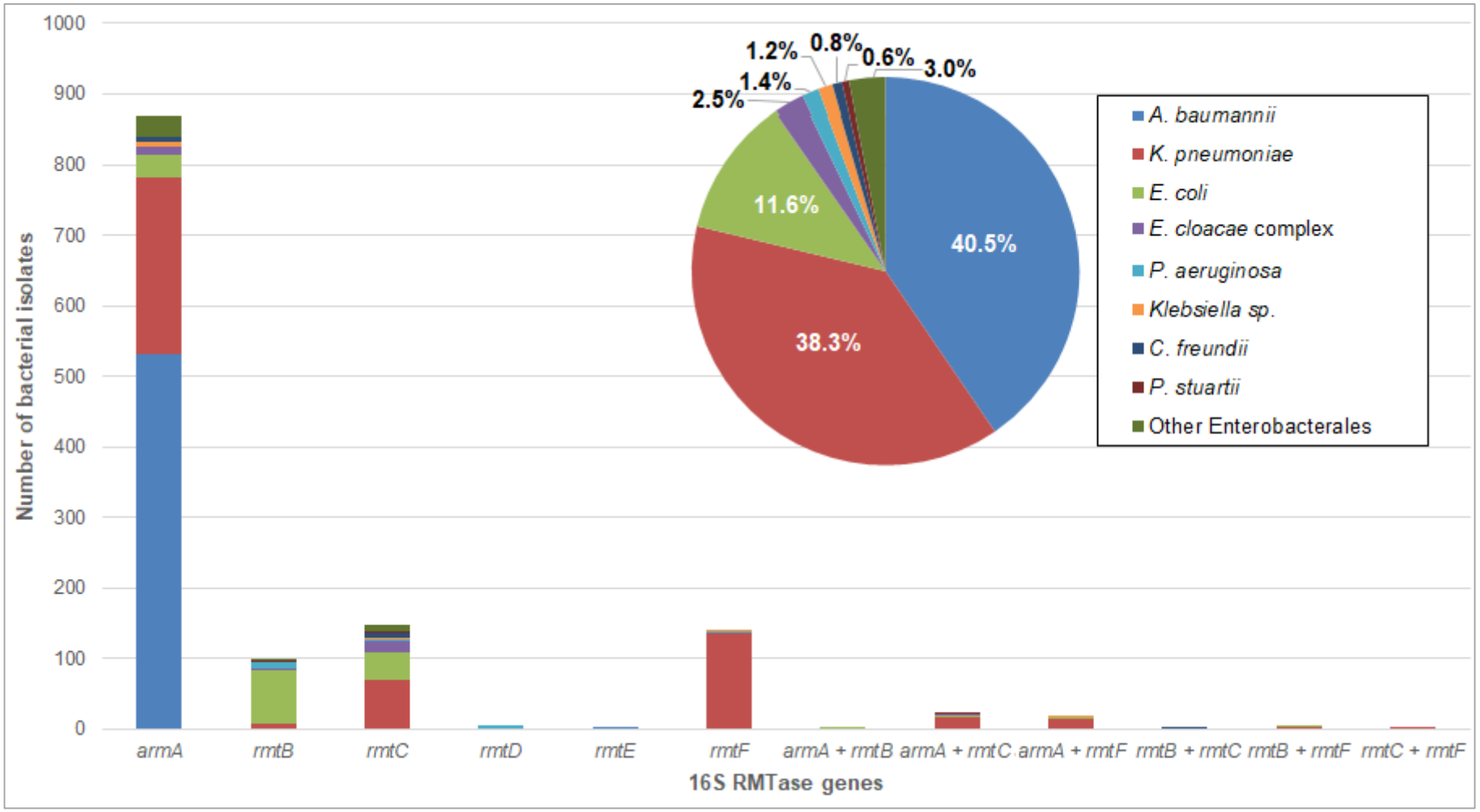


Figure 4.2: Total number of 16S RMTase genes found in pan-aminoglycoside resistant Gram-negative bacteria (n=1,312) from AMRHAI's strain collection, 2003-2015. **'**Other Enterobacterales' includes *Enterobacter* sp. (n=6), *K. oxytoca* (n=6), *P. mirabilis* (n=6), *P. rettgeri* (n=5), *K. aerogenes* (n=4), *C. koseri* (n=2), *Klebsiella terrigena* (n=2), *M. morganii* (n=2), S. *marcescens* (n=2), *Citrobacter* sp. (n=1), *Citrobacter sedlakii* (n=1), *E. hermannii* (n=1), *Klebsiella ornithinolytica* (n=1), and *Providencia* sp. (n=1). The pie chart shows the total percentage of 16S RMTase-positive species.

```
rmtE1      ATGAATATTGATGAAATGGTTGCAGAGGTTCTGTCGAGCAAAAAATATACAAGCGTTGAC 60
rmtE3      ATGAATATTGATGAAATGGCTGCAGAGGTTCTGTCGAGCAAAAAATATACAAGCGTTGAC 60
rmtE2      ATGAATATTGATGAAATGGCTGCAGAGGTTCTGTCGAGCAAAAAATATACAAGCGTTGAC 60
           ********** ********** ********** ********** **********

rmtE1      CCTGCTGTTGTTAGGCGTGTTTGTATGGAAACAGCACCTAAATATCCGAAGAAAAAAGAA 120
rmtE3      CCTGCTGTTGTTAGGCGTGTTTGTATGGAAACAGCACCTAAATATCCGAAGAAAAAAGAA 120
rmtE2      CCTGCTGTTGTTAGGCGTGTTTGTATGGAAACAGCACCTAAATATCCGAAGAAAAAAGAA 120
           ********** ********** ********** ********** **********

rmtE1      GCAATAAAAGCGGTAAAAAATGAACTGCATATCATCCATGAGGTTTTTTTGCAAAACGAA 180
rmtE3      GCAATAAAAGCGGTAAAAAAAGAACTGCATATCATCCATGAGGTTTTTTTGCAAAACGAA 180
rmtE2      GCAATAAAAGCGGTAAAAAATGAACTGCATATCATCCATGAGGTTTTTTTGCAAAACGAA 180
           ********** ********** ********** ********** **********

rmtE1      TGTTACAAAAATGCGCTTTCATTTTTATCACAGCTATCCTTAGATTTTAATAATGCGCAA 240
rmtE3      TGTTACAAAAATGCGCTTTCATTTTTATCACAGCTATCCTTAGATTTTAATAATGCGCAA 240
rmtE2      TGTTACAAAAATGCGCTTTCATTTTTATCACAGCTATCCTTAGATTTTAATAATGCGCAA 240
           ********** ********** ********** ********** **********

rmtE1      TTGATCGATATTACTATGCAAATCATGCAATCACATACATCAACCAAAGAGAGATTAGGC 300
rmtE3      TTGATCGATATTACTATGCAAATCATGCAATCACATACATCAACCAAAGAGAGATTAGGC 300
rmtE2      TTGATCGATATTACTATGCAAATCATGCAATCACATACATCAACCAAAGAGAGATTAGGC 300
           ********** ********** ********** ********** **********

rmtE1      GACATTGAAGCGGTTTGTTCATTCTTAAGCACCCATATATCCAAAGAGGGTTCCGTGATG 360
rmtE3      GACATTGAAGCGGTTTGTTCATTCTTAAGCACCCATATATCCAAAGAGGGTTCCGTGATG 360
rmtE2      GACATTGAAGCGGTTTGTTCATTCTTAAGCACCCATATATCCAAAGAGGGTTCCGTGATG 360
           ********** ********** ********** ********** **********

rmtE1      GACATCGGTTGTGGCTTCAATCCATTTGCGTTGCCATTACTGCACGAGTTCCCGGCAACG 420
rmtE3      GACATCGGTTGTGGCTTCAATCCATTTGCGTTGCCATTACTGCACGAGTTCCCGGCAACG 420
rmtE2      GACATCGGTTGTGGCTTCAATCCATTTGCGTTGCCATTACTGCACGAGTTCCCGGCAACG 420
           ********** ********** ********** ********** **********

rmtE1      TATTATGCTTATGATATATGTTCAGAAGGAATTAACATCCTAAACAAATATTTCTCCATC 480
rmtE3      TATTATGCTTATGATATATGTTCAGAAGGAATTAACATCCTAAACAAATATTTCTCCATC 480
rmtE2      TATTATGCTTATGATATATGTTCAGAAGGAATTAACATCCTAAACAAATATTTCTCCATC 480
           ********** ********** ********** ********** **********

rmtE1      CTAAAAAAAGGAGAATATAGAGCGGAGCTACTTGATGCCGTGTCTGTTACGCCGAAAGAA 540
rmtE3      CTAAAAAAAGGAGAATATAGAGCGGAGCTACTTGATGCCGTGTCTGTTACGCCGAAAGAA 540
rmtE2      CTAAAAAAAGGAGAATATAGAGCGGAGCTACTTGATGCCGTGTCTGTTACGCCGAAAGAA 540
           ********** ********** ********** ********** **********

rmtE1      AAGGTGGATGTTGCCCTCCTATTCAAGCTATTACCTTTGTTGCAGCAACAGAAAAAAGGT 600
rmtE3      AAGGTGGATGTTGCCCTCCTATTCAAGCTATTACCTTTGTTGCAGCAACAGAAAAAAGGT 600
rmtE2      AAGGTGGATGTTGCCCTCCTATTCAAGCTATTACCTTTGTTGCAGCAACAGAAAAAAGGT 600
           ********** ********** ********** ********** **********

rmtE1      CGAGGTTTCAGCATTTTAGAAGAACTGGACTTTGACAAAGCAATTGTATCTTTTCCAATA 660
rmtE3      CGAGGTTTCAGCATTTTAGAAGAACTGGACTTTGACAAAGCAATTGTATCTTTTCCAATA 660
rmtE2      CGAGGTTTCAGCATTTTAGAAGAACTGGACTTTGACAAAGCAATTGTATCTTTTCCAATA 660
           ********** ********** ********** ********** **********

rmtE1      AAAAGCTTGGGAGGAAAGCAAAAAGGAATGGAAACCTTTTATTCGAATTTGTTTGAAGAA 720
rmtE3      AAAAGCTTGGGAGGAAAGCAAAAAGGAATGGAAACCTTTTATTCGAATTTGTTTGAAGAA 720
rmtE2      AAAAGCTTGGGAGGAAAGCAAAAAGGAATGGAAACCTTTTATTCGAATTTGTTTGAAGAA 720
           ********** ********** ********** ********** **********

rmtE1      AACCTGCCTTCCTCATTGGAAATCATTGAGAAGCAGACTTTTTCAAATGAGATGTTCTAT 780
rmtE3      AACCTGCCTTCCTCATTGGAAATCATTGAGAAGCAGACTTTTTCAAATGAGATGTTCTAT 780
rmtE2      AACCTGCCTTCCTCATTGGAAATCATTGAGAAGCAGACTTTTTCAAATGAGATGTTCTAT 780
           ********** ********** ********** ********** **********

rmtE1      GTGATACAAAACAAAACCAAAAACGGAGGAAATCAATCATGA   822
rmtE3      GTGATACAAAACAAAACCAAAAACGGAGGAAATCAATCATGA   822
rmtE2      GTGATACAAAACAAAACCAAAAACGGAGGAAATCAATCATGA   822
           ********** ********** ********** *****
```

Figure 4.3: Alignment of *rmtE3* with *rmtE1* (GU201947.1) and *rmtE2* (KU130396.1) to identify location of SNPs.

Table 4.2 shows the different specimen types that the 16S RMTase-positive and 16S RMTase-negative bacterial isolates were cultured from. 16S RMTase-producing isolates were more commonly isolated from screening swabs (26.2%, 344/1,312) compared to 13.3% (35/264) 16S RMTase-negative isolates (p <0.05). However, 16S RMTase-negative isolates were more frequently isolated from respiratory (26.1%, 69/264) and tissue and fluid (26.1%, 69/264) samples.

16S RMTase-producing isolates were referred by 161 diagnostic laboratories. The majority (51.4%, 675/1,312) of 16S RMTase-producing isolates were referred by laboratories in London, which was also the region with the highest number of referring laboratories (20.5%, 33/161), followed by the West Midlands (9.7%, 127/1,312). *armA* was the most common 16S RMTase gene identified in each region (Figure 4.4).

16S RMTase genes were not identified in 91.4% (201/220) *P. aeruginosa*, 3.5% (19/550) *A. baumannii* and 5.5% (44/806) Enterobacterales, which consisted of *Klebsiella* spp. (4.2%, 23/551), *E. coli* (6.2%, 10/162), *E. cloacae* complex (13.2%, 5/38), *Citrobacter* spp. (14.3%, 2/14), *S. marcescens* (50.0%, 2/4), *Enterobacter* sp. (14.3%, 1/7) and *Proteus* sp. (100%, 1/1).

Table 4.2: Sites of isolation for the 16S RMTase-positive (n=1,312) and 16S RMTase-negative (n=264) bacterial isolates from AMRHAI's strain collection, 2003-2015.

16S RMTase gene	Species	Specimen type								
		Blood and line tip	Environmental	Faecal	Respiratory	Screening swab	Tissue and fluid	Urine	Unknown	Total
Positive	*A. baumannii*	42	2	-	139	84	130	75	59	531
	Enterobacterales	51	7	10	47	259	127	234	27	762
	P. aeruginosa	1	-	-	1	1	7	8	1	19
Negative	*A. baumannii*	4	-	-	6	1	6	2	-	19
	Enterobacterales	3	-	1	2	13	10	15	-	44
	P. aeruginosa	15	-	-	61	21	53	41	10	201
Total		116	9	11	265	379	333	375	97	1,576

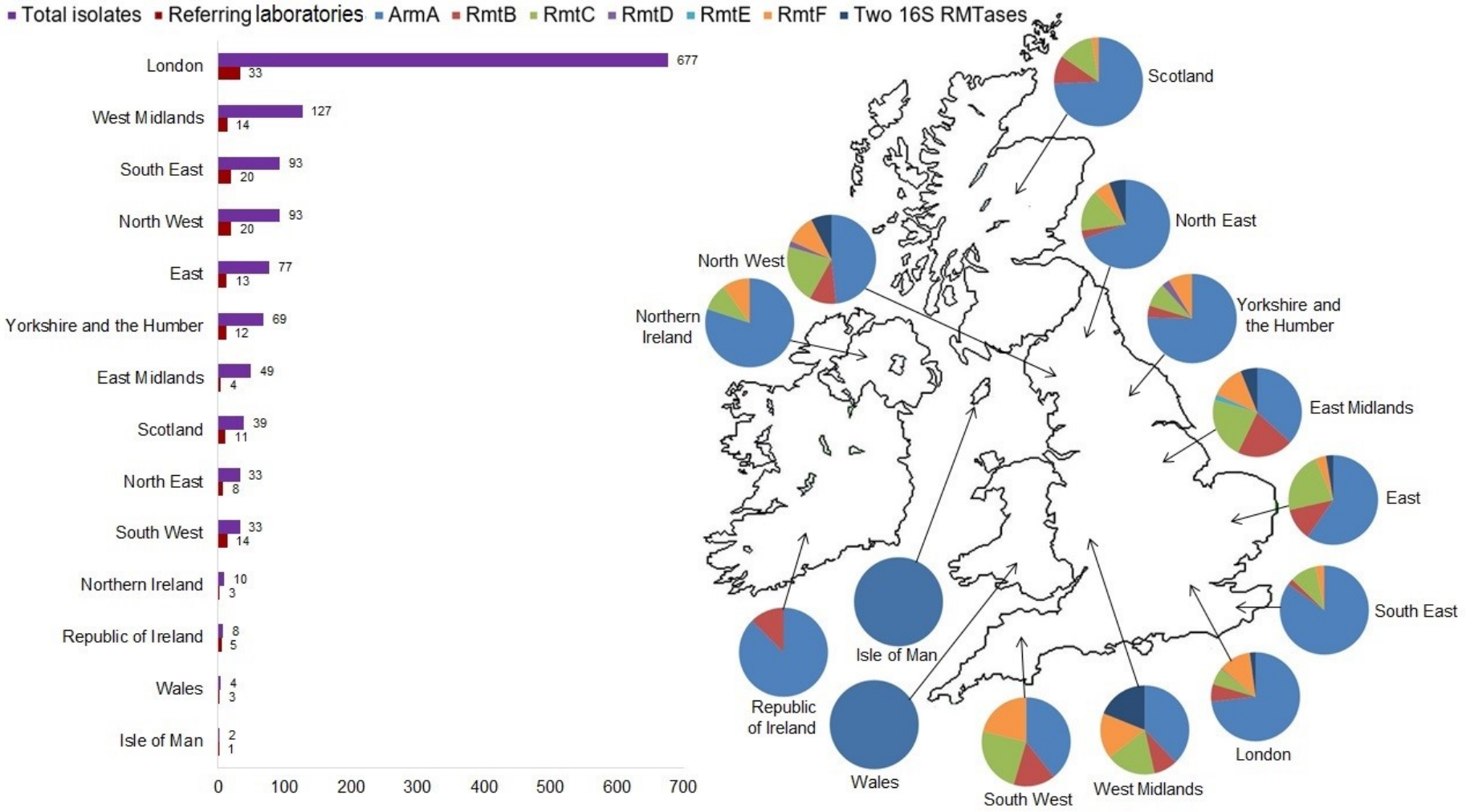


Figure 4.4: The geographical distribution of 16S RMTase-positive Gram-negative bacterial isolates (n=1,312) in the UK and Republic of Ireland, 2003-2015. The total number of 16S RMTase-positive isolates per region, as well as the number of diagnostic laboratories they came from, can be found on the left-hand side. On the right-hand side is the geographical distribution of 16S RMTase-positive isolates. The pie charts indicate diversity of 16S RMTase genes identified from each region according to legend above.

4.4.2 Antibiotic susceptibility

MIC data for 16S RMTase-positive and -negative *A. baumannii* isolates are shown in Table 4.3. Over 97% of 16S RMTase-positive isolates were resistant to ciprofloxacin, imipenem and meropenem but 1.7% (9/531) isolates were resistant to colistin. Additionally, all 16S RMTase-negative isolates showed resistance to ciprofloxacin, imipenem and meropenem but 5.3% (1/19) isolates were resistant to colistin. No EUCAST clinical breakpoints were available for ceftazidime or tigecycline so resistance could not be calculated.

More than 99% of 16S RMTase-positive Enterobacterales isolates (Table 4.4) were resistant to ceftazidime and cefotaxime and 97.3% (712/732) and 92.9% (684/736) were resistant to ertapenem and ciprofloxacin, respectively. However, 86.1% (637/740), 75.5% (558/739), 16.2% (118/729) and 13.8% (101/730) isolates showed resistance to meropenem, imipenem, tigecycline and colistin, respectively. More than 95% of 16S RMTase-negative isolates were resistant to cefotaxime, ceftazidime as well as ertapenem and 90.9% (40/44) isolates were resistance to ciprofloxacin. In contrast to 16S RMTase-positive isolates, lower levels of resistance were found for imipenem (31.8%, 14/44) and meropenem (22.7%, 10/44) but rates of resistance were higher for colistin (31.8%, 14/44) and tigecycline (26.8%, 11/41).

All 16S RMTase-positive *P. aeruginosa* isolates (Table 4.5) showed resistance to ceftazidime as well as ciprofloxacin, and 94.7% (18/19) and 89.5% (17/19) isolates were resistant to imipenem and meropenem, respectively. None of the isolates were resistant to colistin. All 16S RMTase-negative *P. aeruginosa* isolates showed resistance to ceftazidime, but 98.5% (197/200), 93.0% (187/201) and 92.5% (186/201) isolates were resistant to ciprofloxacin, meropenem and imipenem, respectively. Nine percent (18/201) of isolates were resistant to colistin.

MIC values of the other antibiotics tested can be found in Tables 1, 2 and 3 in Appendix 1.

Table 4.3: MIC values for 16S RMTase-positive (n=531) and -negative (n=19) *A. baumannii* isolates from AMRHAI's strain collection, 2003-2015. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	% R
imipenem (0.06-128)	≤2/>8	Positive					2	1	10	71	323	117	7	-	97.6
		Negative								8	9	2		-	100
meropenem (0.06-32)	≤2/>8	Positive				1	1	2	1	47	478[a]			1	99.1
		Negative								4	15[a]			-	100
ceftazidime (0.125-256)	-	Positive							1	1	3	17	198	311	-
		Negative										1	12	6	-
ciprofloxacin (0.125-8)	≤1/>1	Positive						1	530[a]					-	100
		Negative							19[a]					-	100
colistin (0.5-32)	≤2/>2	Positive			319[b]	194	9	3	2	2	2[a]			-	1.7
		Negative			12[b]	6			1					-	5.3
tigecycline (0.25-16)	-	Positive		7[b]	78	41	54	17	8	9[a]				317	-
		Negative			3	3	6	2						5	-

[a] = MIC greater than or equal to indicated value; [b] = MIC less than or equal to indicated value. ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 4.4: MIC values for 16S RMTase-positive (n=762) and -negative (n=44) Enterobacterales isolates from AMRHAI's strain collection, 2003-2015. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	% R
ertapenem (0.125-16)	≤0.5/>1	Positive	4	3	5	8	7	8	17	680[a]				30	97.3
		Negative	2				3	1	9	28[a]				1	95.3
imipenem (0.06-128)	≤2/>8	Positive	4	16	13	18	22	40	68	135	187	138	98	23	75.5
		Negative			2	8	12	6	2	5	7	1	1	-	31.8
meropenem (0.06-32)	≤2/>8	Positive	23	5	6	13	15	18	23	91	546[a]			22	86.1
		Negative	4	2		4	8	10	6		10[a]			-	22.7
cefotaxime (0.125-256)	≤1/>2	Positive				2	1		2	1	16	36	682	22	99.6
		Negative	1				1			1	1	5	35	-	95.5
ceftazidime (0.125-256)	≤1/>4	Positive					1	1	3	3	4	8	720	22	99.7
		Negative	1							1	2	4	36	-	97.7
ciprofloxacin (0.125-8)	≤0.25/>0.5	Positive	39	10	3	12	11	23	638[a]					26	92.9
		Negative	2	1	1	1			39[a]					-	90.9
colistin (0.5-32)	≤2/>2	Positive			439[b]	174	16	12	30	17	42[a]			32	13.8
		Negative			15[b]	12	3	1	1	2	10[a]			-	31.8
tigecycline (0.25-16)	≤1/>2	Positive		99[b]	129	191	192	87	25	6[a]				33	16.2
		Negative		7[b]	6	7	10	8	3					3	26.8

[a] = MIC greater than or equal to indicated value; [b] = MIC less than or equal to indicated value. ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 4.5: MIC values for 16S RMTase-positive (n=19) and -negative (n=201) *P. aeruginosa* isolates from AMRHAI's strain collection, 2003-2015. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	% R
imipenem (0.06-128)	≤4/>8	Positive					1				4	4	10	-	94.7
		Negative				3	1	5	6	17	35	12	122	-	92.5
meropenem (0.06-32)	≤2/>8	Positive						1	1	2	15[a]			-	89.5
		Negative			1	2	3	2	6	20	167[a]			-	93.0
ceftazidime (0.125-256)	≤8/>8	Positive										1	18	-	100
		Negative								4	6	10	179	2	100
ciprofloxacin (0.125-8)	≤0.5/>0.5	Positive							19[a]					-	100
		Negative		1	2	4	8	10	175[a]					1	98.5
colistin (0.5-32)	≤2/>2	Positive			2[b]	13	4							-	0
					16[b]	113	54	6	2	4	6[a]			-	9.0

[a] = MIC greater than or equal to indicated value; [b] = MIC less than or equal to indicated value. ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

4.4.3 Analysis of carbapenemase genes

A total of 94.3% (1,237/1,312) 16S-RMTase-positive isolates were positive for one or more carbapenemase genes (Figure 4.5). *bla*$_{NDM}$ (48.7%, 602/1,237) was the most common carbapenemase gene identified and was associated with all 16S RMTase genes (including two-gene combinations) apart from *rmtE* (Table 4.6). The carbapenemase gene combination *bla*$_{OXA-23-like}$ + *bla*$_{OXA-51-like}$, which was specific to *A. baumannii*, was commonly associated with *armA* (54.1% 493/912), and was the most common carbapenemase combination for this species (92.8%, 493/531). *bla*$_{OXA-48-like}$ was mostly found in *K. pneumoniae* (88.8%, 150/169) and was more frequently associated with *rmtF* (46.7%, 77/165) compared with other 16S RMTase genes (Table 4.6). *bla*$_{VIM}$ was the most common carbapenemase gene identified in 16S RMTase-producing *P. aeruginosa* (47.4%, 9/19).

Analysis of the WGS data (including 449 carbapenemase-producing isolates, 19 16S RMTase-producing *P. aeruginosa* and the *rmtE*-positive *A. baumannii* isolate) also identified *bla*$_{NDM}$ as the most common carbapenemase gene amongst 16S RMTase-producing isolates (77.6%, 364/469), with *bla*$_{NDM-1}$ the most common variant (82.4%, 300/364) followed by *bla*$_{NDM-5}$ (12.1%, 44/364), *bla*$_{NDM-4}$ (3.6%, 13/364) and *bla*$_{NDM-7}$ (1.9%, 7/364) (Table 4.7). *bla*$_{NDM-1}$ was the most common variant associated with all 16S RMTase genes apart from *rmtB*, which was more frequently associated with *bla*$_{NDM-5}$ (54.1%, 40/74) compared with *bla*$_{NDM-1}$ (9.5%, 7/74). *bla*$_{OXA-48-like}$ genes were identified in 29.6% (139/469) isolates including *bla*$_{OXA-232}$ (56.8%, 79/139), *bla*$_{OXA-181}$ (29.5%, 41/139) and *bla*$_{OXA-48}$ (13.7%, 19/139). *bla*$_{OXA-48-like}$ genes were more frequently associated with *rmtF*-positive isolates than *bla*$_{NDM}$ as 64.4% (58/90) isolates had *bla*$_{OXA-48-like}$ genes compared with 40.0% (36/90) *bla*$_{NDM}$-positive isolates, with *bla*$_{OXA-232}$ the most common variant (50.0%, 45/90). The *rmtE* gene was found associated with *bla*$_{OXA-65}$ + *bla*$_{OXA-72}$, which are *bla*$_{OXA-51-like}$ and *bla*$_{OXA-24-like}$ carbapenemases, respectively.

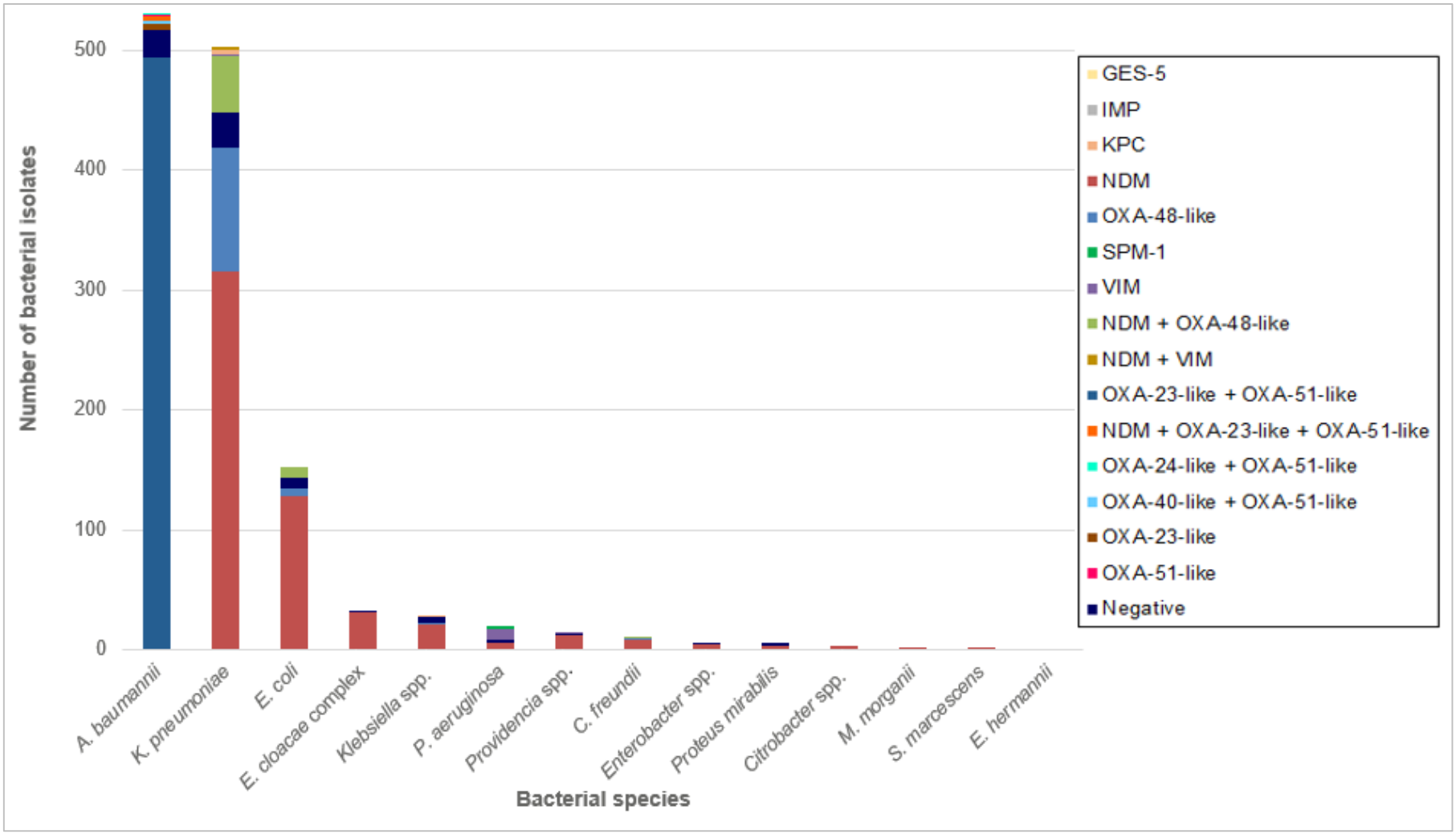


Figure 4.5: Carbapenemases identified in bacterial isolates positive for 16S RMTase genes (n=1,312) from AMRHAI's strain collection, 2003-2015.

Table 4.6: Total number of carbapenemase genes found in 16S RMTase-positive (n=1,312) and 16S RMTase-negative (n=264) isolates from AMRHAI's strain collection, 2003-2015.

16S RMTase gene(s)	Carbapenemase gene(s)																	
	Negative	*bla*$_{NDM}$	*bla*$_{OXA-23-like}$ + *bla*$_{OXA-51-like}$	*bla*$_{VIM}$	*bla*$_{OXA-48-like}$	*bla*$_{NDM}$ + *bla*$_{OXA-48-like}$	*bla*$_{KPC}$	*bla*$_{IMP}$	*bla*$_{OXA-23-like}$	*bla*$_{OXA-24-like}$ + *bla*$_{OXA-51-like}$	*bla*$_{OXA-40-like}$ + *bla*$_{OXA-51-like}$	*bla*$_{NDM}$ + *bla*$_{OXA-23-like}$ + *bla*$_{OXA-51-like}$	*bla*$_{OXA-51-like}$	*bla*$_{OXA-51-like}$ + *bla*$_{OXA-58-like}$	*bla*$_{GES-5}$	*bla*$_{NDM}$ + *bla*$_{VIM}$	*bla*$_{SPM-1}$	Total
Negative	90	5	17	129	6	1	8	6	-	-	-	-	-	2	-	-	-	264
armA	51	250	493	-	31	26	4	-	5	-	3	3	2	-	1	1	-	870
rmtB	5	70	-	7	3	10	1	1	-	-	-	-	-	-	-	-	-	97
rmtC	3	130	-	1	1	12	-	-	-	-	-	-	-	-	-	-	-	147
rmtD	-	1	-	3	-	-	-	-	-	-	-	-	-	-	-	-	1	5
rmtE	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1
rmtF	14	55	-	-	65	6	-	-	-	-	-	-	-	-	-	-	-	140
armA + rmtB	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
armA + rmtC	2	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	23
armA + rmtF	-	4	-	-	11	2	-	-	-	-	-	-	-	-	-	-	-	17
rmtB + rmtC	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
rmtB + rmtF	-	3	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	5
rmtC + rmtF	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
Total	165	546	510	140	118	58	13	7	5	1	3	3	2	2	1	1	1	1,576

Table 4.7: Carbapenemase gene variants found in 16S RMTase-positive bacteria via WGS from AMRHAI's strain collection, 2013-2015.

Carbapenemase gene(s)	16S RMTase gene(s)												Total
	armA	*rmtB*	*rmtC*	*rmtD*	*rmtE*	*rmtF*	*armA* + *rmtB*	*armA* + *rmtC*	*armA* + *rmtF*	*rmtB* + *rmtC*	*rmtB* + *rmtF*	*rmtC* + *rmtF*	
***bla*$_{NDM-1}$**	156	9	55	1	-	30	1	2	-	1	-	1	256
***bla*$_{NDM-4}$**	-	4	3	-	-	-	-	-	-	-	-	-	7
***bla*$_{NDM-5}$**	2	37	-	-	-	-	-	-	-	-	1	-	40
***bla*$_{NDM-7}$**	1	4	1	-	-	-	-	-	-	-	1	-	7
***bla*$_{OXA-48}$**	7	1	-	-	-	1	-	-	1	-	-	-	10
***bla*$_{OXA-181}$**	9	-	1	-	-	8	-	-	5	-	1	-	24
***bla*$_{OXA-232}$**	8	1	-	-	-	43	-	-	-	-	-	-	52
***bla*$_{NDM-1}$ + *bla*$_{OXA-48}$**	1	-	3	-	-	4	-	-	-	-	-	-	8
***bla*$_{NDM-1}$ + *bla*$_{OXA-181}$**	2	-	4	-	-	-	-	-	2	-	-	-	8
***bla*$_{NDM-1}$ + *bla*$_{OXA-232}$**	20	2	3	-	-	2	-	-	-	-	-	-	27
***bla*$_{NDM-4}$ + *bla*$_{OXA-48}$**	-	-	1	-	-	-	-	-	-	-	-	-	1
***bla*$_{NDM-4}$ + *bla*$_{OXA-181}$**	-	5	-	-	-	-	-	-	-	-	-	-	5
***bla*$_{NDM-5}$ + *bla*$_{OXA-181}$**	-	3	-	-	-	-	-	-	-	-	1	-	4
***bla*$_{NDM-1}$ + *bla*$_{VIM-4}$**	1	-	-	-	-	-	-	-	-	-	-	-	1
***bla*$_{OXA-65}$ + *bla*$_{OXA-72}$**	-	-	-	-	1	-	-	-	-	-	-	-	1
***bla*$_{GES-5}$**	1	-	-	-	-	-	-	-	-	-	-	-	1
***bla*$_{IMP-5}$**	-	1	-	-	-	-	-	-	-	-	-	-	1
***bla*$_{KPC-2}$**	1	1	-	-	-	-	-	-	-	-	-	-	2
***bla*$_{KPC-3}$**	1	-	-	-	-	-	-	-	-	-	-	-	1
***bla*$_{SPM-1}$**	-	-	-	1	-	-	-	-	-	-	-	-	1
***bla*$_{VIM-1}$**	-	1	-	-	-	-	-	-	-	-	-	-	1
***bla*$_{VIM-2}$**	-	5	-	3	-	-	-	-	-	-	-	-	8
***bla*$_{VIM-18}$**	-	-	1	-	-	-	-	-	-	-	-	-	1

Analysis of the 16S RMTase-negative isolates (Table 4.6) showed that 65.9% (174/264) harboured carbapenemase genes with bla_{VIM} (74.1%, 129/174) the most common; bla_{NDM} was found in low numbers (3.4%, 6/174) compared with bla_{NDM} in the 16S RMTase-producing isolates (48.7%, 602/1,237). Additionally, bla_{OXA-48} was found at a lower frequency (4.0%, 7/174) compared with bla_{OXA-48} in 16S RMTase-producing bacteria (13.7%, 169/1,237); conversely, bla_{KPC} was found at a higher frequency in 16S RMTase-negative isolates (4.6%, 8/174) compared with 16S RMTase-positive isolates (0.4%, 5/1,237). The differences in occurrence of the total number of carbapenemase genes in 16S RMTase-positive and -negative isolates were found to be statistically significant (p <0.05).

4.4.4 Typing of 16S RMTase-producing bacterial isolates

4.4.4.1 PFGE analysis

A total of 96.0% (510/531) 16S RMTase-producing *A. baumannii* were typed by PFGE (Figure 4.6). A cut-off value of 85% similarity identified 87 distinct PFGE patterns. There were 66 clusters of isolates (ranging from two to 14 isolates) sharing identical profiles, of which 16 PFGE profiles represented clusters from individual laboratories. A total of 89.9% (473/526) isolates were found to belong to International Clone II according to Turton *et al*. [409]. The *rmtE*-positive isolate was confirmed as belonging to ST79 by MLST.

In total 54.6% (83/152) 16S RMTase-producing *E. coli* isolates were typed by PFGE (Figure 4.7), where a cut-off value of 85% similarity identified 67 distinct PFGE patterns. Only two pairs of isolates sharing identical PFGE patterns were identified: two isolates from different patients in the Republic of Ireland, which were *armA* and bla_{NDM} positive, and two isolates from the West Midlands and London, which were *rmtB*- and bla_{NDM}-positive and were from different patients.

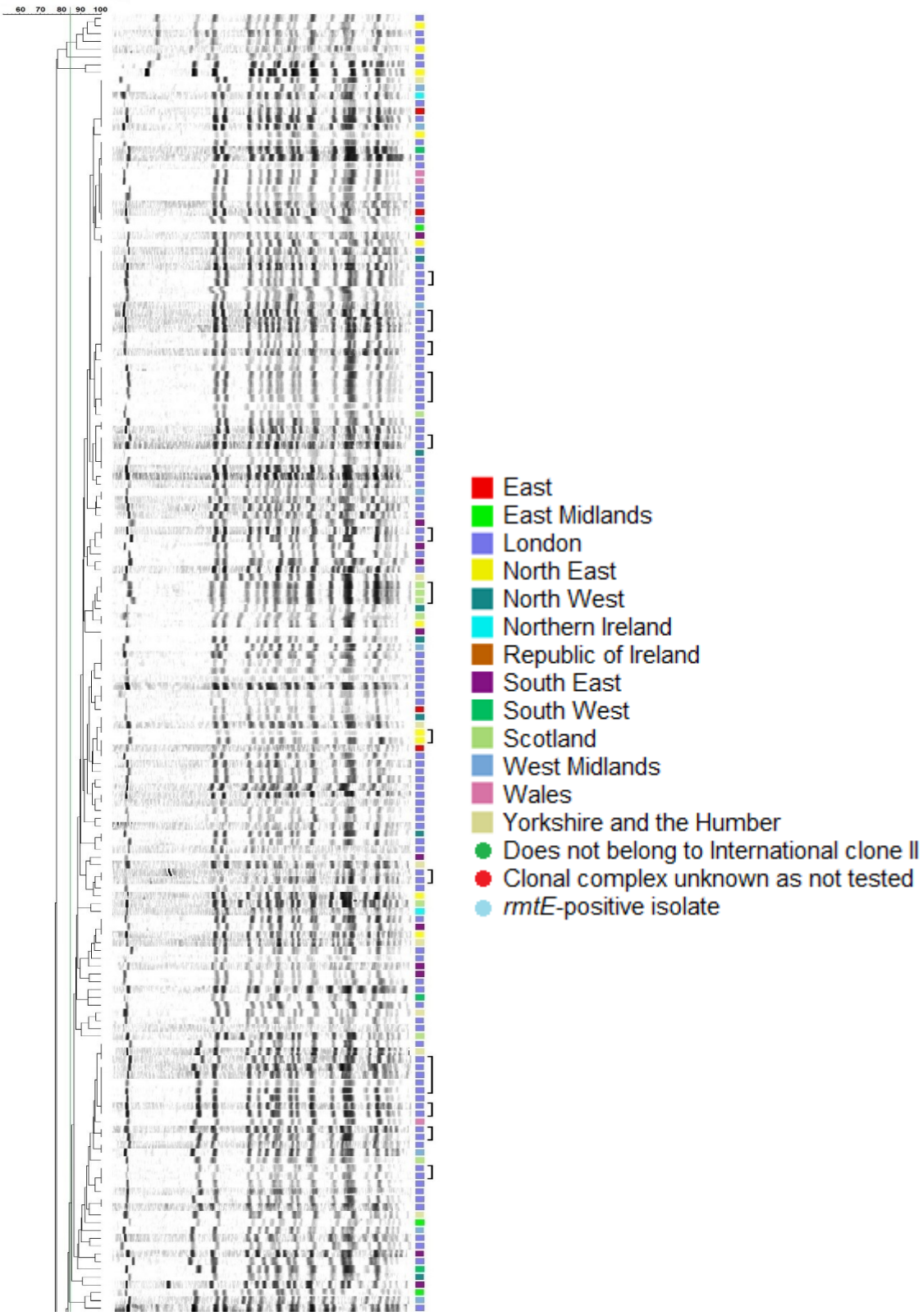


Figure 4.6: PFGE results for 16S RMTase-producing *A. baumannii* isolates (n=510) from AMRHAI's strain collection, 2003-2015. Brackets indicate isolates that were part of hospital clusters. The scale represents percentage of genetic similarity, where the 85% cut-off value to identify distinct PFGE profiles is signified by the green line.

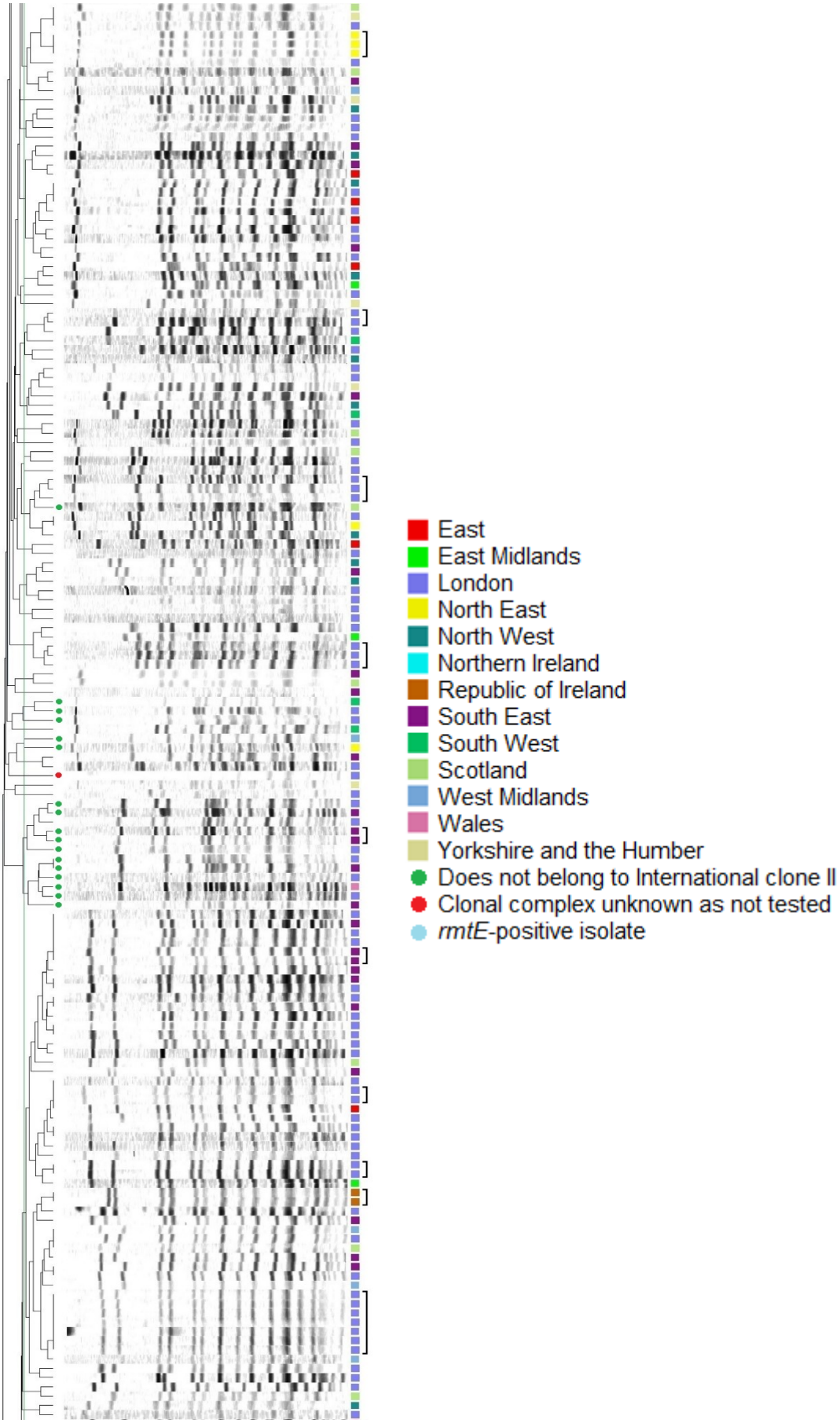


Figure 4.6 continued

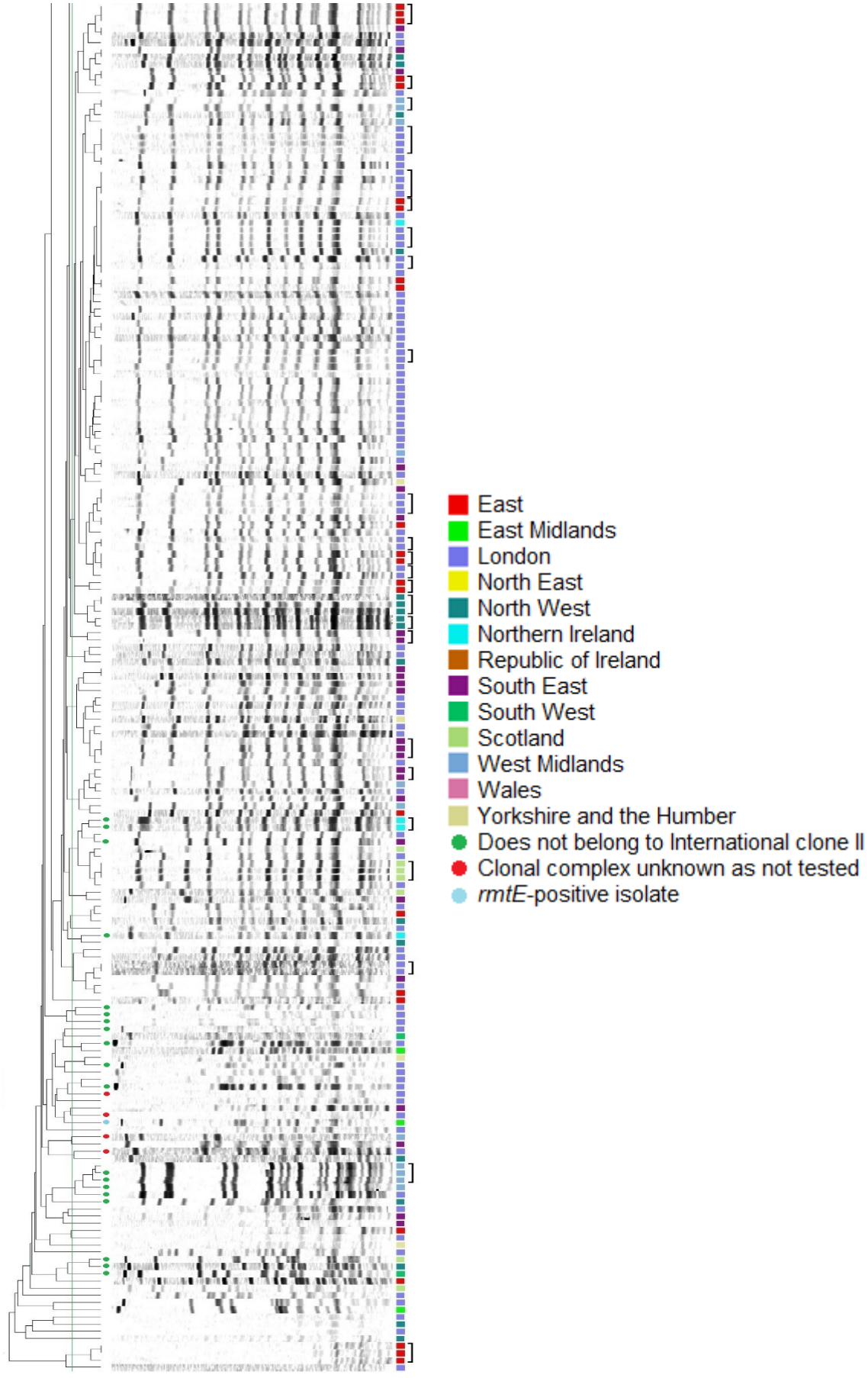


Figure 4.6 continued

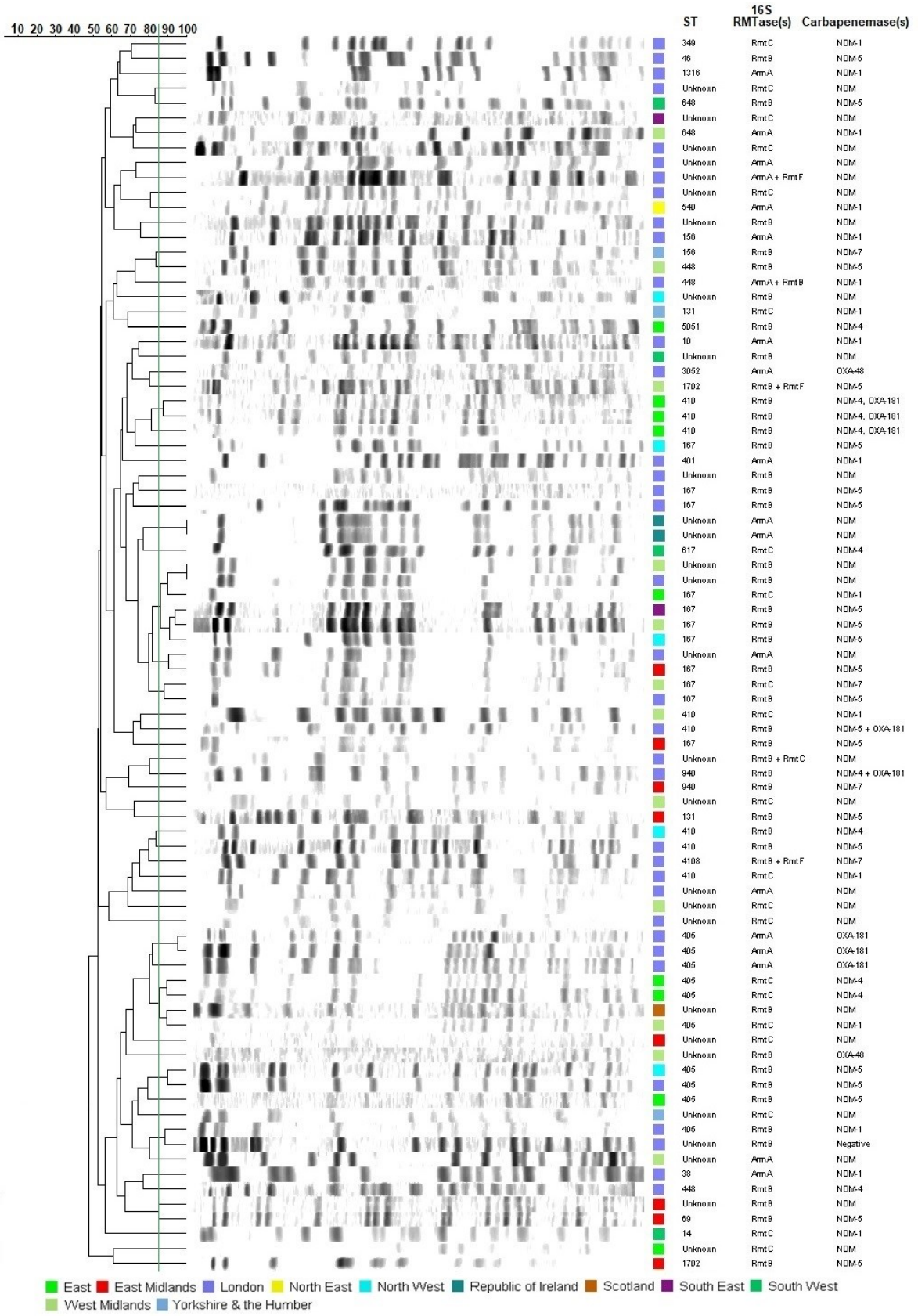


Figure 4.7: PFGE results for 16S RMTase-producing *E. coli* isolates (n=83) from AMRHAI's strain collection, 2003-2015. STs were derived from WGS data. The scale represents percentage of genetic similarity, where the 85% cut-off value to identify distinct PFGE profiles is signified by the green line.

A total of 75.8% (25/33) 16S RMTase-producing *E. cloacae* complex isolates were typed by PFGE (Figure 4.8), where a cut-off value of 85% similarity identified 23 distinct PFGE patterns. Two sets of isolates shared 85% similarity including two *armA*- and *bla*$_{NDM-1}$-positive isolates from Yorkshire and the Humber, which were from the same patient as well as two *rmtB*-positive isolates from the West Midlands, which were from different patients.

Additionally, 81.8% (9/11) 16S RMTase-producing *C. freundii* were typed by PFGE (Figure 4.9) where a cut-off value of 85% similarity identified nine distinct PFGE profiles and 83.3% (5/6) 16S RMTase-producing *K. oxytoca* were typed by PFGE (Figure 4.10), where five distinct PFGE patterns were observed with a cut-off value of 85% similarity.

4.4.4.2 VNTR analysis

VNTR analysis of 16S RMTase-producing *P. aeruginosa* identified 16 different VNTR profiles (Figure 4.11). Three pairs of *P. aeruginosa* isolates shared identical VNTR profiles. Those with profile 10,2,5,4,4,2,6,4,12, which were *rmtB* and *bla*$_{VIM}$ positive, were from the same patient. The isolates with profile 13,2,1,5,2,3,6,5,14, which were *rmtD*- and *bla*$_{VIM}$-positive were from the same laboratory but different patients, whilst those with profile 11,3,2,-,3,1,9,3,11, which were *rmtB* and *bla*$_{NDM}$ positive, were not from the same patient or referring laboratory.

VNTR data were available for 63.5% (319/502) 16S RMTase-producing *K. pneumoniae* isolates, amongst which 48 different VNTR profiles were identified (see Table 4.8 for VNTR profiles found in more than five isolates and see Table 4 in Appendix 1 for the VNTR profiles found in the remaining isolates). Several VNTR profiles were involved in outbreaks including profiles 6,3,4,0,1,1,3,1 and 6,3,4,0,1,1,4,1, where 62.2% (23/37) and 6.7% (3/45) *armA* and *bla*$_{NDM-1}$-positive ST14 *K. pneumoniae* isolates with these profiles, respectively, were part of an outbreak in a multicentre group of London hospitals [418]. Other strain clusters were identified, which were referred from the same laboratories such as *armA* + *rmtC*-positive (100%, 14/14) and *rmtC*-positive isolates (100%, 1/1) with profile 3,6,5,1,2,1,3,2 from the West

Midlands; *rmtF*-positive isolates (100%, 7/7) with profile 4,5,3,5,2,2,2,3 from London and *rmtC*-positive isolates (66.7%, 6/9) with profile 5,3,5,14,14,2,4,2 from London.

4.4.4.3 MLST

MLST data were obtained from the 450 carbapenemase-producing isolates that underwent WGS and were available for *K. pneumoniae* (n=315), *E. coli* (n=87), *E. cloacae* complex (n=18), *C. freundii* (n=9) and *K. oxytoca* (n=1), where 32, 25, 12, six and one STs were identified, respectively. MLST data was not available for *K. aerogenes*, *K. ornithinolytica*, *E. hermannii, M. morganii*, *P. mirabilis*, *P. rettgeri* and *P. stuartii* due to no MLST schemes being currently available. Additionally, MLST data were obtained for 16S RMTase-producing *P. aeruginosa* isolates (n=19) and the *rmtE*-positive *A. baumannii* isolate following WGS.

The most common STs identified in *K. pneumoniae* (Table 4.9) were ST14 (35.6%, 112/315), ST231 (15.6%, 49/315) and ST147 (10.5%, 33/315), which represented 61.6% (194/315) of the *K. pneumoniae* isolates. *K. pneumoniae* ST14 was mainly associated with *armA* (97.3%, 109/112) and $bla_{\text{NDM-1}}$ (90.2%, 101/112) and was mainly referred by 15 laboratories in London (80.4%, 90/112). Variation was observed within ST14 isolates that underwent VNTR analysis (Table 4.8) as three different VNTR profiles (6,3,4,0,1,1,4,1 [n=45], 6,3,4,0,1,1,3,1 [n=37] and 6,3,4,0,1,1,2,1 [n=18]) were identified. Most *K. pneumoniae* ST231 isolates were referred by 11 laboratories in London (59.2%, 29/49) and were mainly associated with *rmtF* (77.6%, 38/49) and $bla_{\text{OXA-232}}$ (63.3%, 31/49). No variation was found amongst ST231 isolates that had undergone VNTR analysis as only one VNTR profile was identified (Table 4.8). ST147 was mostly identified in London (54.5%, 18/33), where it was referred by seven laboratories and was frequently associated with *rmtF* (33.3%, 11/33) as well as $bla_{\text{NDM-1}}$ (72.7%, 24/33). Variation was observed in ST147 isolates that had undergone VNTR analysis as two VNTR profiles (5,3,5,14,14,2,4,2 [n=43] and 5,3,5,14,1,2,4,2 [n=1]) were identified (Table 4.8). The association between ST and carriage of a 16S RMTase gene can be seen in Figure 4.12.

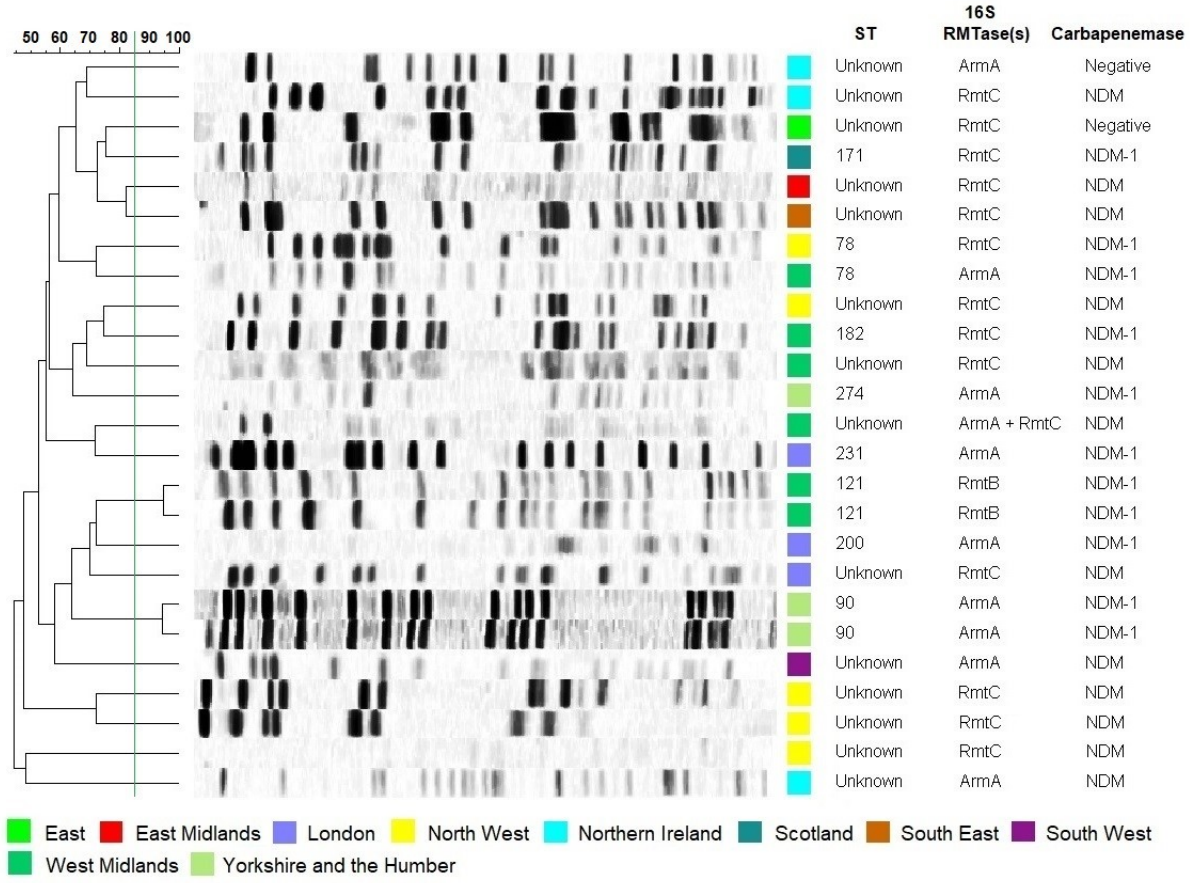


Figure 4.8: PFGE results for 16S RMTase-producing *E. cloacae* complex isolates (n=25) from AMRHAI's strain collection, 2003-2015. STs were derived from WGS data. The scale represents percentage of genetic similarity, where the 85% cut-off value to identify distinct PFGE profiles is signified by the green line.

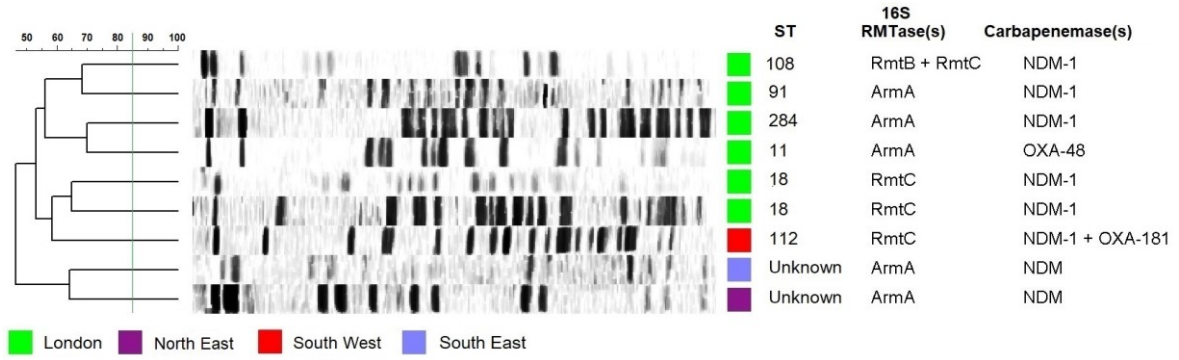


Figure 4.9: PFGE results for 16S RMTase-producing *C. freundii* isolates (n=9) from AMRHAI's strain collection, 2003-2015. STs were derived from WGS data. The scale represents percentage of genetic similarity, where the 85% cut-off value to identify distinct PFGE profiles is signified by the green line.

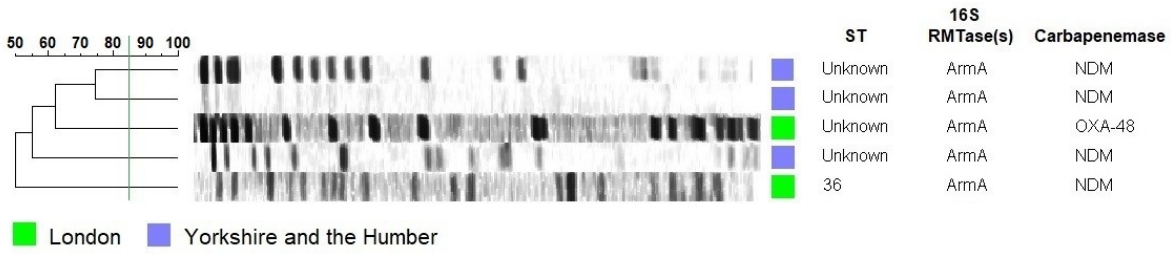


Figure 4.10: PFGE results for 16S RMTase-producing *K. oxytoca* isolates (n=5) from AMRHAI's strain collection, 2003-2015. STs were derived from WGS data. The scale represents percentage of genetic similarity, where the 85% cut-off value to identify distinct PFGE profiles is signified by the green line.

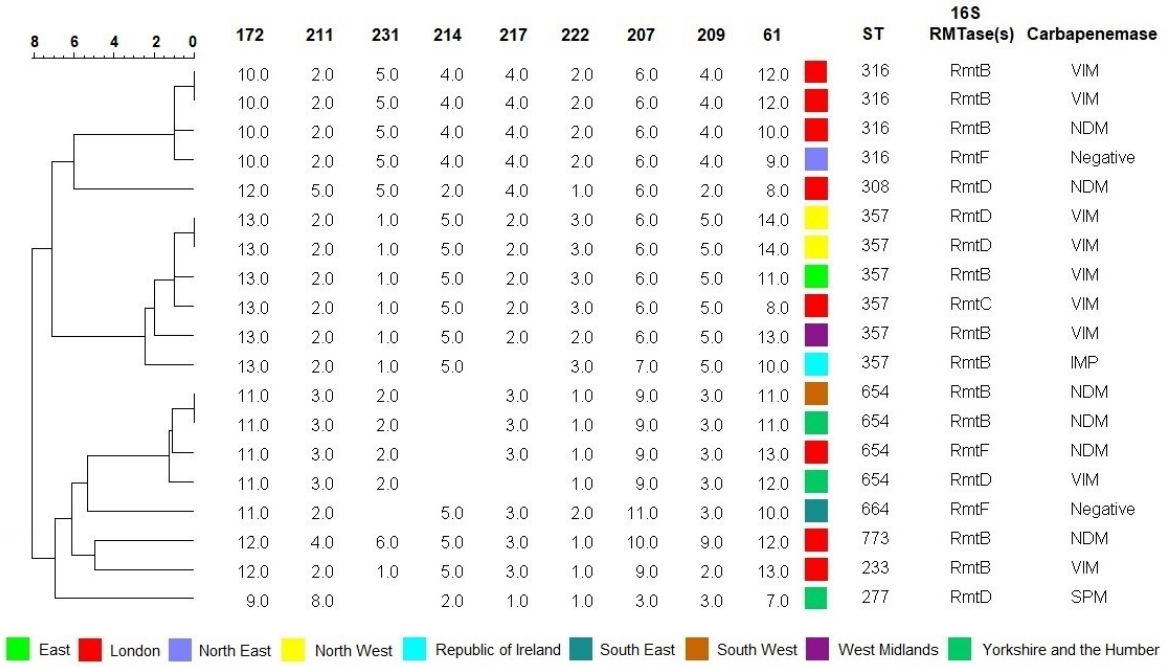


Figure 4.11: VNTR analysis of 16S RMTase-producing *P. aeruginosa* isolates (n=19) from AMRHAI's strain collection, 2003-2015. STs were inferred from WGS data. Gaps in the VNTR profile indicate loci that failed to amplify. The scale represents the number of differences in loci.

Table 4.8: VNTR profiles found in ≥5 *K. pneumoniae* isolates (n=319) producing 16S RMTases from AMRHAI's strain collection, 2003-2015.

Number of isolates	Inferred ST	VNTR profile (A,E,H,J,K,D,N1,N2)	16S RMTase(s) (no. of isolates)	Carbapenemase(s) (no. of isolates)	Geographical distribution (no. of isolates)
56	231	3,6,5,1,2,1,3,2	RmtF (30)	OXA-232 (20)	London (13), North West (3) and South West (2), East (1), and East Midlands (1),
				Negative (1)	London (1)
				NDM-1 (4) and NDM (5)	West Midlands (9)
			ArmA + RmtF (7)	OXA-48-like (3) and OXA-181 (1)	North West (3) and London (1)
				NDM + OXA-181 (2)	London (1) and North West (1)
				NDM (1)	West Midlands (1)
			ArmA (4)	OXA-181 (3)	London (3)
				NDM (1)	London (1)
			RmtC (1)	NDM (1)	West Midlands (1)
			ArmA + RmtC (14)	NDM (14)	West Midlands (14)
45	14	6,3,4,0,1,1,4,1	ArmA (40)	NDM-1 (12) and NDM (6)	London (9), Yorkshire and the Humber (5), West Midlands (2) and East Midlands (2)
				NDM-1 + OXA-232 (16) and NDM + OXA-48-like (1)	London (8), West Midlands (5), East (1), North West (1), Republic of Ireland (1) and South East (1)
				OXA-232 (2) and OXA-48 (2)	East (1), North West (1), South East (1) and Yorkshire and the Humber (1)
				Negative (1)	South East (1)
			RmtB (1)	OXA-232 (1)	London (1)
			RmtC (3)	NDM (2) and NDM-1 (1)	London (2) and North West (1)
			RmtF (1)	NDM (1)	London (1)

Table 4.8 continued

Number of isolates	Inferred ST	VNTR profile (A,E,H,J,K,D,N1,N2)	16S RMTase(s) (no. of isolates)	Carbapenemase(s) (no. of isolates)	Geographical distribution (no. of isolates)
43	147	5,3,5,14,14,2,4,2	ArmA (16)	NDM (9) and NDM-1 (4)	London (9), North West (1), South East (2) and South West (1)
				NDM-1 + OXA-181 (2)	London (2)
				OXA-232 (1)	East (1)
			RmtF (15)	NDM-1 + OXA-48 (1)	London (1)
				NDM (3) and NDM-1 (3)	London (2), North West (1) West Midlands (2) and Yorkshire and the Humber (1)
				OXA-48-like (5) and OXA-181 (2)	London (4), South East (1) and West Midlands (2)
				Negative (1)	London (1)
			RmtC (9)	NDM-1 (5), NDM (3) and NDM-1 + OXA-181 (1)	London (9)
			RmtB + RmtF (1)	NDM-5 + OXA-181 (1)	London (1)
			RmtC + RmtF (1)	NDM (1)	West Midlands (1)
			RmtB (1)	KPC-2 (1)	East (1)
37	14	6,3,4,0,1,1,3,1	ArmA (37)	NDM-1 (34) and NDM (2)	London (36)
				Negative (1)	London (1)
18	14	6,3,4,0,1,1,2,1	ArmA (17)	NDM-1 (15) and NDM (2)	London (17)
			ArmA + RmtF (1)	NDM (1)	London (1)
17	11 (4) and unknown (5)	3,5,2,13,2,1,3,4	RmtF (10)	NDM (5) and NDM-1 (4)	London (6), West Midlands (1), East Midlands (1) and South West (1)
	11 (1)			NDM-1 + OXA-48 (1)	West Midlands (1)
	340 (1) and unknown (4)		RmtC (6)	NDM (4) and NDM-1 (1)	East Midlands (3), London (1) and West Midlands (1)
	11 (1)			OXA-181 (1)	London (1)
	11 (1)		ArmA + RmtF (1)	OXA-48 (1)	London (1)

Table 4.8 continued

Number of isolates	Inferred ST	VNTR profile (A,E,H,J,K,D,N1,N2)	16S RMTase(s) (no. of isolates)	Carbapenemase(s) (no. of isolates)	Geographical distribution (no. of isolates)
16	15	3,3,3,0,1,1,4,1	ArmA (10)	NDM-1 (7) NDM (3)	London (4), North West (3), South East (2) and West Midlands (1)
			RmtC (4)	NDM-1 (4)	Yorkshire and the Humber (2), East Midlands (1) and West Midlands (1)
			RmtF (1)	NDM-1 (1)	London (1)
			ArmA + RmtC (1)	NDM (1)	West Midlands (1)
9	336	4,5,3,5,2,2,2,3	RmtF (7)	NDM (5) and NDM-1 (2)	London (7)
			ArmA (1)	NDM (1)	Yorkshire and the Humber (1)
			RmtC (1)	NDM (1)	West Midlands (1)
8	11	13,5,2,13,2,1,3,4	ArmA (4)	NDM-1 (3) and OXA-181 (1)	London (4)
			RmtC (2)	NDM-1 (2)	East (2)
			RmtF (1)	Negative (1)	London (1)
			ArmA + RmtF (1)	OXA-48-like (1)	West Midlands (1)
6	437	3,5,2,13,2,1,3,3	RmtF (5)	OXA-232 (3)	London (3)
				NDM (1)	London (1)
				NDM-1 + OXA-232 (1)	London (1)
			ArmA (1)	NDM (1)	North West (1)
6	515	3,2,2,5,2,1,3,3	ArmA (6)	NDM-1 (6)	London (6)
5	101	5,4,1,1,-,2,4,4	RmtC (2)	NDM-1 (1) and NDM + OXA-48-like (1)	South West (2)
			RmtF (2)	NDM-1 (1) and OXA-232 (1)	London (1) and West Midlands (1)
			ArmA (1)	OXA-48-like (1)	London (1)
5	11	-,5,2,13,2,1,3,4	RmtC (3)	NDM-1 (3)	London (2) and North West (1)
	1680		ArmA (1)	NDM-1 (1)	London (1)
	2208		RmtF (1)	NDM-1 (1)	London (1)

Loci that failed to amplify are designated with '-'; isolates belonging to ST11, ST340, ST1680 and ST2208 share similar VNTR profiles (*,5,2,13,2,1,3,4) where locus A differs as they belong to clonal complex 11.

Table 4.9: The most common STs found in 16S RMTase-producing *K. pneumoniae* isolates (n=315) as well as their association with carbapenemases and geographical distribution.

ST (no. of isolates)	16S RMTase (no. of isolates)	Carbapenemase (no. of isolates)	Geographical distribution (no. of laboratories)
14 (112)	ArmA (109)	NDM-1 (78)	London (13)
		NDM-1 + OXA-232 (20)	London (8), East (1), North West (1), South West (1) and West Midlands (1)
		OXA-232 (7)	London (4), South East (1) and Yorkshire and the Humber (1)
		OXA-48 (2)	East (1)
		NDM-1 + VIM-4 (1)	London (1)
		OXA-181 (1)	London (1)
	RmtB (1)	OXA-232 (1)	London (1)
	RmtC (2)	NDM-1 (1)	London (1)
		NDM-1 + OXA-181 (1)	East (1)
231 (49)	ArmA (5)	NDM-1 (2)	London (2)
		OXA-181 (3)	London (2)
	ArmA + RmtF (6)	OXA-181 (4)	London (2) and North West (1)
		NDM-1 + OXA-181 (2)	London (1) and North West (1)
	RmtF (38)	OXA-232 (31)	London (21), East Midlands (2), South West (2), Yorkshire and the Humber (2), East (1) and North West (1)
		NDM-1 (7)	West Midlands (3) and Yorkshire and the Humber (1)
147 (33)	ArmA (10)	NDM-1 (7)	London (2), North West (1), South East (1) and South West (1)
		NDM-1 + OXA-181 (2)	London (1)
		OXA-232 (1)	East (1)
	RmtB (1)	KPC-2 (1)	East (1)
	RmtB + RmtF (2)	NDM-5 + OXA-181 (1)	London (1)
		OXA-181 (1)	North East (1)
	RmtC (9)	NDM-1 (9)	London (2), North West (1), South West (1) and Scotland (1)

Table 4.9 continued

ST (no. of isolates)	16S RMTase (no. of isolates)	Carbapenemase (no. of isolates)	Geographical distribution (no. of laboratories)
147 (33)	RmtF (11)	NDM-1 (5)	London (2), Yorkshire and the Humber (2) and South East (1)
		OXA-181 (5)	London (2), South East (1), South West (1) and Yorkshire and the Humber (1)
		NDM-1 + OXA-48 (1)	London (1)

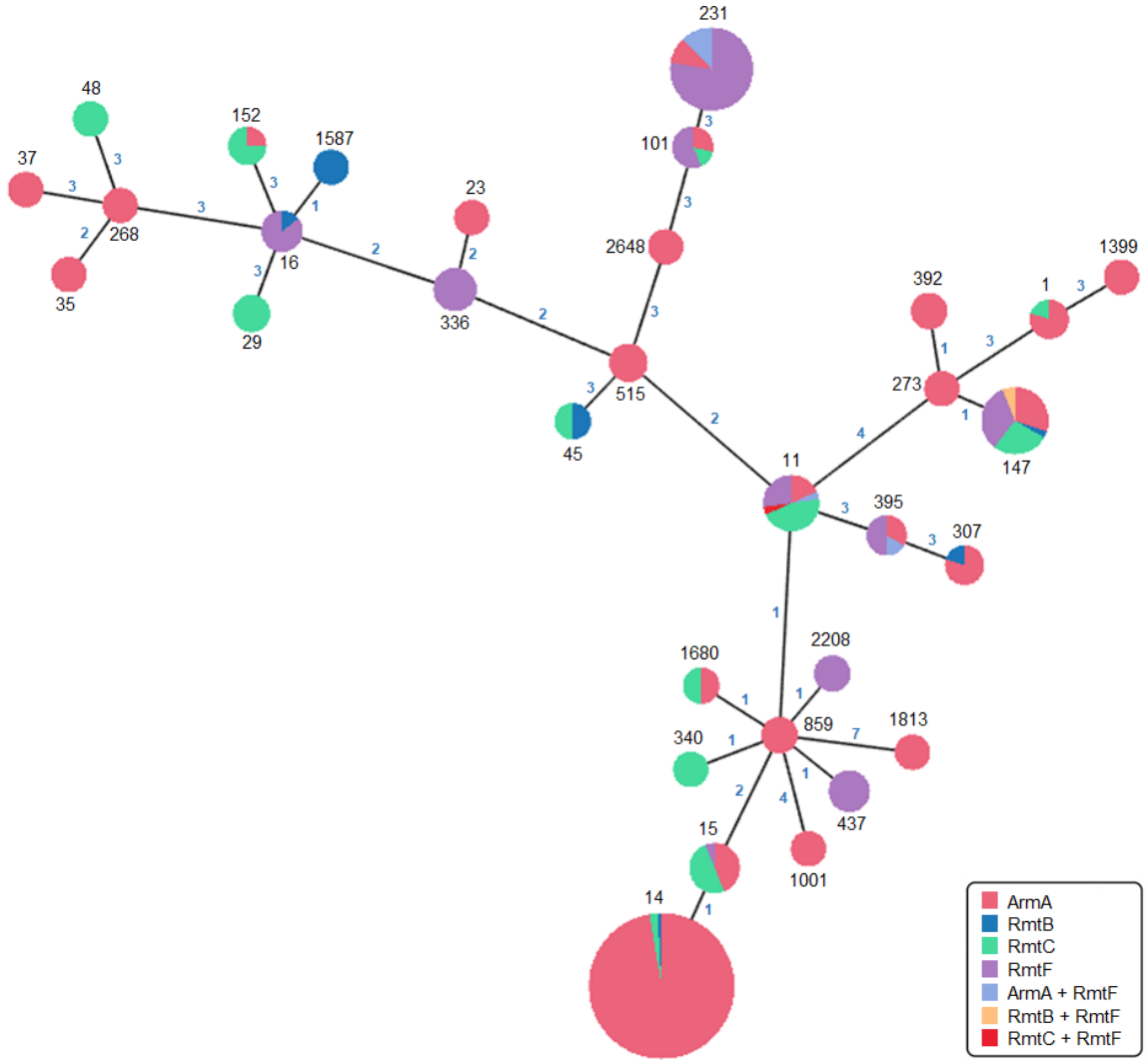


Figure 4.12: eBURST diagram of 16S RMTase-producing *K. pneumoniae* isolates (n=311) with WGS data showing the specific associations between STs and 16S RMTases. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of single locus variants (SLVs) between STs (black font).

The most common *E. coli* STs (Table 4.10) were ST167 (21.8%, 19/87), ST405 (21.8%, 19/87) and ST410 (12.6%, 11/87). *E. coli* ST167 was mainly identified in London (36.8%, 7/19) and referred by six laboratories and was mostly associated with *rmtB* (84.2%, 16/19) as well as $bla_{\text{NDM-5}}$ (84.2%, 16/19). *E. coli* ST405 isolates were mostly submitted by seven laboratories in London (57.9%, 11/19) and mainly associated with *rmtB* (57.9%, 11/19) and $bla_{\text{NDM-5}}$ (47.4%, 9/19), whilst ST410 was mainly isolated in London (36.4%, 4/11) by four laboratories and associated with *rmtB* (81.8%, 9/11) and $bla_{\text{NDM-4}}$ (45.5%, 5/11). The association between ST and carriage of an 16S RMTase can be seen in Figure 4.13.

The two most common *E. cloacae* complex STs were ST78 (16.7%, 3/18) followed by ST90 (16.7%, 3/18), which were associated with a range of 16S RMTases and were isolated from different regions (Table 4.11). The association between ST and carriage of a 16S RMTase gene can be seen in Figure 4.14.

The most common *C. freundii* ST (Table 4.12) identified was ST18 (22.2%, 2/9), which was only found associated with *armA* (100%, 2/2) and $bla_{\text{NDM-1}}$ (100%, 2/2) and was isolated by two London laboratories. One *C. freundii* isolate had a novel ST and another isolate had an unknown ST as MLST failed. The association between ST and carriage of an 16S RMTase gene can be seen in Figure 4.15.

Three of the four *K. oxytoca* isolates belonged to novel STs. ST36 was the only identified ST, which was isolated in London and found to be *armA* and $bla_{\text{NDM-1}}$ positive.

Eight *P. aeruginosa* STs (inferred from WGS data) were associated with 16S RMTases, with ST357 (31.6%, 6/19) the most common ST. STs did not appear to be restricted to specific regions nor did they have a specific association with 16S RMTase genes when more than one isolate belonged to a ST (Figure 4.11).

Table 4.10: The most common STs found in 16S RMTase-producing *E. coli* isolates (n=87) as well as their association with carbapenemases and geographical distribution.

ST (no. of isolates)	16S RMTase (no. of isolates)	Carbapenemase (no. of isolates)	Geographical distribution (no. of laboratories)
167 (19)	RmtB (17)	NDM-5 (17)	London (7), North West (3), East Midlands (2), West Midlands (2), East (1), South East (1) and South West (1)
	RmtC (2)	NDM-1 (1)	East (1)
		NDM-7 (1)	West Midlands (1)
405 (19)	RmtB (11)	NDM-5 (9)	London (2), West Midlands (2), East (1), North West (1) and Scotland (1)
		NDM-1 (2)	London (2)
	ArmA (4)	OXA-181 (4)	London (2)
	RmtC (4)	NDM-1 (2)	London (1) and West Midlands (1)
		NDM-4 (2)	East (2)
410 (11)	RmtB (9)	NDM-4 (2)	North West (1)
		NDM-4 + OXA-181 (3)	East (3)
		NDM-5 (1)	London (1)
		NDM-5 + OXA-181 (3)	London (2) and North West (1)
	RmtC (2)	NDM-1 (2)	London (1) and West Midlands (1)

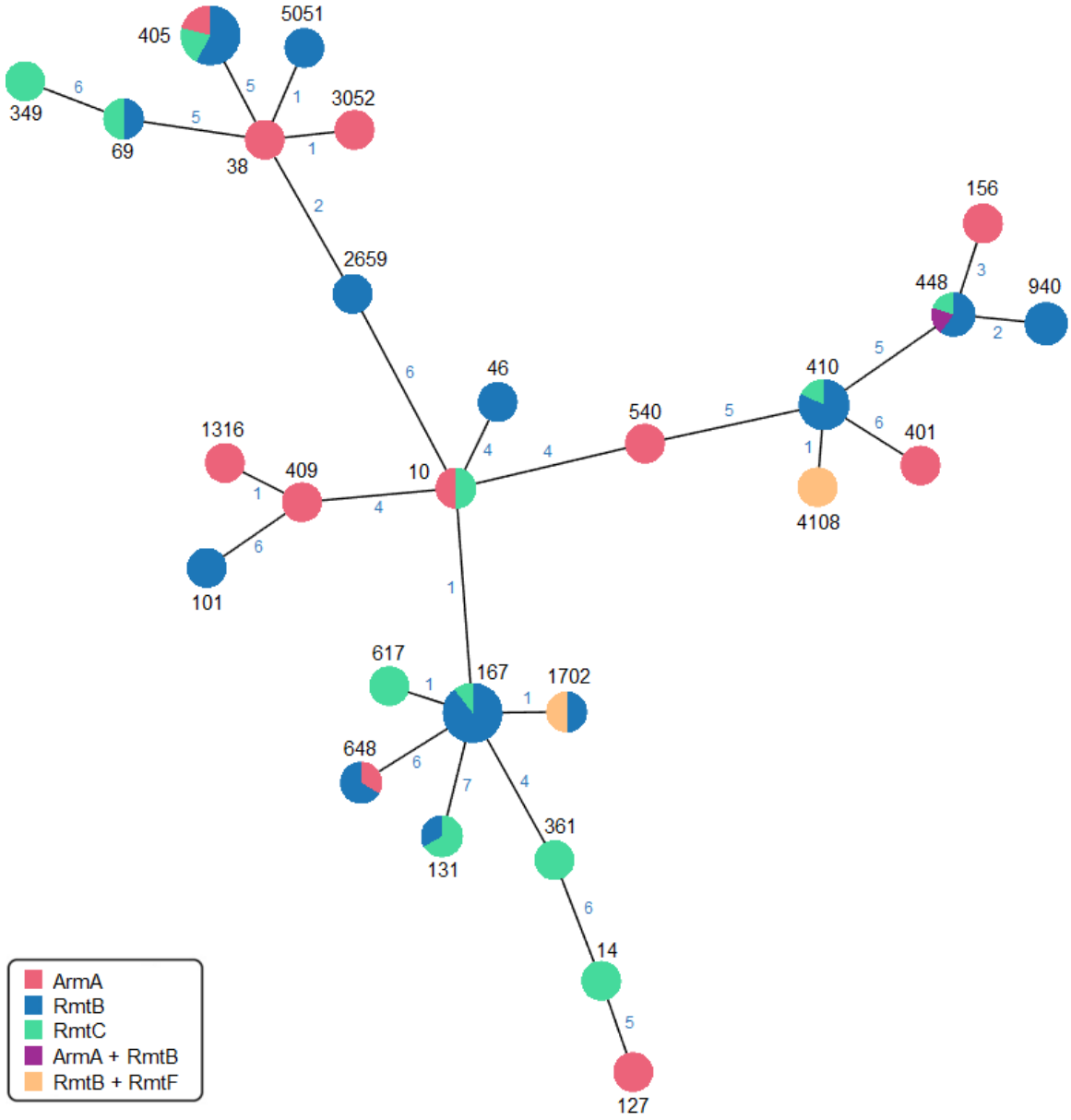


Figure 4.13: eBURST diagram of 16S RMTase-producing *E. coli* isolates (n=87) with WGS data showing the specific associations between STs and 16S RMTases. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of SLVs between STs (black font).

Table 4.11: The most common STs found in 16S RMTase-producing *E. cloacae* complex isolates (n=18) as well as the associated 16S RMTases, carbapenemases and the geographical distribution.

ST (no. of isolates)	16S RMTase (no. of isolates)	Carbapenemase (no. of isolates)	Geographical distribution (no. of laboratories)
78	ArmA (1)	NDM-1 (1)	West Midlands (1)
	RmtB (1)	NDM-1 (1)	East Midlands (1)
	RmtC (1)	NDM-1 (1)	North West (1)
90	ArmA (2)	NDM-1 (2)	Yorkshire and the Humber (2)
	RmtC (1)	NDM-1 (1)	West Midlands (1)

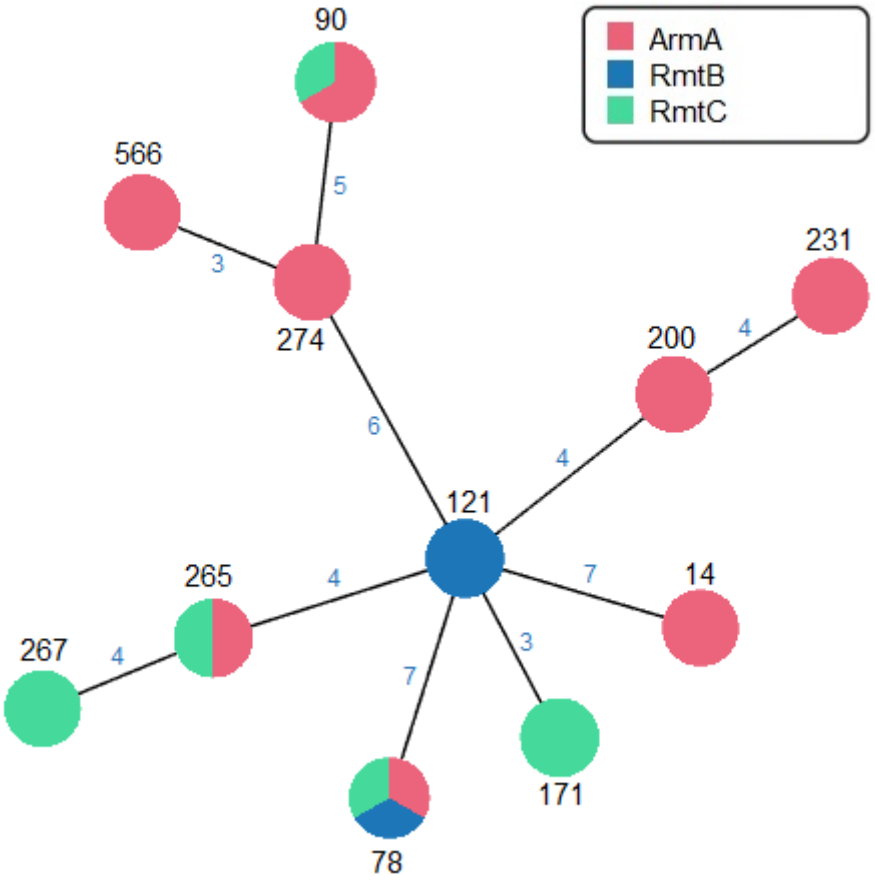


Figure 4.14: eBURST diagram of 16S RMTase-producing *E. cloacae* complex isolates (n=18) with WGS data showing the specific associations between STs and 16S RMTases. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of SLVs between STs (black font).

Table 4.12: The most common STs found in 16S RMTase-producing *C. freundii* isolates (n=8) as well as the associated 16S RMTases, carbapenemases and the geographical distribution.

ST (no. of isolates)	16S RMTase (no. of isolates)	Carbapenemase (no. of isolates)	Geographical distribution (no. of laboratories)
18 (2)	RmtC (2)	NDM-1 (2)	London (2)
11 (1)	ArmA (1)	OXA-48 (1)	London (1)
91 (1)	ArmA (1)	NDM-1 (1)	London (1)
108 (1)	ArmA (1)	NDM-1 (1)	London (1)
112 (1)	RmtC (1)	NDM-1 + OXA-181 (1)	South West (1)
284 (1)	ArmA (1)	NDM-1 (1)	London (1)
Novel ST (1)	ArmA (1)	NDM-1 (1)	North East (1)

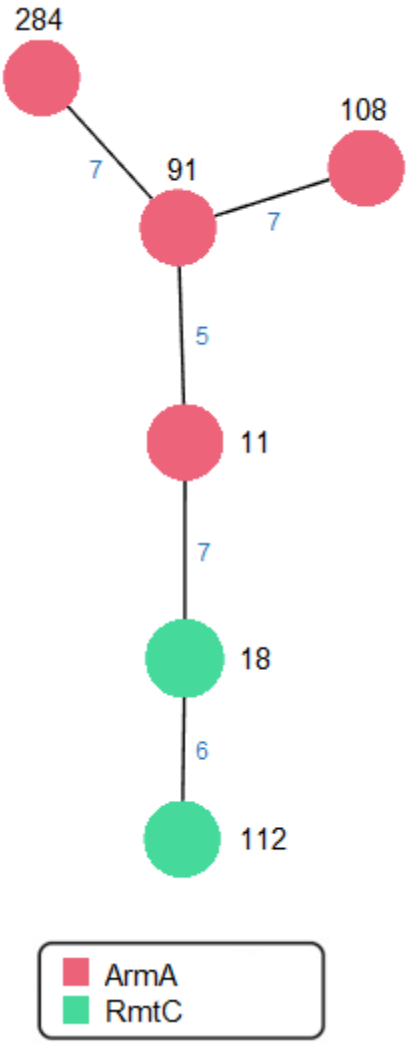


Figure 4.15: eBURST diagram of 16S RMTase-producing *C. freundii* isolates (n=7) with WGS data showing the specific associations between STs and 16S RMTases. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of SLVs between STs (black font).

4.4.5 Analysis of AMRHAI's WGS data from carbapenemase-positive bacterial isolates

WGS data from carbapenemase-positive bacterial isolates from AMRHAI's strain collection, the 16S RMTase-producing *P. aeruginosa* and the *rmtE*-positive *A. baumannii* isolate were analysed for the presence of ESBL genes, acquired PMQR genes and chromosomal mutations associated with fluoroquinolone resistance.

4.4.5.1 Analysis of ESBL genes

ESBL genes were identified in 82.5% (387/469) isolates with WGS data (Table 4.13). ESBLs belonging to the $bla_{CTX\text{-}M}$ family were the most common as they were identified in 91.0% (352/387) isolates, including 85.7% (270/315) *K. pneumoniae*, 71.3% (62/87) *E. coli*, 55.6% (10/18) *E. cloacae* complex and 37.5% (3/8) *C. freundii* isolates. These genes were not identified in any *P. aeruginosa* isolate or the *rmtE*-positive *A. baumannii* isolate. $bla_{CTX\text{-}M\text{-}15}$ was the most common variant as it was found in 98.3% (346/352) isolates and was mainly associated with *rmtF* (87.8%, 79/90), followed by *armA* (76.2%, 160/210), *rmtC* (68.1%, 49/72), *rmtB* (59.5%, 44/74) and isolates harbouring two 16S RMTase genes (76.5%, 13/17). Within the most commonly identified STs, $bla_{CTX\text{-}M\text{-}15}$ was identified in 100% (49/49), 90.9% (30/33) and 83.0% (93/112) *K. pneumoniae* STs 231, 147 and 14 isolates, respectively; 100% (19/19), 81.8% (9/11) and 42.1% (8/19) *E. coli* ST405, ST410 and ST167 isolates, respectively, as well as 100% (2/2) *C. freundii* ST18 isolates. This gene was not found in the two most common *E. cloacae* complex STs (ST78 and ST90).

The second most common ESBL gene family was bla_{SHV}, which was identified in 34.8% (163/469) isolates including 100% (1/1) *K. ornithinolytica*, 50.0% (2/4) *K. oxytoca*, 48.9% (154/315) *K. pneumoniae* isolates, 16.7% (3/18) *E. cloacae* complex, 14.3% (1/7) *P. stuartii*, 12.5% (1/8) *C. freundii* and 1.1% (1/87) *E. coli* isolates. These genes were not identified in *C. sedlakii*, *E. hermannii, K. aerogenes*, *M. morganii*, *P. mirabilis*, *P. rettgeri*, *P. aeruginosa* isolates or the *rmtE*-positive *A. baumannii* isolate.

Table 4.13: Total number of ESBL genes identified in 16S RMTase-producing isolates (n=1,312) with WGS data.

ESBL gene	16S RMTase gene(s)											Total
	armA	*rmtB*	*rmtC*	*rmtD*	*rmtF*	*armA + rmtB*	*armA + rmtC*	*armA + rmtF*	*rmtB + rmtC*	*rmtB + rmtF*	*rmtC + rmtF*	
***bla*$_{\text{CTX-M-1}}$**	-	1	-	-	-	-	-	-	-	-	-	1
***bla*$_{\text{CTX-M-9}}$**	1	-	-	-	-	-	-	-	-	-	-	1
***bla*$_{\text{CTX-M-14}}$**	-	-	-	-	2	-	-	-	-	-	-	2
***bla*$_{\text{CTX-M-15}}$**	160	44	49	-	79	1	-	8	-	3	1	345
***bla*$_{\text{CTX-M-27}}$**	-	1	-	-	-	-	-	-	-	-	-	1
***bla*$_{\text{CTX-M-55}}$**	1	1	-	-	-	-	-	-	-	-	-	2
***bla*$_{\text{CTX-M-123}}$**	-	1	-	-	-	-	-	-	-	-	-	1
***bla*$_{\text{SHV-5}}$**	-	1	-	-	-	-	-	-	-	-	-	1
***bla*$_{\text{SHV-12}}$**	3	-	4	-	2	-	-	-	1	-	-	10
***bla*$_{\text{SHV-39}}$**	39	-	5	-	11	-	-	1		-	-	56
***bla*$_{\text{SHV-100}}$**	53	1	6	-	33	-	-	3	-	-	-	96
***bla*$_{\text{GES-9}}$**	-	-	-	-	1	-	-	-	-	-	-	1
***bla*$_{\text{PME-1}}$**	-	1	-	1	-	-	-	-	-	-	-	2
***bla*$_{\text{SFO-1}}$**	-	1	-	-	-	-	-	-	-	1	-	2
***bla*$_{\text{VEB-1}}$**	-	4	-	2	-	-	-	-	-	-	-	6
***bla*$_{\text{VEB-6}}$**	1	-	-	-	-	-	-	-	-	-	-	1
Total	258	56	64	3	128	1	-	12	1	4	1	528

bla$_{SHV-100}$ was the most common variant identified as it was found in 58.9% (96/163) isolates and appeared to be highly associated with *rmtF* (36.7%, 33/90), followed by *armA* (25.2%, 53/210), *rmtC* (8.3%, 6/72), *rmtB* (1.4%, 1/74) and isolates harbouring two 16S RMTase genes (17.6%, 3/17). Within the most frequently identified STs, *bla*$_{SHV-100}$ was associated with *K. pneumoniae* ST231 (75.5%, 37/49) and ST14 (35.7%, 40/112) as well as *E. coli* ST410 (9.1%, 1/11).

bla$_{VEB}$ ESBL genes were identified in 1.5% (7/469) isolates including *P. mirabilis* (33.3%, 1/3), *P. aeruginosa* (21.1%, 4/19), *P. stuartii* (14.3%, 1/7) and *K. pneumoniae* (0.3%, 1/315), *bla*$_{VEB-1}$ (85.7%, 6/7) was the most common variant and was found to be associated with *rmtD* (40.0%, 2/5) followed by *rmtB* (5.4%, 4/74). Additionally, it was identified in *P. aeruginosa* ST357 (66.7%, 4/6) and in *K. pneumoniae* ST147 (3.0%, 1/33).

4.4.5.2 Analysis of PMQR genes and chromosomal mutations conferring fluoroquinolone resistance

Chromosomal mutations in the quinolone resistance determining regions (QRDRs) of the genes *gyrA* and *parC* (Table 4.14) were identified in 81.7% (383/469) isolates including *K. oxytoca* (100%, 4/4), *E. coli* (89.7%, 78/87), *K. pneumoniae* (89.2%, 281/315), *C. freundii* (88.9%, 8/9) and *E. cloacae* complex (66.7%, 12/18). Mutations were not identified in *K. aerogenes*, *E. hermannii*, *K. ornithinolytica*, *M. morganii*, *P. mirabilis*, *Providencia* spp., *P. aeruginosa* or the *rmtE*-positive *A. baumannii* isolate.

A total of 99.7% (382/383) isolates had mutations in *gyrA* where substitutions at codons 83 and 87 in GyrA were identified in 100% (382/382) and 62.6% (239/382) isolates, respectively. A total of 96.6% (370/383) isolates had mutations in *parC* including 96.6% (369/382) isolates with *gyrA* mutations. In the isolates with *gyrA* mutations, substitutions at codons 80 and 84 of ParC were identified in 97.8% (361/369) and 4.6 % (17/369) isolates, respectively. Additional substitutions included S57T (0.8%, 3/382) and L88Q (0.8%, 3/382). One *rmtB*-positive *E. coli* isolate belonging to ST69 had the substitution S80I but did not have any mutation in *gyrA*.

The most common combination of GyrA and ParC substitutions was S83I and S80I (35.8%, 132/369) and was only identified in *C. freundii* (55.6%, 5/9), *E. cloacae* complex (50.0%, 9/18) and *K. pneumoniae* (37.5%, 118/315). This combination was found in 100% (3/3) *E. cloacae* complex ST78, 98.0% (48/49) *K. pneumoniae* ST231 and 93.9% (31/33) *K. pneumoniae* ST147 isolates.

PMQR determinants were identified in 88.7% (416/469) isolates (Table 4.15). PMQR determinants were found in 100% (9/9) *C. freundii*, 99.7% (314/315) *K. pneumoniae*, 85.7% (6/7) *P. stuartii*, 77.8% (14/18) *E. cloacae* complex, 63.2% (55/87) *E. coli*, 57.9% (11/19) *P. aeruginosa*, 50.0% (2/4) *K. oxytoca* and single *K. aerogenes*, *K. ornithinolytica*, *M. morganii*, *P. rettgeri* and *P. mirabilis* isolates. None were found in the *rmtE*-positive *A. baumannii* or *E. hermanni.*

The most common PMQR determinant gene identified was *aac(6')-Ib-cr* (83.9%, 349/416), which was found in 100% (1/1) *K. aerogenes*, 100% (1/1) *M. morganii*, 100% (1/1) *P. rettgeri,* 100% (1/1) *P. mirabilis*, 92.7% (51/55) *E. coli*, 85.7% (12/14) *E. cloacae* complex, 84.1% (264/314) *K. pneumoniae*, 66.7% (6/9) *C. freundii*, 66.7% (4/6) *P. stuartii,* 63.6% (7/11) *P. aeruginosa* and 50.0% (1/2) *K. oxytoca*. It was highly associated with *rmtF* (98.9%, 89/90), followed by *rmtC* (83.3%, 60/72), *armA* (71.0%, 149/210), *rmtB* (45.9%, 34/74), *rmtD* (20.0%, 1/5) and was found in 94.1% (16/17) isolates with two 16S RMTase genes. Out of the most commonly identified STs *aac(6')-Ib-cr* was identified in 100% (2/2) *C. freundii* ST18, 89.8% (44/49) *K. pneumoniae* ST231, 84.8% (95/112) *K. pneumoniae* ST14, 81.8% (9/11) *E. coli* ST410, 78.8% (26/33) *K. pneumoniae* ST147, 66.7% (2/3) *E. cloacae* complex ST78, 57.9% (11/19) *E. coli* ST405, 50.0% (2/4) *P. aeruginosa* ST654, 42.1% (8/19) *E. coli* ST167, 33.3% (1/3) *E. cloacae* complex ST90 and 16.7% (1/6) *P. aeruginosa* ST357 isolates.

Table 4.14: Substitutions in QRDRs of GyrA and ParC found in 16S RMTase-producing isolates (n=1,312) with WGS data.

Species	*armA*	*rmtB*	*rmtC*	*rmtF*	Two genes	Total	GyrA position		ParC positions		
C. freundii	3	-	-	-	-	3	S83I				
	1	-	3	-	1	5	S83I		S80I		
E. cloacae complex	2	3	4	-	-	9	S83I		S80I		
	1	-	-	-	-	1	S83F	D87A	S80I		
	1	-	-	-	-	1	S83F	D87A	S80R		
	1	-	-	-	-	1	S83Y	D87G	S80I		
E. coli	-	1	-	-	-	1			S80I		
	6	46	11	-	2	65	S83L	D87N	S80I		
	1	1	1	-	-	3	S83L	D87N	S80I	S57T	
	1	-	1	-	-	2	S83L	D87N	S80I	E84G	
	-	2	2	-	-	4	S83L	D87N	S80I	E84V	
	-	2	-	-	1	3	S83L	D87N	S80I	E84G	L88Q
K. oxytoca	1	-	-	-	-	1	S83I				
	2	-	-	-	-	2	S83T				
	1	-	-	-	-	1	S83T	D87Y			
K. pneumoniae	-	-	-	1	-	1	S83I				
	28	2	12	66	10	118	S83I		S80I		
	3	-	-	-	-	3	S83F				
	10	-	21	1	1	33	S83F	D87A	S80I		
	1	-	-	-	-	1	S83F	D87E	S80I		
	-	2	-	6	-	8	S83F	D87N	E84K		
	1	-	-	-	-	1	S83Y				
	3	-	-	1	-	4	S83Y	D87A	S80I		
	102	1	2	1	-	106	S83Y	D87G	S80I		
	2	-	1	2	-	5	S83Y	D87N	S80I		
	-	1	-	-	-	1	D87Y				
Total	171	61	58	78	15	383					

Table 4.15: Total number of PMQR genes found in 16S RMTase-producing isolates (n=1,312) with WGS data.

PMQR gene	16S RMTase gene(s)						Total
	armA	***rmtB***	***rmtC***	***rmtD***	***rmtF***	**Two genes**	
aac(6')-Ib-cr	149	34	60	1	89	16	349
qnrB1	103	4	20	-	28	6	161
qnrB4	1	-	-	-	1	-	2
qnrB7	1	2	-	-	-	-	3
qnrB9	6	-	-	-	-	1	7
qnrB17	1	-	-	-	-	-	1
qnrB19	-	-	1	-	-	-	1
qnrB35	1	-	-	-	-	-	1
qnrB38	1	-	-	-	-	-	1
qnrD1	2	3	1			2	8
qnrA1	5	-	2	-	-	-	7
qnrS1	29	9	9	-	46	8	101
qepA1	-	3	-	-	5	-	8
oqxA	170	6	41	-	86	11	314
oqxB	170	6	41	-	85	11	313
Total	639	67	175	1	340	55	1,277

A total of 69.5% (289/416) isolates harboured *qnr* genes including 100% (9/9) *C. freundii*, 100% (2/2) *K. oxytoca*, 100% (1/1) *K. aerogenes*, 100% (1/1) *K. ornithinolytica*, 100% (1/1) *M. morganii*, 100% (1/1) *P. mirabilis*, 85.7% (12/14) *E. cloacae* complex, 83.3% (5/6) *P. stuartii*, 74.2% (233/314) *K. pneumoniae*, 36.4% (20/55) *E. coli* and 36.4% (4/11) *P. aeruginosa* isolates. The most common variant was *qnrB1* (54.2%, 161/297), which was highly associated with *armA* (49.0%, 103/210), followed by *rmtF* (31.1%, 28/90), *rmtC* (27.8%, 20/72), *rmtB* (5.4%, 4/74) and 35.3% (6/17) isolates with two 16S RMTase genes. This gene was found in 100% (2/2) *C. freundii* ST18, 69.6% (78/112) *K. pneumoniae* ST14, 66.7% (2/3) *E. cloacae* complex ST78, 54.5% (18/33) *K. pneumoniae* ST147 and 22.4% (11/49) *K. pneumoniae* ST231 isolates

Other identified PMQR determinants (Table 4.15) included genes for efflux pumps QepA and OqxAB. The gene *qepA1* was found in 2.2% (9/416) isolates including *M. morganii* (100%, 1/1), *K. pneumoniae* (2.2%, 7/314), and *E. coli* (1.8%, 1/55) and was only identified in *rmtF* (5.6%, 5/90) and *rmtB*-positive (5.4%, 4/74) isolates. It was also identified in 10.2% (5/49) *K. pneumoniae* ST231 isolates. Genes *oqxA* and *oqxB* were found in 100% (314/314) and 99.7% (313/314) *K. pneumoniae* isolates, where they are intrinsic, but also found in 5.6% (1/18) *E. cloacae complex* isolates.

4.4.6 Analysis of PCR-negative pan-aminoglycoside-resistant bacteria

Sixty-three isolates (44 Enterobacterales and 19 *A. baumannii*) that were 16S RMTase-negative but exhibited pan-aminoglycoside resistance (MICs amikacin >64 mg/L, gentamicin >32 mg/L and tobramycin >32 mg/L) were analysed further to determine if they harboured novel 16S RMTase genes. The 16S RMTase-negative *P. aeruginosa* isolates were not analysed further due to 16S RMTase genes being rarely reported in *P. aeruginosa* compared to *A. baumannii* and Enterobacterales, and AMEs and efflux pumps being reported as the most common mechanisms of aminoglycoside resistance in this species [419-421].

A total of 78.9% (15/19) *A. baumannii* and 9.1% (4/44) Enterobacterales, including two *C. freundii* and two *K. pneumoniae* isolates, grew on all agar plates containing 256 mg/L

amikacin, gentamicin or tobramycin. These isolates were sent for WGS, where species was confirmed and the most common STs identified were ST624 (33.3%, 5/15) for *A. baumannii* and ST405 (50.0%, 1/2) and ST750 (50.0%, 1/2) for *K. pneumoniae*. Only one International Clone II isolate (ST2) was identified in this collection of *A. baumannii* isolates (Table 4.16).

The most common AMEs identified were *aph(3')-VIa* and *ant(2")-Ia* as they were identified in 78.9% (15/19) and 73.7% (14/19) isolates, respectively, but were only found in *A. baumannii*. All four Enterobacterales harboured the genes *aac(3)-IIa*, *aac(6')-Ib-cr* and *aph(3')-VIb*. All of the isolates had AME gene combinations that could account for resistance to all three aminoglycosides. No previously described 16S RMTase genes were identified in these isolates (i.e. there were no 16S RMTase PCR false-negatives).

In order to see if any isolates harboured novel 16S RMTase genes, amino acid sequences from individual contigs from each isolate were analysed using Pfam 31.0 to see if any shared identify with the ribosomal RNA methyltransferase family FmrO (Pfam ID: PF07091) or Methyltransf_4 family (Pfam ID: PF02390). None of the amino acid sequences from the contigs matched to either of the protein families.

Table 4.16: Genes encoding AMEs identified in 16S RMTase-negative isolates (n=19) that demonstrate high-level resistance to amikacin, gentamicin and tobramycin from AMRHAI's strain collection, 2003-2015.

Species (individual isolates)	ST	AME genes						
		aph(3')-VIa[a]	***ant(2")-Ia***[bc]	***aac(3)-IIa***[bc]	***aac(6')-Ib-cr***[ac]	***aph(3')-VIb***[a]	***aac(3)-IId***[bc]	***aac(6')-Ib***[ac]
C. freundii	-	-	-	1	1	1	-	-
C. freundii	-	-	-	1	1	1	-	-
K. pneumoniae	405	-	-	1	1	1	-	-
K. pneumoniae	750	-	-	1	1	1	-	-
A. baumannii	1	2	1	-	-	-	-	-
A. baumannii	1	2	1	-	-	-	-	-
A. baumannii	1	1	1	-	1	-	-	1
A. baumannii	2	1	-	1	-	-	-	-
A. baumannii	16	1	1	-	-	-	1	-
A. baumannii	23	1	1	-	-	-	-	-
A. baumannii	94	2	1	-	-	-	-	-
A. baumannii	94	2	1	-	-	-	-	-
A. baumannii	113	1	1	-	-	-	-	-
A. baumannii	624	1	1	-	-	-	-	-
A. baumannii	624	1	1	-	-	-	-	-
A. baumannii	624	1	1	-	-	-	-	-
A. baumannii	624	1	1	-	-	-	-	-
A. baumannii	624	1	1	-	-	-	-	-
A. baumannii	718	1	1	-	-	-	-	-
Total		19	14	5	5	4	1	1

Enzymes confer resistance to [a] = amikacin; [b] = gentamicin and [c] = tobramycin.

4.4.7 Patient demographics

4.4.7.1 Patients with 16S RMTase-producing isolates

16S RMTase-producing isolates originated from 1,134 patients, of which 1,012 (89.2%) patients had single isolates and 122 (10.8%) patients had multiple isolates. Amongst patients with multiple isolates, 66 (54.1%) had bacterial isolates belonging to different species, 29 (23.8%) had isolates obtained from different anatomical sites and 27 (22.1%) patients had isolates belonging to the same species but with different MIC profiles.

Most patients were inpatients (69.8%, 792/1,134), though patient origin was unknown for 211 (18.6%) patients. Gender and age were known for 98.3% (1,115/1,134) and 98.1% (1,113/1,134) patients, respectively. The average age was found to be 60.6 years, ranging from one day to 95 years and most patients were male (65.7%, 732/1,115).

Only 14.6% (165/1,134) patients had a record of any foreign travel history (Table 4.17), where the most common country visited was India (41.8%, 69/165), followed by Pakistan (10.3%, 17/165) and Thailand (9.7%, 16/165) (Figure 4.16). Travel history was unknown for 838 (73.9%) patients and 131 (11.6%) patients had no travel history. Five patients had visited more than one country, where patients had visited China + Hong Kong (0.6%, 1/165), China + Thailand (0.6%, 1/165), Dubai + India (0.6%, 1/165), India + Kenya (0.6%, 1/165) and Thailand + Myanmar (0.6%, 1/165) and one (0.6%) patient visited an unknown location.

Fifty patients (30.3%) were previously hospitalised abroad, of which 42.0% (21/50) patients had been hospitalised in India, followed by 12.0% (6/50) patients in Thailand, 8.0% (4/50) paitents in Greece, 6.0% (3/50) patients in Egypt, 6.0% (3/50) patients in Italy, 6.0% (3/50) patients in Pakistan and single patients were hospitalised in Bangladesh, China, Croatia, Equatorial Guinea, Indonesia, Kuwait, South Korea, Sri Lanka and Turkey.

Table 4.17: Countries visited by patients with 16S RMTase-positive (n=165) and -negative (n=17) bacterial isolates between 2003-2015.

Country	Patients with 16S RMTase-positive bacteria (n=165)	Patients with 16S RMTase-negative bacteria (n=17)
India	69 (41.8%)	4 (23.5%)
Pakistan	17 (10.3%)	1 (5.9%)
Thailand	16 (9.7%)	2 (11.8%)
Greece	9 (5.5%)	1 (5.9%)
Kuwait	6 (3.6%)	3 (17.6%)
Egypt	6 (3.6%)	1 (5.9%)
Italy	6 (3.6%)	-
Bangladesh	5 (3.0%)	-
China	3 (1.8%)	-
Turkey	3 (1.8%)	-
South Africa	-	3 (17.6%)
Middle East	2 (1.2%)	-
Sri Lanka	2 (1.2%)	-
Vietnam	2 (1.2%)	-
Albania	1 (0.6%)	-
Brazil	1 (0.6%)	-
Corfu	1 (0.6%)	-
Croatia	1 (0.6%)	-
Cyprus	1 (0.6%)	-
Democratic Republic of the Congo	1 (0.6%)	-
Dubai	1 (0.6%)	1 (5.9%)
Equatorial Guinea	1 (0.6%)	-
Fiji	1 (0.6%)	-
Hong Kong	1 (0.6%)	-
Indonesia	1 (0.6%)	-
Kenya	1 (0.6%)	-
Macedonia	-	1 (5.9%)
Myanmar	1 (0.6%)	-
Mauritius	1 (0.6%)	-
Montenegro	1 (0.6%)	-
Nigeria	1 (0.6%)	-
Oman	1 (0.6%)	-
Romania	1 (0.6%)	-
Saudi Arabia	1 (0.6%)	-
Singapore	1 (0.6%)	-
South Korea	1 (0.6%)	-
Syrian Arab Republic	1 (0.6%)	-
Tunisia	1 (0.6%)	-
United Arab Emirates	-	1 (5.9%)

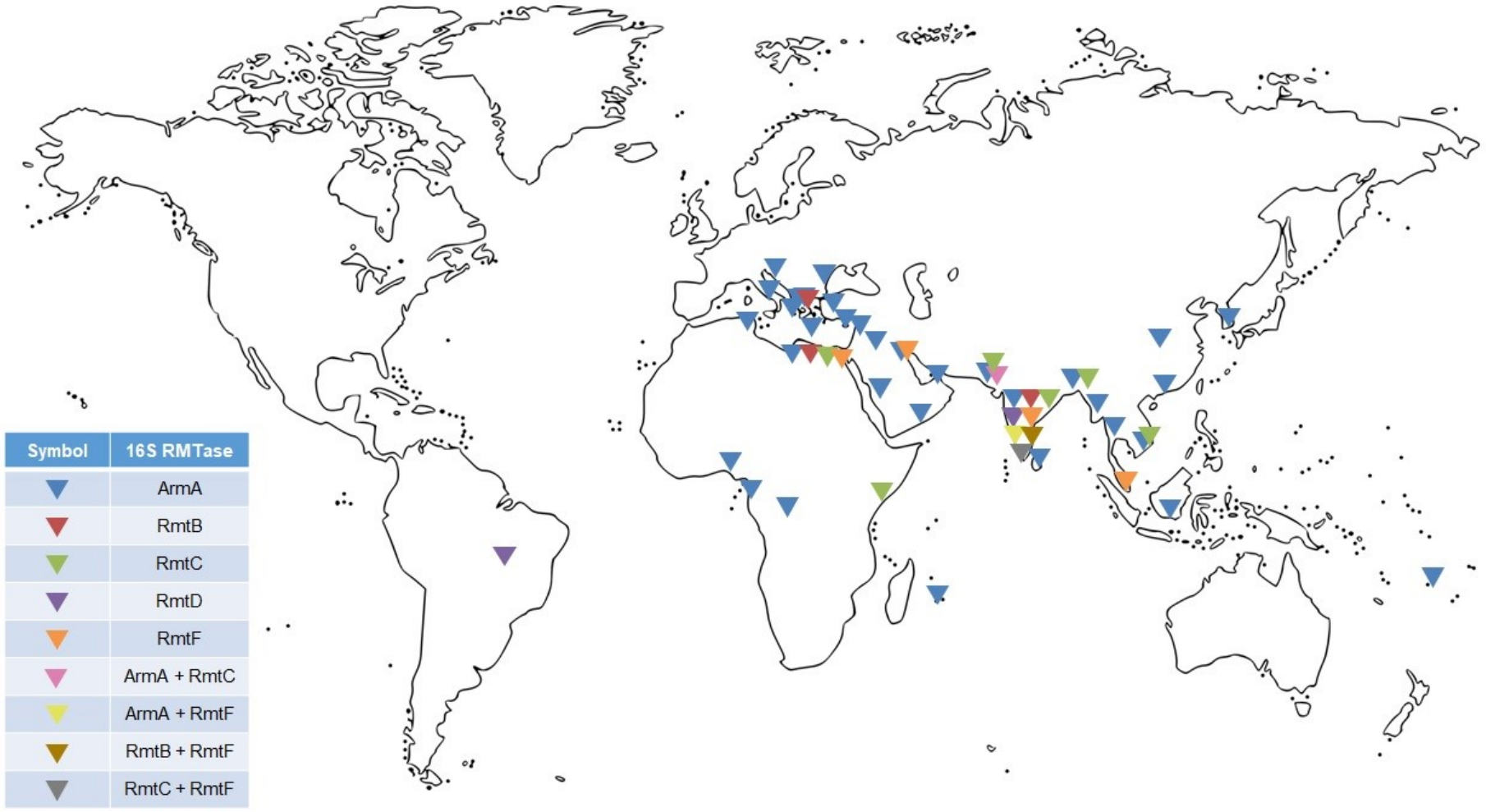


Figure 4.16: Countries visited by patients with 16S RMTase-producing bacterial isolates (n=165) between 2003-2015.

4.4.7.2 Patients with 16S RMTase-negative isolates

16S RMTase-negative isolates were collected from 257 patients, where 97.3% (250/257) had single isolates and 2.7% (7/257) patients had multiple isolates. All seven patients had isolates belonging to the same species but with different MIC profiles.

Patient types were known for 82.5% (212/257) patients, where the majority were inpatients (80.7%, 171/212), though patient origin was unknown for 45 (17.5%) patients. Age and gender was known for 99.2% (255/257) and 98.8% (254/257) patients, respectively. Most patients were male (54.3%, 138/254) and the median age of patients was 61 years with a range of zero days to 101 years.

Travel history was unknown for 77.4% (199/257) patients, 15.9% (41/257) patients had no history of travel but 6.6% (17/257) patients had a record of travel history. The most common country visited was India (17.6%, 3/17) (Table 4.17). Additionally, one (5.9%) patient travelled to Dubai and India.

4.5 Discussion

In this study, 83.2% of pan-aminoglycoside-resistant Gram-negative bacterial isolates from the AMRHAI Reference Unit's strain archive spanning 2003-2015 were positive for 16S RMTase genes. *armA* (69.5%, 912/1,312) was the most commonly identified 16S RMTase gene, which is also the most frequently reported 16S RMTase gene worldwide, followed by *rmtB* [127,149]. However, in this study *rmtB* (8.1%) was found at a lower frequency than *rmtC* (13.3%) and *rmtF* (12.6%). This may be due to *rmtC* having a higher association with bla_{NDM} (96.0%, 168/175) than *rmtB* (83.0%, 88/106) as well as *rmtF* having a higher association with $bla_{\text{OXA-48-like}}$ (52.1%, 86/165) compared to *rmtB* (14.2%, 15/106) and/or to bacterial clones or plasmids harbouring *rmtC* and *rmtF* being more prevalent than those associated with *rmtB*. No *rmtA*, *rmtG*, *rmtH* or *npmA* genes were identified.

Two-gene combinations were only identified in 4.0% of 16S RMTase-producing isolates, demonstrating that they are currently rare in the UK, particularly in England, where the majority

of bacterial isolates (98.2%, 1,288/1,312) were obtained from. Isolates harbouring more than one 16S RMTase have been reported elsewhere [172,180,185,189,197,204,212,272,291,422,423] and appear to be frequently identified in India [180,204,291] as rates of 70.2% (40/57) [180], 35.0% (21/60) [291] and 31.6% (6/19) [204] 16S RMTase-producing isolates with multiple 16S RMTase genes have been identified. The most common two-gene combination in this strain collection was *armA* + *rmtC*, which was mainly found in *K. pneumoniae* but has also been reported in *E. cloacae* complex in Australia [185]; *E. coli* in India [291], New Zealand [198], Pakistan [272] and Saudi Arabia [189]; *K. oxytoca* in Saudi Arabia [189] and *P. aeruginosa* in India [291].

Overall, 94.5% pan-aminoglycoside-resistant Enterobacterales harboured 16S RMTase genes, of which the majority were *K. pneumoniae* (65.9%, 502/762). *armA*, *rmtC* and *rmtF* were mainly associated with *K. pneumoniae*, whereas *rmtB* was mainly identified in *E. coli*. The high prevalence of 16S RMTase genes in these two species is concerning as *K. pneumoniae* and *E. coli* are leading causes of nosocomial and community infections and are known for their accumulation and dissemination of antibiotic resistance genes [424], meaning these species are likely to be contributing to the spread of 16S RMTase genes in the hospital and community settings.

In total, 96.5% of pan-aminoglycoside-resistant *A. baumannii* harboured 16S RMTase genes, with *armA* found in all but one isolate, which was positive for *rmtE3*. *armA* is the most frequently reported 16S RMTase gene in *A. baumannii* worldwide [149] and has been identified frequently in MDR *A. baumannii* from India (87.0%, 47/54) [202], Iran (94.4%, 17/18) [400] and Vietnam (82.8%, 77/93) [150]. Other 16S RMTase genes reported in *A. baumannii*, albeit very rarely, are *rmtA* [179,425], *rmtB* [252], *rmtC* [202,252] and *rmtD* [179]. An *rmtE*-positive *A. baumannii* was recently reported in Mexico (accession number: NZ_ULHD01000074.1); however, to our knowledge this study is the first to report *rmtE3* in an *A. baumannii* isolate in Europe. The host isolate from this study belonged to ST79, which has previously been reported associated with *rmtC* [196].

In contrast to the Enterobacterales and *A. baumannii*, 16S RMTase genes were only found in 8.6% of *P. aeruginosa* isolates. High-level pan-aminoglycoside resistance in *P. aeruginosa* is therefore likely due to alternative resistance mechanisms such as impermeability, MexXY-OprM efflux pumps or a combination of AMEs [426-428]. In this study only *rmtB*, *rmtC*, *rmtD*, and *rmtF* were identified in this species; however, *armA*, *rmtA*, *rmtE*, *rmtG* and *npmA* have previously been reported in *P. aeruginosa* elsewhere [131,181,295,296,429]. In this study, several rarely reported 16S RMTase gene variants were identified in *P. aeruginosa*, including *rmtB4*, *rmtD3* and *rmtF2*, which were initially reported in Nepal [144], Myanmar [140] and Nepal [144], respectively. Most patients had an unknown travel history so it is not known if they had travelled to Nepal, Myanmar or countries where these variants have been subsequently identified such as *rmtD3* in Poland [275]. However, three (33.3%) patients with *rmtB4*-positive *P. aeruginosa* had previously travelled to India, indicating that *rmtB4*-positive *P. aeruginosa* are circulating outside of Nepal. Additionally, one patient with a *rmtD3*-positive *P. aeruginosa* isolate had travelled to India and another had not travelled outside of the UK or the Republic of Ireland, indicating that *rmtD3*-positive *P. aeruginosa* are circulating outside of Myanmar and Poland. *rmtF2* was identified in one ST664 isolate, which was the same ST associated with two of the three *rmtF2*-positive isolates found in Nepal by Tada *et al*. [144], whilst *rmtB4* and *rmtD3* were associated with different STs than previously reported [140,144,275]. *rmtB4* and *rmtD3* were first identified in strains isolated in 2011 [144] and 2014 [140], respectively but in this study these genes were identified in strains isolated in 2008 and 2003, respectively, meaning that these variants have been circulating in *P. aeruginosa* longer than previously recognised.

In this study, 16S RMTase genes were found to be highly associated with carbapenemase genes, with 94.3% of 16S RMTase-positive isolates harbouring carbapenemase genes compared to only 65.9% of 16S RMTase-negative isolates. It therefore seems likely that 16S RMTase genes are spreading in the UK, particularly in England, due to their association with carbapenemase genes. bla_{NDM} was the most common carbapenemase gene identified in 16S

RMTase-producing isolates and was found associated with *armA*, *rmtB* and *rmtC*; *rmtD* was associated with *bla*$_{VIM}$ and *rmtF* with *bla*$_{OXA-48-like}$ carbapenemase genes, particularly *bla*$_{OXA-232}$. The latter association has only been reported previously with *rmtF* in Switzerland [265]. In contrast, this is the first report of *bla*$_{VIM}$ associated with *rmtD* in *P. aeruginosa*.

Analysis of WGS data identified that 16S RMTase genes were frequently associated with other antibiotic resistance genes such as ESBL genes, particularly *bla*$_{CTX-M-15,}$ (76.7%) and PMQR genes such as *aac(6')-Ib-cr* (74.2%) and *qnrB1* (39.6%). The frequency with which these genes, as well as those encoding carbapenemases, are associated with 16S RMTase genes suggests that they may be co-located on MGEs such as plasmids, which will be investigated in Chapter 7 of this thesis. Plasmids co-harbouring 16S RMTase genes alongside other resistance genes such as *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib-cr* or *qnrB* have previously been reported in Kenya [430], China [162,315,431] and Egypt [205]. Co-location of 16S RMTase genes with other resistance genes on plasmids would allow co-selection of resistance by use of antibiotics of different classes as well as the spread of these genes to other strains of bacteria, therefore promoting multidrug resistance.

In this study, multiple 'high-risk' bacterial clones were found associated with 16S RMTase-positive isolates. *A. baumannii* International Clone II, which has spread globally [432], has been found highly associated with *armA* and *bla*$_{OXA-23}$ [150,151,239,433], which explains the high prevalence of *armA* and *bla*$_{OXA-23}$-positive *A. baumannii* in this collection of isolates. *K. pneumoniae* ST14, a 'high-risk' clone known to carry *bla*$_{NDM-1}$ [44,434], was frequently associated in this study with *armA*, (97.3%) with many (90.8%) isolates also positive for *bla*$_{NDM-1}$. *K. pneumoniae* ST14 isolates co-producing *armA* and *bla*$_{NDM-1}$ have been previously reported in India [435], Oman [436] and Pakistan [437]. In this study, *rmtB* was frequently identified in *E. coli* ST167, an internationally disseminated clone associated with *bla*$_{NDM}$ [438,439], and ST405, a worldwide disseminated clone known to harbour *bla*$_{CTX-M-15}$ and *bla*$_{NDM}$ [44,440,441]. All ST167 isolates and 81.8% ST405 isolates were positive for *bla*$_{NDM-5}$; the association of *rmtB* with *bla*$_{NDM-5}$ has been previously reported in ST167 in China [438,442]

and ST405 in Italy [443]. *P. aeruginosa* ST357, a 'high-risk' clone known to be associated with IMP and VIM carbapenemases [387,444,445], was found to harbour *rmtB1*, *rmtC* and *rmtD3* 16S RMTase genes in this collection of isolates. The association of 16S RMTase genes with 'high-risk' bacterial clones suggests they have the potential to spread globally due to these bacterial clones being highly antibiotic resistant and/or virulent [446,447].

PFGE and VNTR analysis showed that there was evidence of diversity within the bacterial species and within STs, indicating that different strains carrying 16S RMTase genes are circulating within the UK. This means that clonal expansion may not be the sole reason behind the emergence of 16S RMTases and that other factors may be contributing to their spread, such as dissemination of plasmids or other MGEs.

Antibiotic susceptibility testing showed that 16S RMTase-producing bacteria exhibit high levels of resistance to carbapenems, 3rd generation cephalosporins and fluoroquinolones, which are the three main antibiotic classes used to treat Gram-negative infections [58,59]. Fortunately, lower levels of resistance were found for tigecycline (42.3%) and colistin (8.6%), which are last line antibiotics for MDR Gram-negative infections [448-450]. These results show that 16S RMTase-producing bacteria are frequently MDR, leaving few therapeutic options.

Analysis of patient demographic data showed that most patients with a travel history who had 16S RMTase-producing bacteria had travelled to India, Pakistan and Thailand. However, these data are unreliable as 73.9% (838/1,134) patients had an unknown travel history due to referring laboratory staff not completing sample request forms. It is therefore unclear how many other 16S RMTase-producing bacteria originated from patients with a history of travel. Interestingly, 30.3% (50/165) patients who had travelled abroad also received medical treatment in these countries, particularly in India (42.0%, 21/50) and Thailand (12.0%, 6/50), which are known reservoirs of carbapenemase genes [451-453]. Therefore, these countries are also likely reservoirs of 16S RMTase genes and receiving medical treatment in these countries could be a possible risk factor for the acquisition of 16S RMTase-producing bacteria, which will be investigated in the prospective surveillance study conducted in Chapter 6.

The main limitation to this study is that this strain collection is heavily biased toward carbapenemase-producing bacteria due to the AMRHAI Reference Unit focussing on surveillance of carbapenem-resistant bacteria. Therefore, the high number of 16S RMTase-producers identified does not truly represent the occurrence of 16S RMTase and carbapenemase genes among Enterobacterales in the UK. Another limitation of this study was that 16S RMTase-positive bacteria were mainly isolated from England (98.2%, 1,288/1,312) as the AMRHAI Reference Unit is not the reference laboratory for the Republic of Ireland, Scotland and Wales. Therefore, this may explain why few 16S RMTase-positive isolates originated from these countries and means that these countries are not accurately represented in this study.

In conclusion, this study identified a high number of 16S RMTase genes in pan-aminoglycoside-resistant bacteria referred to the AMRHAI Reference Unit between 2003-2015 and identified an association between 16S RMTase and carbapenemase genes, which may be contributing to their spread in the UK. However, due to the biased nature of this strain collection towards carbapenemase-producing bacteria an unbiased strain collection needs to be investigated to determine a more accurate prevalence of 16S RMTase genes in the UK, which will be explored in the following chapter.

Chapter 5: Retrospective screening of clinical isolates from the BSAC Resistance Surveillance Project's Bacteraemia Programme for 16S RMTase genes

5.1 Introduction

In the previous chapter the occurrence of 16S RMTase genes in the AMRHAI Reference Unit's strain collection was determined. However, due to the Reference Unit's focus on surveillance of exceptional antibiotic resistance (primarily to the carbapenems) its strain collection is biased towards carbapenemase-producing bacteria. Analysis of an unbiased strain collection, such as the strain collection from the BSAC Resistance Surveillance Project, is therefore needed to determine a more accurate prevalence of 16S RMTase-producing bacteria within the UK.

The BSAC Resistance Surveillance Project was set up in 1999 to conduct national long-term surveillance of antimicrobial resistance in the UK, which had not been conducted before and had been deemed essential by the Standing Medical Advisory Committee (SMAC) Sub-Group on Antimicrobial Resistance due to concern over rising antimicrobial resistance levels at the time [454,455].

The BSAC Resistance Surveillance Project is split into two surveillance programmes, the Respiratory Programme and the Bacteraemia Programme, which were initiated in 1999 and 2001, respectively [455,456]. The former was created to study the main bacterial species causing lower respiratory tract infections such as *S. pneumoniae* and *Haemophilus influenzae* and the latter was set up to study bacterial isolates causing bloodstream infections including *E. coli*, *K. pneumoniae* and *P. aeruginosa* [455].

Twenty to 25 clinical laboratories are recruited annually across the UK and Ireland for both programmes; each laboratory sends the first 10-20 consecutive non-duplicate isolates per bacterial species chosen for each programme [455,456]. These isolates are screened against a standard panel of antibiotics to determine resistance levels for each species, providing data on the epidemiology of, and trends in, antimicrobial resistance over time in both the community and hospital settings [455,456].

As consecutive isolates are screened, selection bias is avoided, which may otherwise lead to over-representation of highly antibiotic-resistant or unusual strains [457] meaning that it is an

ideal strain collection in which to study the occurrence of 16S RMTase-producing bacteria. In this study, WGS data derived from Gram-negative bacteria isolated in the UK and Ireland that were collected as part of the Bacteraemia Programme spanning 2001-2013 were screened for the presence of 16S RMTase genes.

5.2 Aims

The aim of this study was to identify the occurrence of 16S RMTase genes in Gram-negative bacterial isolates from the BSAC Resistance Surveillance Project's Bacteraemia Programme between 2001-2013 using WGS data generated by the Wellcome Trust Sanger Institute.

5.3 Methods

5.3.1 Analysis of WGS data from *A. baumannii*, Enterobacterales and *P. aeruginosa* isolates from the BSAC Resistance Surveillance Project

A list of 1,566 bacterial isolates from the Bacteraemia Programme of the BSAC Resistance Surveillance Project from 2001-2013 was provided by the Wellcome Trust Sanger Institute. These isolates had previously undergone WGS via Hiseq 2500 platform (Illumina) due to being MDR (classified as resistance to a minimum of three antibiotic classes) to analyse antibiotic resistance determinants and population structure via other studies supported by the Wellcome Trust Sanger Institute [458-461]. These isolates were not previously screened for 16S RMTase genes and no data was available other than the laboratories the bacteria were isolated from and MICs.

5.3.2 Determination of MICs

MICs were previously determined by the AMRHAI Reference Unit at PHE (Colindale) using the BSAC agar dilution method and EUCAST guidelines (see Section 2.3). Table 5.1 shows the antibiotics screened against *A. baumannii*, Enterobacterales and *P. aeruginosa* isolates.

Table 5.1: The range in concentration (mg/L) of antibiotics used in the agar dilution method to determine MICs as well as the target species.

Antibiotic +/- inhibitor	Range tested (mg/L)	Tested species
Aminoglycosides		
amikacin	2-64	*P. aeruginosa*
gentamicin	0.25-256	*A. baumannii* and Enterobacterales
	1-16	*P. aeruginosa*
tobramycin	1-16	*P. aeruginosa*
Penicillins		
amoxicillin	4-512	Enterobacterales
amoxicillin + clavulanic acid[a]	0.5-128	Enterobacterales
piperacillin + tazobactam[b]	0.5-512	*A. baumannii* and Enterobacterales
	4-128	*P. aeruginosa*
ticarcillin + clavulanic acid[a]	8-128	*P. aeruginosa*
2nd generation cephalosporins		
cefoxitin	0.5-256	Enterobacterales
cefuroxime	1-256	Enterobacterales
3rd generation cephalosporins		
cefotaxime	0.015-512	Enterobacterales
ceftazidime	0.03-512	*A. baumannii* and Enterobacterales
	1-64	*P. aeruginosa*
Carbapenems		
imipenem	0.06-128	*A. baumannii* and Enterobacterales
	0.25-16	*P. aeruginosa*
meropenem	0.25-16	*P. aeruginosa*
Monobactams		
aztreonam	1-64	*P. aeruginosa*
Fluoroquinolones		
ciprofloxacin	0.015-256	*A. baumannii* and Enterobacterales
	0.25-4	*P. aeruginosa*
Tetracyclines		
minocycline	0.12-64	*A. baumannii* and Enterobacterales
tetracycline	1-256	*A. baumannii* and Enterobacterales
Glycylcyclines		
tigecycline	0.06-32	*A. baumannii* and Enterobacterales
Polymyxins		
colistin	0.5-32	Enterobacterales
	0.5-16	*P. aeruginosa*

[a] = Concentration of clavulanic acid was 2 mg/L; [b] = Concentration of tazobactam was 4 mg/L

5.3.3 Bacterial typing and identification of antibiotic resistance genes

MLST data were derived from the WGS data using the SRST2 program version 0.2.0 [40], which was also used to identify antibiotic resistance genes using the incorporated ARG-ANNOT database [38]. MLST and antibiotic resistance gene content were confirmed using MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/) [415] and ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/) [39], respectively. eBURST diagrams were generated using the PHYLOViZ online tool (http://www.phyloviz.net/) [410].

5.3.4 Analysis of patient demographic information

No patient demographic data were available for the bacterial isolates as patient identifiable information is not collected as part of the BSAC Resistance Surveillance Project.

5.3.5 Study approval

Permission to work with the bacterial isolates was granted by Professor Julian Parkhill and Professor Sharon Peacock at the Wellcome Trust Sanger Institute.

5.4 Results

5.4.1 Bacterial isolates

Bacterial isolates included *E. cloacae* complex (23.6%, 369/1,566), *K. pneumoniae* (20.6%, 323/1,566), *P. aeruginosa* (18.7%, 293/1,566) *E. coli* (17.3%, 271/1,566), *S. marcescens* (13.7%, 214/1,566), *K. oxytoca* (3.1%, 48/1,566), *Enterobacter sakazakii* (0.9%, 14/1,566), *A. baumannii* (0.8%, 13/1,566), *K. aerogenes* (0.8%, 12/1,566), *Serratia liquefaciens* (0.4%, 6/1,566), *Enterobacter amnigenus* (0.1%, 2/1,566) and *Enterobacter gergoviae* (0.1%, 1/1,566).

These isolates were submitted from 49 laboratories spanning 13 regions within the UK and Ireland, with the largest number of isolates submitted by Wales (11.0%, 173/1,566) followed by the Republic of Ireland (10.1%, 158/1,566) and London (8.6%, 134/1,566). The origin of 13.7% (215/1,566) isolates was unknown (Figure 5.1).

5.4.2 Identification of 16S RMTase genes

Only 0.3% (4/1,566) of isolates were positive for 16S RMTase genes. The 16S RMTase genes identified were *armA* and *rmtC* + *rmtF*, where *armA* was identified in two *K. pneumoniae* isolates (which were isolated from the East of England in 2007 and the West Midlands in 2008) and one *A. baumannii* isolate (from London in 2004). The gene combination *rmtC* + *rmtF* was found in one *K. pneumoniae* isolate from the North West of England in 2009.

5.4.3 Antibiotic susceptibility

MIC data for the 16S RMTase-positive and -negative *A. baumannii*, Enterobacterales and *P. aeruginosa* isolates can be found in Tables 5.2-5.4.

The single *armA*-positive *A. baumannii* isolate was resistant to ciprofloxacin and imipenem. All 16S RMTase-negative isolates were resistant to ciprofloxacin but none of the isolates were resistant to imipenem. No EUCAST clinical breakpoints were available for ceftazidime so resistance could not be calculated. MIC values of the other antibiotics tested can be found in Table 5 in Appendix 1.

All 16S RMTase-positive Enterobacterales were resistant to cefotaxime, ceftazidime and ciprofloxacin but only one isolate was resistant to imipenem. No 16S RMTase-positive isolates were resistant to tigecycline. None of the 16S RMTase-positive isolates were tested against colistin so resistance is unknown. A total of 76.4% (960/1,257), 75.1% (824/1,097) and 52.6% (661/1,257) isolates were resistant to ciprofloxacin, cefotaxime and ceftazidime, respectively. However, only 13.4% (162/1,209), 0.5% (6/1,257) and 1.2% (1/83) isolates were resistant to tigecycline, imipenem and colistin, respectively. MIC values of the other antibiotics tested can be found in Table 6 in Appendix 1.

Susceptibility testing data for the *P. aeruginosa* isolates is summarised in Table 5.4. MIC values of the other antibiotics tested can be found in Table 7 in Appendix 1.

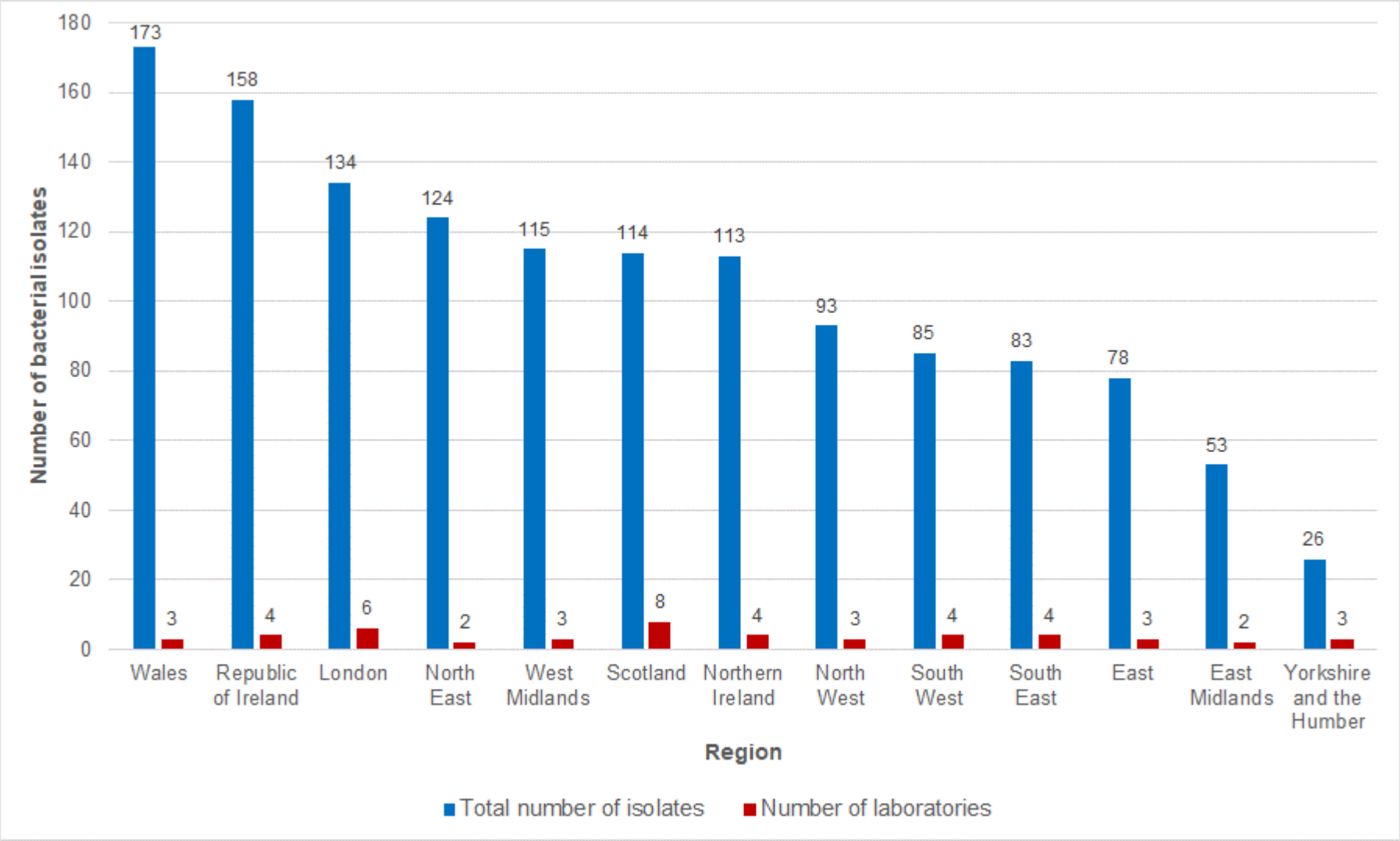


Figure 5.1: Total number of isolates (n=1,566) collected by the Bacteraemia Programme of the BSAC Resistance Surveillance Project with WGS data from each region and the number of submitting laboratories between 2001-2013.

Table 5.2: MIC values for 16S RMTase-positive (n=1) and -negative (n=12) *A. baumannii* isolates from the Bacteraemia Programme of the BSAC Resistance Surveillance Project, 2001-2013. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	% R
imipenem (0.03-128)	≤2/>8	Positive								1				-	100
		Negative	3		4		4		1					-	0
ceftazidime (0.03-512)	-	Positive											1	-	-
		Negative						2	1	5		2	2	-	-
ciprofloxacin (0.015-256)	≤1/>1	Positive											1	-	100
		Negative						2		1		2	7	-	100

ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 5.3: MIC values for 16S RMTase-positive (n=3) and -negative (n=1,257) Enterobacterales isolates from the Bacteraemia Programme of the BSAC Resistance Surveillance Project, 2001-2013. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTases	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	% R
imipenem (0.03-128)	≤2/>8	Positive		2						1				-	33.3
		Negative	412	349	370	97	12	6	5		2	3	1	-	0.5
cefotaxime (0.015-512)	≤1/>2	Positive											3	-	100
		Negative	113	64	53	27	16	38	70	154	98	64	400	160	75.1
ceftazidime (0.03-512)	≤1/>4	Positive									1		2	-	100
		Negative	30	120	147	163	98	38	46	102	85	93	335	-	52.6
ciprofloxacin (0.015-256)	≤0.25/>0.5	Positive										2	1	-	100
		Negative	171	59	67	169	140	108	67	65	71	94	246	-	76.4
colistin (0.25-16)	≤2/>2	Positive												3	-
		Negative		4	54	24				1				1,174	1.2
tigecycline (0.06-32)	≤1/>2	Positive				2	1							-	0
		Negative	33	144	293	311	266	113	40	8	1			48	13.4

ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 5.4: MIC values for 16S RMTase-negative *P. aeruginosa* isolates (n=293) from the Bacteraemia Programme of the BSAC Resistance Surveillance Project, 2001-2013. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTases	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	% R
imipenem (0.25-6)	≤4/>8	Negative		13	2	44	122		13	90				9	31.7
meropenem (0.25-16)	≤2/>8	Negative		97	35	46	8	28	18	54				7	18.9
ceftazidime (1-64)	≤8/>8	Negative				62	43	87	8	32	7	46		8	29.8
ciprofloxacin (0.25-4)	≤0.5/>0.5	Negative		177	12	14	11	70						9	33.5
colistin (0.5-16)	≤2/>2	Negative			276	1	6		1	1				8	0.7

ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

5.4.4 MLST

MLST data were available for *A. baumannii*, *E. cloacae* complex, *E. coli*, *K. oxytoca*, *K. pneumoniae* and *P. aeruginosa* isolates. Analysis of the 16S RMTase-producing isolates found that 66.7% (2/3) *K. pneumoniae* isolates belonged to ST231, which were positive for *armA* and *rmtC* + *rmtF*. Additionally, a single *K. pneumoniae* isolate belonged to ST15, which was positive for *armA*. The single 16S RMTase-producing *A. baumannii* isolate belonged to ST2, which belongs to International Clone II. The association of all identified STs for *A. baumannii* and *K. pneumoniae* with and without 16S RMTase genes can be seen in Figures 5.2 and 5.3.

Analysis of the STs of 16S RMTase-negative isolates (Table 5.5) found there were 105 *E. cloacae* complex STs, where ST108 (8.2%, 30/366) was the most common; 94 *K. pneumoniae* STs with ST15 (9.4%, 30/320) and ST874 (9.4%, 30/320) the most common; 86 *P. aeruginosa* STs with ST253 (8.2%, 24/293) the most common; 39 *E. coli* STs, where ST131 (64.6%, 175/271) was the most common; 13 *K. oxytoca* STs, where ST2 (27.7%, 13/47) was the most frequent ST identified and five STs were identified in *A. baumannii* where ST2 (41.7%, 5/12) was the most frequently identified.

Out of the STs identified in the 16S RMTase-producing isolates, *A. baumannii* ST2 (41.7%, 5/12) and *K. pneumoniae* ST15 (9.4%, 30/320) were the most common STs found in the 16S RMTase-negative isolates. However, *K. pneumoniae* ST231, which was the most common *K. pneumoniae* ST in the 16S RMTase-positive isolates (66.7%, 2/3), was only identified in 0.6% (2/320) 16S RMTase-negative isolates.

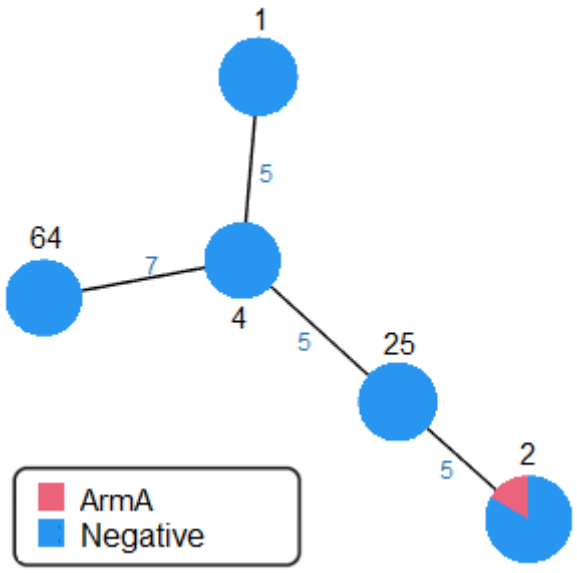


Figure 5.2: eBURST diagram of 16S RMTase-positive (n=1) and -negative (n=12) *A. baumannii* isolates. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of SLVs between STs (black font).

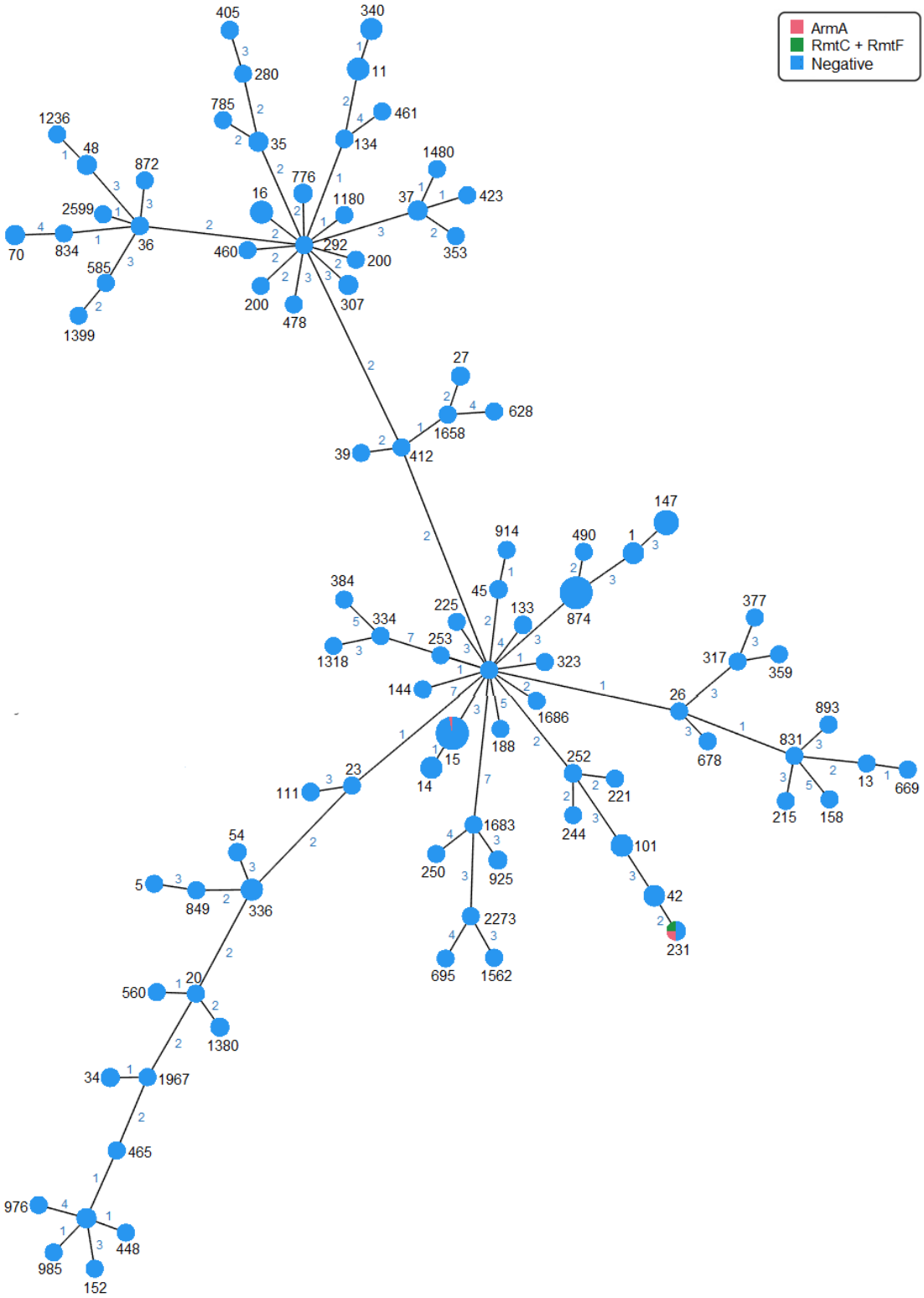


Figure 5.3: eBURST diagram of 16S RMTase-positive (n=3) and -negative (n=320) *K. pneumoniae* isolates. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of SLVs between STs (black font).

Table 5.5: Total STs identified for 16S RMTase-positive and -negative *A. baumannii*, *E. cloacae* complex, *E. coli*, *K. oxytoca*, *K. pneumoniae* and *P. aeruginosa* isolates from the Bacteraemia Programme of the BSAC Resistance Surveillance Project, 2001-2013.

Species	Number of isolates	16S RMTase (no. of isolates)	STs (no. of isolates if >1)	Number of submitting laboratories	Number of regions
A. baumannii	13	*armA* (1)	2	1	1
		Negative (12)	2 (5), 1 (2), 25 (2), 4, 64 and unknown	10	7
E. cloacae complex	369	Negative (369)	13, 23, 24 (9), 45, 46, 50 (11), 62, 65 (15), 66 (4), 68 (18), 78 (19), 88, 90 (13), 93 (5), 94 (11), 97 (3), 102 (4), 104 (4), 106 (3), 108 (30), 110 (10), 114 (9), 116 (3), 118 (4), 120 (3), 125, 128, 133 (24), 134 (2), 135 (8), 138, 141 (2), 144 (2), 146, 151 (2), 162, 166, 168 (4), 171 (11), 175 (2), 177, 182 (2), 190 (2), 198 (2), 200 (2), 204, 233, 264 (2), 267, 269, 278 (4), 286 (8), 295 (2), 304, 346, 395, 421, 422, 435, 461, 519, 544, 566, 598, 609, 610, 636 (2), 742 (2), 767, 772, 773, 774, 776, 777, 778, 779 (2), 780 (2), 782 (2), 784, 785, 786, 787, 788 (2), 790, 792, 793, 794, 795, 796, 797, 798 (4), 799 (2), 800 (2), 801, 802, 803 (2), unknown (37) and failed	37	14
E. coli	271	Negative (271)	10 (4), 12 (3), 23 (3), 38 (8), 44, 46, 69 (5), 73, 88 (4), 131 (175), 156, 167 (4), 205, 224, 315, 354 (10), 393 (9), 404, 405 (5), 410 (2), 448, 617 (2), 635, 648 (11), 746, 1193, 1284, 1394, 2450, 5291, 5446, 5456, 5489, 5504, 5527, 5562, 5641, 5667, failed and unknown	46	14
K. oxytoca	47	Negative (47)	2 (13), 11 (2), 36 (4), 40, 50 (2), 58, 85, 88, 92, 105, 141, 153, 176 (5) and unknown (13)	21	12

Table 5.5 continued

Species	Number of isolates	16S RMTase (no. of isolates)	STs (no. of isolates if >1)	Number of submitting laboratories	Number of regions
K. pneumoniae	323	*armA* (2)	15, 231	2	2
		rmtC + *rmtF* (1)	231	1	1
		Negative (320)	1 (8), 5, 11 (10), 13, 14 (9), 15 (30), 16 (11), 17 (4), 20, 23, 26, 27 (3), 29 (5), 34 (3), 35 (5), 36 (2), 37 (5), 39, 42 (8), 45 (2), 48 (5), 54 (2), 70 (5), 101 (10), 111 (2), 133 (2), 134 (2), 144, 147 (15), 152, 158, 163, 188, 200, 215, 221, 225, 231 (2), 244, 250, 252 (2), 253, 280, 292, 307 (5), 317, 323, 334, 336 (9), 340 (9), 353, 359, 377, 384, 405 (2), 412, 423, 448, 460, 461, 465, 478, 490, 560, 585, 628, 669, 678, 695, 776 (4), 785, 831, 834, 849, 872, 874 (30), 893, 914, 925 (3), 976, 985, 1180, 1236, 1318, 1380 (3), 1399, 1480, 1562, 1658, 1683, 1686, 1967, 2004, 2273, 2599, and unknown (40)	38	14
P. aeruginosa	293	Negative (293)	17, 27 (2), 108, 110, 111 (2), 137, 146, 155 (5), 167, 170, 175 (22), 179 (5), 205, 207 (8), 217 (4), 231, 235 (7), 242, 244 (7), 245 (3), 252, 253 (24), 254 (3), 257, 260 (4), 267, 270. 273, 274 (3), 298 (2), 308 (7), 309 (4), 313, 316 (2), 319 (2), 334, 348 (7), 357 (8), 358, 360, 379, 389, 395 (9), 408, 446 (3), 455, 498, 508, 527, 532, 554, 560 (4), 589, 591, 598, 633 (2), 664 (2), 667, 672, 698, 699, 701, 815, 977 (2). 1027, 1053, 1068, 1086, 1149, 1203, 1223, 1232, 1247, 1248 (3), 1320, 1326, 1400, 1412, 1556 (8), 1753, 1806, 1848, 1858, 2012, 2044 and 2100	23*	12*

* = The region and submitting laboratory was unknown for 215 *P. aeruginosa* isolates.

5.4.5 Identification of antibiotic resistance genes

5.4.5.1 Analysis of carbapenemase genes

Analysis of 16S RMTase-producing isolates showed that 50.0% (2/4) had carbapenemase genes, where the *armA*-positive *A. baumannii* isolate was positive for bla_{OXA-23} + bla_{OXA-66} and the *rmtC* + *rmtF*-positive *K. pneumoniae* ST231 isolate was positive for bla_{NDM-1} (Table 5.6). In contrast, only 1.9% (29/1,562) 16S RMTase-negative isolates were positive for carbapenemases, including 83.3% (10/12) *A. baumannii*, 1.4% (17/1,257) Enterobacterales and 0.7% (2/293) *P. aeruginosa* isolates. The carbapenemase gene bla_{KPC-4} (31.0%, 9/29) was the most common and was mainly identified in *E. cloacae* complex (88.9%, 8/9) as well as *K. aerogenes* (11.1%, 1/9). The carbapenemase genes bla_{NDM-1} and bla_{OXA-23} were not found in 16S RMTase-negative isolates.

Table 5.6: Total number of carbapenemase genes found in 16S RMTase-positive (n=4) and 16S RMTase-negative (n=1,562) isolates from the BSAC Resistance Surveillance Project's Bacteraemia Programme between 2001-2013.

Carbapenemase genes	Bacterial Isolate(s)					
	16S RMTase-negative isolates	***A. baumannii* ST2 (*armA*)**	***K. pneumoniae* ST231 (*armA*)**	***K. pneumoniae* ST15 (*armA*)**	***K. pneumoniae* ST231 (*rmtC* + *rmtF*)**	**Total**
Negative	1,533	-	1	1	-	1,535
***bla*$_{IMP-34}$**	1	-	-	-	-	1
***bla*$_{KPC-4}$**	9	-	-	-	-	9
***bla*$_{NDM-1}$**	-	-	-	-	1	1
***bla*$_{NMC-A}$**	1	-	-	-	-	1
***bla*$_{OXA-48}$**	1	-	-	-	-	1
***bla*$_{OXA-64}$**	2	-	-	-	-	2
***bla*$_{OXA-66}$**	6	-	-	-	-	6
***bla*$_{OXA-69}$**	2	-	-	-	-	2
***bla*$_{OXA-23}$ + *bla*$_{OXA-66}$**	-	1	-	-	-	1
***bla*$_{VIM-1}$**	1	-	-	-	-	1
***bla*$_{VIM-2}$**	2	-	-	-	-	2
***bla*$_{VIM-4}$**	4	-	-	-	-	4
Total	1,562	1	1	1	1	1,566

5.4.5.2 Analysis of ESBL genes

ESBL genes were found in all 16S RMTase-producing *K. pneumoniae* isolates and 35.9% (561/1,562) 16S RMTase-negative isolates (Table 5.7). *bla*$_{\text{CTX-M-15}}$ was the most common ESBL gene identified in both 16S RMTase-producing and -negative bacteria as it was found in 100% (3/3) and 47.1% (264/561) isolates, respectively. Most *bla*$_{\text{CTX-M-15}}$ genes were found associated with *K. pneumoniae* (44.6%, 144/323). Additional ESBL genes were identified in the 16S RMTase-producing *K. pneumoniae* isolates including *bla*$_{\text{SHV-55}}$ (66.7%, 2/3) and *bla*$_{\text{SHV-100}}$ (33.3%, 1/3).

5.4.5.3 Analysis of PMQR genes and chromosomal mutations conferring fluoroquinolone resistance

PMQR genes were identified in all 16S RMTase-producing isolates, apart from the *armA*-positive *A. baumannii* isolate, as well as 52.0% (812/1,562) 16S RMTase-negative isolates (Table 5.8). The most common PMQR gene identified was *oqxB*, which is intrinsic to *K. pneumoniae* and was identified in all 16S RMTase-producing *K. pneumoniae* isolates (100%, 3/3) as well as 16S RMTase-negative *Klebsiella* spp. (90.5%, 334/369) and *Enterobacter* spp. (21.4%, 85/397). Additionally, *qnrB1* was identified in 66.7% (2/3) 16S RMTase-producing *K. pneumoniae* isolates, which were *armA* and *rmtC* + *rmtF*-positive and belonged to ST231. In contrast, *qnrB1* was found in 4.2% (34/812) 16S RMTase-negative isolates. *aac(6')-Ib-cr* was identified in one (33.3%) 16S RMTase-producing *K. pneumoniae* isolate, which was *armA*-positive and belonged to ST231 and was also identified in 31.4% (255/812) 16S RMTase-negative isolates.

No chromosomal mutations in the QRDRs of *gyrA* and/or *parC* genes were identified in the 16S RMTase-producing isolates. In contrast, chromosomal mutations were only identified in 16S RMTase-negative *E. coli* isolates (95.9%, 260/271) (see Table 8 in Appendix 1).

Table 5.7: Total number of ESBL genes found in 16S RMTase-positive (n=4) and 16S RMTase-negative (n=1,562) isolates from the BSAC Resistance Surveillance Project's Bacteraemia Programme between 2001-2013.

ESBL genes	Bacterial Isolate(s)					
	16S RMTase-negative isolates	***A. baumannii* ST2 (*armA*)**	***K. pneumoniae* ST231 (*armA*)**	***K. pneumoniae* ST15 (*armA*)**	***K. pneumoniae* ST231 (*rmtC* + *rmtF*)**	**Total**
Negative	1,001	1	-	-	-	1,002
***bla*$_{CTX-M-3}$**	4	-	-	-	-	4
***bla*$_{CTX-M-9}$**	28	-	-	-	-	28
***bla*$_{CTX-M-14}$**	6	-	-	-	-	6
***bla*$_{CTX-M-15}$**	264	-	1	1	1	267
***bla*$_{CTX-M-16}$**	1	-	-	-	-	1
***bla*$_{CTX-M-26}$**	4	-	-	-	-	4
***bla*$_{CTX-M-27}$**	3	-	-	-	-	3
***bla*$_{CTX-M-36}$**	1	-	-	-	-	1
***bla*$_{CTX-M-55}$**	1	-	-	-	-	1
***bla*$_{OXA-129}$**	9	-	-	-	-	9
***bla*$_{SHV-2}$**	1	-	-	-	-	1
***bla*$_{SHV-5}$**	7	-	-	-	-	7
***bla*$_{SHV-8}$**	2	-	-	-	-	2
***bla*$_{SHV-12}$**	144	-	-	-	-	144
***bla*$_{SHV-13}$**	2	-	-	-	-	2
***bla*$_{SHV-27}$**	3	-	-	-	-	3
***bla*$_{SHV-28}$**	3	-	-	-	-	3
***bla*$_{SHV-31}$**	7	-	-	-	-	7
***bla*$_{SHV-38}$**	2	-	-	-	-	2
***bla*$_{SHV-39}$**	15	-	-	-	-	15
***bla*$_{SHV-40}$**	5	-	-	-	-	5

Table 5.7 continued

ESBL genes	Bacterial Isolate(s)					
	16S RMTase-negative isolates	*A. baumannii* ST2 (*armA*)	*K. pneumoniae* ST231 (*armA*)	*K. pneumoniae* ST15 (*armA*)	*K. pneumoniae* ST231 (*rmtC* + *rmtF*)	Total
bla_{SHV-41}	1	-	-	-	-	1
bla_{SHV-55}	-	-	1	1	-	2
bla_{SHV-67}	14	-	-	-	-	14
bla_{SHV-70}	3	-	-	-	-	3
bla_{SHV-98}	2	-	-	-	-	2
bla_{SHV-99}	2	-	-	-	-	2
$bla_{SHV-100}$	46	-	-	-	1	47
$bla_{SHV-110}$	2	-	-	-	-	2
$bla_{SHV-122}$	1	-	-	-	-	1
$bla_{SHV-148}$	17	-	-	-	-	17
bla_{TEM-3}	2	-	-	-	-	2
bla_{TEM-20}	1	-	-	-	-	1
bla_{TEM-30}	23	-	-	-	-	23
bla_{TEM-33}	19	-	-	-	-	19
$bla_{TEM-104}$	3	-	-	-	-	3
$bla_{TEM-106}$	1	-	-	-	-	1
Total	1,650	1	2	2	2	1,657

Table 5.8: Total number of PQMR genes found in 16S RMTase-positive (n=4) and 16S RMTase-negative (n=1,562) isolates from the BSAC Resistance Surveillance Project's Bacteraemia Programme between 2001-2013.

PQMR genes	Bacterial Isolate(s)					
	16S RMTase-negative isolates	***A. baumannii* ST2 (*armA*)**	***K. pneumoniae* ST231 (*armA*)**	***K. pneumoniae* ST15 (*armA*)**	***K. pneumoniae* ST231 (*rmtC* + *rmtF*)**	**Total**
Negative	750	1	-	-	-	751
aac(6')-Ib-cr	255	-	1	-	-	256
qnrA1	97	-	-	-	-	97
qnrB1	34	-	1	-	1	64
qnrB2	18	-	-	-	-	18
qnrB4	3	-	-	-	-	3
qnrB7	5	-	-	-	-	5
qnrB19	1	-	-	-	-	1
qnrB38	1	-	-	-	-	1
qnrS1	19	-	-	-	-	19
oqxA	305	-	1	1	1	308
oqxB	419	-	1	1	1	422
qepA1	1	-	-	-	-	1
Total	1,653	1	3	2	3	1,690

5.5 Discussion

In this study, just four isolates out of 1,566 (0.3%) Gram-negative bacterial isolates from the BSAC Resistance Surveillance Project's Bacteraemia Programme between 2001-2013 were positive for 16S RMTase genes. The most commonly identified 16S RMTase gene was *armA* (75.0%, 3/4) but the two-gene combination *rmtC* + *rmtF* (25.0%, 1/4) was also identified, with the majority of genes (*armA* [n=2] and *rmtC* + *rmtF* [n=1]) being found in *K. pneumoniae* (75.0%, 3/4).

Although only a small number of 16S RMTase-producing isolates were identified, this study supports the hypothesis that 16S RMTase-producing bacteria had a higher association with carbapenemases (50.0%, 2/4) compared with 16S RMTase-negative bacteria (1.5%, 23/1,562). Carbapenemases are not frequently reported in bloodstream infections as only 3.6% (109/3,000) of CPE referred to the AMRHAI Reference Unit in 2017 originated from bloodstream infections [94]. Therefore, this explains why the prevalence of 16S RMTase genes was so low in this strain collection compared to the AMRHAI Reference Unit's strain collection (83.2%, 1,312/1,576), which was biased towards carbapenemase-producing bacteria (see Chapter 4). Additionally, this study suggests that KPC carbapenemases are more common in 16S RMTase-negative bacteria compared with 16S RMTase-positive bacteria, which appear to have a higher association with metallo-β-lactamase carbapenemases such as NDM [127,149,304].

The most commonly acquired ESBL and PMQR genes associated with 16S RMTase genes were $bla_{\text{CTX-M-15}}$, *aac(6')-Ib-cr* and *qnrB1*, which were also the most commonly identified genes found in 16S RMTase-producing bacteria from the AMRHAI Reference Unit (see Chapter 4). This suggests that these genes may be associated with 16S RMTase genes on MGEs such as plasmids, as previously reported in the literature [193,205,315,431], which could further facilitate their spread between bacteria and promote multidrug resistance but this needs to be investigated further (see Chapter 7).

Analysis of MICs identified that 16S RMTase-producing bacteria exhibit multidrug resistance as the 16S RMTase-producing *A. baumannii* and *K. pneumoniae* isolates were highly resistant to 3rd generation cephalosporins and fluoroquinolones, with 50.0% (2/4) isolates exhibiting resistance to carbapenems. This supports findings from the AMRHAI Reference Unit's strain collection where 16S RMTase-producing isolates exhibited high-level resistance to carbapenems, 3rd generation cephalosporins and fluoroquinolones but were generally susceptible to colistin (91.4%, 1,170/1,280) and 83.9% (791/943) isolates showed susceptibility to tigecycline (see Chapter 4). In this study colistin was not screened against the 16S RMTase-producing isolates but susceptibility to tigecycline was observed in all 16S RMTase-producing *K. pneumoniae* isolates. As aminoglycosides, β-lactams and fluoroquinolones are the most commonly used antibiotics to treat Gram-negative bacterial infections [58,59], MIC data from the BSAC Resistance Surveillance Project, as well as the AMRHAI strain collection, indicate that last-line antibiotics will be the most useful in treating 16S RMTase-producing *A. baumannii*, Enterobacterales or *P. aeruginosa*, where colistin can be used for all of these species and tigecycline can be used for all but the latter as tigecycline is inactive against *P. aeruginosa* [462].

'High-risk' clones were found associated with 16S RMTase genes including *A. baumannii* International Clone II, which was also the most predominant clone identified in 16S RMTase-producing *A. baumannii* (89.2%, 473/530) from the AMRHAI Reference Unit (see Chapter 4). This clone is known to have disseminated worldwide and carries the genes *armA*, $bla_{\text{OXA-23}}$ and intrinsically-encoded $bla_{\text{OXA-51-like}}$ genes [150,151,239,432,433]. Additionally, *armA* was found in *K. pneumoniae* ST15, which is an international 'high-risk' clone [463] that has previously been reported to carry *armA* in addition to $bla_{\text{NDM-1}}$ in France [464] and Lebanon [240]. However, no carbapenemases were found in ST15 isolates in this study. Lastly, *K. pneumoniae* ST231, which was the most common clone identified with 16S RMTase genes in this study and was one of the most common in the AMRHAI Reference Unit strain collection (15.6%, 49/315), was found associated with *armA* and the two-gene combination *rmtC* + *rmtF*,

where the *rmtC* + *rmtF* isolate was positive for bla_{NDM-1}. This clone is mainly associated with $bla_{OXA-232}$ [265,465,466] but has been known to carry bla_{NDM-1} in the UK [434] and the USA [467] and, according to Momin *et al*. [465], *K. pneumoniae* ST231 may be an emerging 'high-risk' clone disseminating throughout Southeast Asia. To date, it has only been reported to carry *rmtF* [265] meaning, to our knowledge, this is one of the first reports of an ST231 isolate carrying *armA* (which was also identified in ST231 isolates from the AMRHAI Reference Unit, see Chapter 4) or *rmtC* in combination with *rmtF*.

In contrast to the 16S RMTase-producing *P. aeruginosa* isolates from the AMRHAI Reference Unit's strain collection (see Chapter 4), the 'high-risk' clone *P. aeruginosa* ST253 was found to be the most common ST identified, which has spread globally and is mainly found in cystic fibrosis patients [468,469]. The *P. aeruginosa* 'high-risk' clones identified with 16S RMTase genes in the AMRHAI Reference Unit's strain collection (see Chapter 4) were either not identified in this strain collection (such as ST654, ST773, ST233, and ST277) or were found in low numbers such as ST357 (2.7%, 8/293). As the 16S RMTase-producing *P. aeruginosa* isolates from the AMRHAI Reference Unit were not isolated from blood, this suggests that the 'high-risk' clones associated with 16S RMTase genes did not play a key role in bacteraemia during 2001-2013, which is supported by these clones being absent or present in low numbers in this strain collection.

One limitation of this study is that only a small subset of bacterial isolates from the Bacteraemia Programme were studied as only 8.4% (1,566/18,633) isolates from 2001-2013 had WGS data readily available for analysis [470]. As the samples were randomly selected throughout the UK and were unbiased in terms of being selected for a specific resistance mechanism (such as carbapenemase production) the prevalence is a good indicator of the prevalence of 16S RMTase genes. However, these isolates were collected from 2001-2013 and were only from bloodstream infections so additional studies are needed to calculate the current prevalence of 16S RMTase genes in the UK. Another limitation of this study is that no patient demographic data were available for this study meaning that patient characteristics such as travel history

could not be used to identify possible risk factors for the acquisition of 16S RMTase-producing bacteria. Therefore, additional studies need to be conducted where patient demographic data are available to identify possible risk factors for the acquisition of 16S RMTase-producing bacteria.

In conclusion, this study identified a low number of 16S RMTase genes in Gram-negative isolates from the Bacteraemia Programme of the BSAC Resistance Surveillance Project, which were primarily carbapenemase gene-negative. The inability to identify many 16S RMTase- and carbapenemase-producing bacteria suggests that these genotypes may be genetically linked. The following chapter will investigate the current prevalence of 16S RMTase genes in the UK as well as identify potential risk factors for the acquisition of 16S RMTase-producing bacteria using a prospective study protocol. Additionally, it will aim to determine any linkage between carbapenemase and 16S RMTase genes.

Chapter 6: A prospective surveillance study to determine the period prevalence of 16S RMTase-producing bacteria in the UK.

6.1 Introduction

To date, there has been no systematic surveillance for 16S RMTase-producing bacteria within the UK. In the previous chapter, data arising from analysis of isolates from the BSAC Resistance Surveillance Project identified a prevalence rate of 0.3% (4/1,566). However, this prevalence rate may be outdated as the isolates were from 2001-2013 and bacterial isolates were all from bloodstream infections. As such, a new study was conducted in order to identify the current prevalence of 16S RMTase-producing bacteria in the UK.

Previous studies have identified the prevalence of 16S RMTases to be lower within Europe compared with Asia [127], as rates of 16S RMTase genes in non-duplicate Enterobacterales isolates were 0.12% (19/15,386), 0.15% (6/4,080) and 3.9% (38/985) in Belgium [153], Greece [471] and China [472], respectively. Other studies have focused on bacteria harbouring specific resistance genes such as ESBLs: rates of 16S RMTase genes in ESBL-producing Enterobacterales were 1.3% (5/373) [215] in France, compared with Saudi Arabia [189], which had a prevalence rate of 99.1% (111/112). High rates have also been found in carbapenemase-producing *K. pneumoniae* isolates including 35.1% (26/74) [473] in China and 70.4% (19/27) in Vietnam [199].

Additionally, studies have identified high levels of 16S RMTase genes in amikacin-resistant bacteria, with rates of 97.5% (193/198) in Gram-negative bacteria in China [157], 85.7% (30/35) in *E. coli* and *K. pneumoniae* in Taiwan [263] and 78.1% (75/96) in *A. baumannii* in Nepal [242]. Lee *et al*. [125] highlighted the usefulness of screening Gram-negative bacteria with high-level amikacin resistance (MIC ≥1024 mg/L) to select for those producing 16S RMTases, which was supported by Doi and Arakawa [304] who suggested using >256 mg/L amikacin to detect 16S RMTases. Amikacin is a good screening tool for the detection of 16S RMTases as it was designed to be resistant to AMEs. Therefore, high-level amikacin resistance should be indicative of 16S RMTase production, with the exception of AMEs that may cause amikacin resistance such as AAC(6')-Ib [90].

This study was therefore conducted using high-level amikacin resistance (MICs >256 mg/L) to screen for 16S RMTase-producing Gram-negative bacteria from hospital laboratories within the UK in order to calculate the prevalence of 16S RMTase genes. Additionally, this study aimed to determine possible risk factors for the acquisition of 16S RMTase-producing bacterial isolates using anonymized questionnaires with the potential that the resulting data might be used to inform guidelines for clinicians to identify 'at-risk' patients.

6.2 Aims

The aims of this study were to: i) conduct a prospective six-month surveillance study involving UK hospital laboratories to determine the period prevalence of 16S RMTase-producing bacteria in the UK and, ii) identify possible risk factors for the acquisition of 16S RMTase-producing bacteria.

6.3 Methods

The six-month study ran from May 1st to October 31st 2016. Participating laboratories were asked to send Gram-negative bacterial isolates that demonstrated high-level amikacin resistance by local susceptibility testing methods so that they could be screened for 16S RMTase genes.

6.3.1 Ethical approval

Dr Elizabeth Coates, PHE Head of Research Governance, confirmed that ethical approval was not required for this study as it involved work carried out routinely by PHE's AMRHAI Reference Unit, therefore making it an enhanced surveillance study (see Figure 1 in Appendix 1).

6.3.2 Design of study questionnaire and study protocol

A questionnaire (Figure 6.1) was designed to send to participating hospital laboratories in order to obtain information regarding the bacterial isolate and the patient's antibiotic and travel history. A study protocol (Figure 6.2) was also distributed in order to provide background information regarding 16S RMTases and outline the requirements of the study, i.e. that

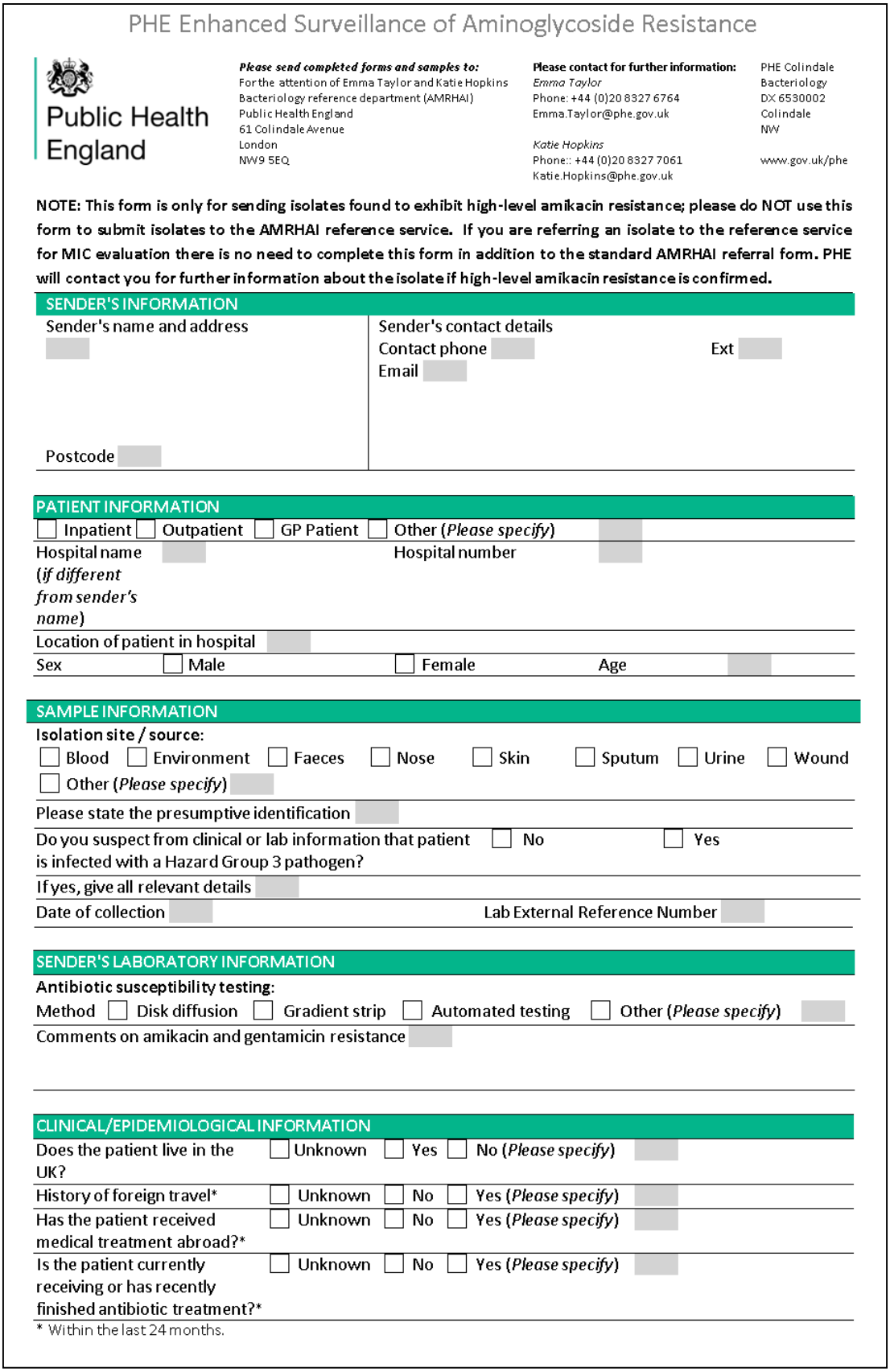
anonymised Gram-negative isolates (Enterobacterales, *A. baumannii* and *P. aeruginosa*) with the following antibiotic susceptibility results were to be sent for screening: no zone of inhibition (30 µg amikacin discs); or amikacin gradient strip MIC >256 mg/L or those at the top of the MIC range when using automated susceptibility testing methods.

6.3.3 Identification of UK hospitals for study recruitment

A list of 110 laboratories in England that screened bacterial isolates (*E. coli, Serratia* spp., *Klebsiella* spp*., Enterobacter* spp. and *Citrobacter* spp.) with the aminoglycosides amikacin, gentamicin and tobramycin from January to October 2015 was taken from PHE's Second Generation Surveillance System (SGSS) [474] and provided by Professor Alan Johnson from PHE's Department of Healthcare Associated Infections and Antimicrobial Resistance. The laboratories were ranked, based on the number of *E. coli* isolates screened for amikacin resistance per annum (as *E. coli* is one of the most common causes of bacterial infections in the hospital and community settings [424]), and the top 30 performing the most tests were approached to participate in the study.

6.3.4 Hospital laboratory recruitment

Consultant microbiologists and laboratory managers from the top 30 hospital laboratories that screen with amikacin on the list were sent email invitations to participate in the study along with the study questionnaire (Figure 6.1), protocol (Figure 6.2) and approval letter (see Figure 1 in Appendix 1). Staff from Public Health Wales, Health Protection Scotland and Belfast Health and Social Care Trust, Belfast City Hospital were contacted in order to recruit hospital laboratories in the devolved administrations.



PHE Enhanced Surveillance of Aminoglycoside Resistance

Public Health England

Please send completed forms and samples to:
For the attention of Emma Taylor and Katie Hopkins
Bacteriology reference department (AMRHAI)
Public Health England
61 Colindale Avenue
London
NW9 5EQ

Please contact for further information:
Emma Taylor
Phone: +44 (0)20 8327 6764
Emma.Taylor@phe.gov.uk

Katie Hopkins
Phone:: +44 (0)20 8327 7061
Katie.Hopkins@phe.gov.uk

PHE Colindale
Bacteriology
DX 6530002
Colindale
NW

www.gov.uk/phe

NOTE: This form is only for sending isolates found to exhibit high-level amikacin resistance; please do NOT use this form to submit isolates to the AMRHAI reference service. If you are referring an isolate to the reference service for MIC evaluation there is no need to complete this form in addition to the standard AMRHAI referral form. PHE will contact you for further information about the isolate if high-level amikacin resistance is confirmed.

SENDER'S INFORMATION

Sender's name and address

Postcode

Sender's contact details
Contact phone Ext
Email

PATIENT INFORMATION

☐ Inpatient ☐ Outpatient ☐ GP Patient ☐ Other (*Please specify*)

Hospital name (*if different from sender's name*) Hospital number

Location of patient in hospital

Sex ☐ Male ☐ Female Age

SAMPLE INFORMATION

Isolation site / source:

☐ Blood ☐ Environment ☐ Faeces ☐ Nose ☐ Skin ☐ Sputum ☐ Urine ☐ Wound
☐ Other (*Please specify*)

Please state the presumptive identification

Do you suspect from clinical or lab information that patient is infected with a Hazard Group 3 pathogen? ☐ No ☐ Yes

If yes, give all relevant details

Date of collection Lab External Reference Number

SENDER'S LABORATORY INFORMATION

Antibiotic susceptibility testing:

Method ☐ Disk diffusion ☐ Gradient strip ☐ Automated testing ☐ Other (*Please specify*)

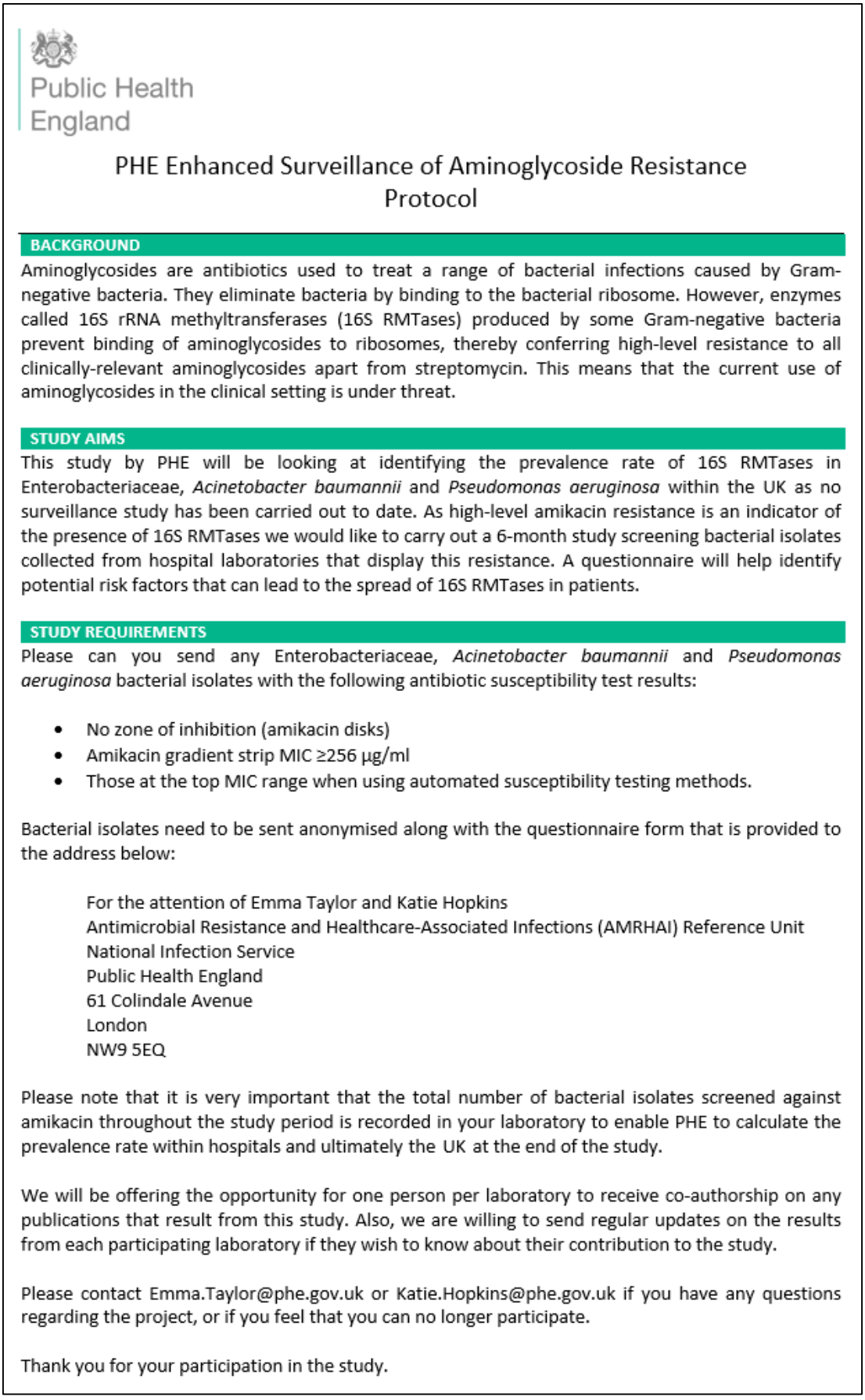
Comments on amikacin and gentamicin resistance

CLINICAL/EPIDEMIOLOGICAL INFORMATION

Does the patient live in the UK?	☐ Unknown	☐ Yes	☐ No (*Please specify*)	
History of foreign travel*	☐ Unknown	☐ No	☐ Yes (*Please specify*)	
Has the patient received medical treatment abroad?*	☐ Unknown	☐ No	☐ Yes (*Please specify*)	
Is the patient currently receiving or has recently finished antibiotic treatment?*	☐ Unknown	☐ No	☐ Yes (*Please specify*)	

* Within the last 24 months.

Figure 6.1: Questionnaire sent to participating hospital laboratories.



Public Health England

PHE Enhanced Surveillance of Aminoglycoside Resistance Protocol

BACKGROUND

Aminoglycosides are antibiotics used to treat a range of bacterial infections caused by Gram-negative bacteria. They eliminate bacteria by binding to the bacterial ribosome. However, enzymes called 16S rRNA methyltransferases (16S RMTases) produced by some Gram-negative bacteria prevent binding of aminoglycosides to ribosomes, thereby conferring high-level resistance to all clinically-relevant aminoglycosides apart from streptomycin. This means that the current use of aminoglycosides in the clinical setting is under threat.

STUDY AIMS

This study by PHE will be looking at identifying the prevalence rate of 16S RMTases in Enterobacteriaceae, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* within the UK as no surveillance study has been carried out to date. As high-level amikacin resistance is an indicator of the presence of 16S RMTases we would like to carry out a 6-month study screening bacterial isolates collected from hospital laboratories that display this resistance. A questionnaire will help identify potential risk factors that can lead to the spread of 16S RMTases in patients.

STUDY REQUIREMENTS

Please can you send any Enterobacteriaceae, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* bacterial isolates with the following antibiotic susceptibility test results:

- No zone of inhibition (amikacin disks)
- Amikacin gradient strip MIC ≥256 µg/ml
- Those at the top MIC range when using automated susceptibility testing methods.

Bacterial isolates need to be sent anonymised along with the questionnaire form that is provided to the address below:

For the attention of Emma Taylor and Katie Hopkins
Antimicrobial Resistance and Healthcare-Associated Infections (AMRHAI) Reference Unit
National Infection Service
Public Health England
61 Colindale Avenue
London
NW9 5EQ

Please note that it is very important that the total number of bacterial isolates screened against amikacin throughout the study period is recorded in your laboratory to enable PHE to calculate the prevalence rate within hospitals and ultimately the UK at the end of the study.

We will be offering the opportunity for one person per laboratory to receive co-authorship on any publications that result from this study. Also, we are willing to send regular updates on the results from each participating laboratory if they wish to know about their contribution to the study.

Please contact Emma.Taylor@phe.gov.uk or Katie.Hopkins@phe.gov.uk if you have any questions regarding the project, or if you feel that you can no longer participate.

Thank you for your participation in the study.

Figure 6.2: Study protocol sent to participating hospital laboratories.

6.3.5 Identification of 16S RMTase genes

Amikacin-resistant isolates sent directly for the study or sent to PHE reference services for antimicrobial susceptibility testing by the participating laboratories during the study period were screened for 16S RMTase genes. Isolates were deduplicated if they were from the same patients but were kept in the study if they were different species or from different anatomical sites. The isolates, along with control strains (Table 6.1), were streaked onto nutrient agar plates to test for viability. Subsequently, they were streaked onto MH agar plates supplemented with 16 mg/L amikacin (Sigma Aldrich) to confirm resistance to amikacin based on the EUCAST clinical breakpoint for Enterobacterales, *A. baumannii* and *P. aeruginosa* [379] and 256 mg/L amikacin to screen for potential 16S RMTase production. Isolates that exhibited high-level amikacin resistance (MIC >256 mg/L) underwent DNA extraction (see Section 2.4.1.3) and were screened with PCR multiplexes 1 and 2 (see Sections 2.4.1.4 and 2.4.1.6) to identify 16S RMTase genes *armA*, *rmtA-rmtH* and *npmA*. Additionally, isolates that exhibited amikacin resistance (MIC >16 mg/L) but not high-level amikacin resistance (MIC >256 mg/L) were screened by PCR to confirm the lack of 16S RMTase genes.

6.3.6 Identification of carbapenemase genes

The carbapenemase genes bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48\text{-}like}$ and bla_{VIM} were identified as previously described in Section 2.4.2, and 16S RMTase-producing isolates that were sent for WGS (see Section 2.6) were screened for carbapenemase genes as described in Section 6.3.9. *A. baumannii* isolates that were sent to the AMRHAI Reference Unit for carbapenemase testing alongside this study were screened for $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ genes as previously described [404] by the AMRHAI Reference Unit.

6.3.7 Determination of MICs

MICs were determined using the BSAC agar dilution method and EUCAST guidelines (see Section 2.3).

Table 6.1: Positive control bacterial strains used in this study.

Bacterial strain	16S RMTase gene	Amikacin MIC (mg/L)	Provider
E_coli_armA	*armA*	>256	AMRHAI
*K_pneumoniae*_8	-	8	AMRHAI
*K_pneumoniae*_16	-	16	AMRHAI
*K_pneumoniae*_32	-	32	AMRHAI

6.3.8 Analysis of 16S RMTase gene-negative isolates

PCR-negative isolates were streaked onto three sets of MH agar plates (Media Services, PHE Microbiology Services Colindale) supplemented with 256 mg/L amikacin, gentamicin or tobramycin and incubated overnight at 37 °C. Isolates that grew on all three plates were sent for WGS as described in Section 2.6 and analysis of the data was conducted as described in Section 6.3.9.

To screen for potentially novel 16S RMTase genes, DNA sequences of contigs were analysed using the 'sequence search' tool from Pfam 31.0 (https://pfam.xfam.org/) [411] to determine whether DNA within the contigs encoded proteins belonging to the same protein families as known 16S RMTases. A six-frame translation was performed by Pfam 31.0 to generate a set of protein sequences, where the presence of the ribosomal RNA methyltransferase family FmrO (Pfam ID: PF07091), which ArmA and RmtA-RmtH belong to, as well as the Methyltransf_4 family (Pfam ID: PF02390), which NpmA belongs to, could be identified. Two searches were carried out using different E-value cut-offs, where the default E-value of 1.0 was used as well as an E-value of 1×10^{-5}, where the latter provided a more stringent search.

6.3.9 WGS and analysis of WGS data

WGS was carried out on all isolates that displayed high-level amikacin resistance (MIC >256 mg/L) to determine MLST profiles and the genetic environment of the 16S RMTase genes as well as to determine if 16S RMTase-negative isolates harboured novel 16S RMTase genes (see Section 6.3.8) as the PCR primers used may not be able to amplify their genes. WGS was carried out as described in Section 2.6.

Purity of the DNA sequences were checked using KmerFinder 3.0 (https://cge.cbs.dtu.dk/services/KmerFinder/) [412-414], antibiotic resistance genes were identified using ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/) [39] and the ARG-ANNOT database [38] via the read mapping-based tool SRST2

(https://github.com/katholt/srst2) [40]. MLST was conducted using MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/) [415].

6.3.10 Determination of risk factors for the acquisition of 16S RMTase-producing bacteria

Potential risk factors for the acquisition of 16S RMTase-producing bacteria were identified from questionnaire responses using statistical analysis. The likelihood-ratio test was performed using Stata MP version 15 (StataCorp LP, College Station, Texas, USA) to calculate *p* values in single variable and multivariable analyses, where a *p* value ≤0.05 was considered statistically significant. Odds ratios and 95% CIs were calculated following the fit of the logistic regression model. Odds ratios were used to identify potential risk factors as an odds ratio greater than one indicates higher odds of acquiring an 16S RMTase-producing bacterial infection.

The multivariable analysis was performed to determine the association of variables with the acquisition of 16S RMTase-producing bacteria and to control for potential confounders, which are variables that may influence the effect of the dependent and independent variables, such as age. Variables with a *p* value ≤0.2 from the single-variable analysis were selected for inclusion in the multivariable model. Variables were kept in the final multivariable model if their removal from the preliminary multivariable model altered the significance of the remaining variables and/or altered odds ratios by 10.0% or more.

6.3.11 Calculation of period prevalence of 16S RMTases in the UK

The period prevalence of 16S RMTases was calculated for individual laboratories and species using the total number of *A. baumannii*, Enterobacterales and *P. aeruginosa* screened against amikacin over the study period for each individual laboratory. The total number of isolates tested throughout the study period was provided by the individual laboratories. These numbers were combined in order to calculate the period prevalence of 16S RMTases in the UK.

6.4 Results

6.4.1 Hospital laboratory recruitment

Of the 30 hospital laboratories contacted to take part in the sentinel survey, 18 (60.0%) agreed to participate (Table 6.2). The 18 laboratories were situated in London (22.2%, 4/18), Scotland (22.2%, 4/18), the North West of England (16.7%, 3/18), the East of England (11.1%, 2/18), the East Midlands (11.1%, 2/18), the West Midlands (11.1%, 2/18) and Wales (5.6%, 1/18). The geographical distribution of these laboratories can be seen in Figure 6.3. Four laboratories were excluded from the study; three laboratories (East_2, London_2 and West Midlands_1) had forgotten to participate during the study period, even though reminders were sent throughout the study period, and communication was lost with East_Midlands_2.

6.4.2 Bacterial isolates

Twelve (85.7%) participating laboratories submitted 271 bacterial isolates. However, 83 (30.6%) isolates had to be excluded from the study as 55 (66.3%) isolates were not amikacin resistant (MIC >16 mg/L), 14 (16.9%) isolates were non-viable, 13 (15.7%) isolates were a species that were not being collected and one (1.2%) isolate was not from the correct timeframe (Figure 6.4). Two laboratories did not have any amikacin-resistant isolates to send during the study period (West Midlands_2 and Scotland_4).

The remaining 188 isolates eligible for inclusion in the study were: *K. pneumoniae* (34.6%, 65/188), *P. aeruginosa* (19.7%, 37/188), *E. coli* (21.8%, 41/188), *A. baumannii* (8.5%, 16/188), *E. cloacae* complex (5.3%, 10/188), *C. freundii* (4.8%, 9/188), *K. oxytoca* (2.1%, 4/188), *S. marcescens* (1.6%, 3/188), *Citrobacter amalonaticus* (0.5%, 1/188), *M. morganii* (0.5%, 1/188) and *P. stuartii* (0.5%, 1/188). The isolates were sent from London (62.2%, 117/188), the North West of England (16.0%, 30/188), Scotland (11.7%, 22/188), the East of England (6.9%, 13/188), the East Midlands (2.1%, 4/188) and Wales (1.1%, 2/188) (Figure 6.5).

Table 6.2: List of the 18 hospital laboratories participating in the prospective surveillance study of 16S RMTases.

Country	Laboratory	Number of *E. coli* screened with amikacin from January-October 2015
England	East_1	20,479
	London_1	5,945
	West Midlands_1	2,273
	London_2	2,044
	London_3	1,923
	North West_1	1,738
	London_4	1,674
	East Midlands_1	1,469
	East Midlands_2	1,359
	East_2	1,290
	North West_2	1,380
	North West_3	1,150
	West Midlands_2	1,142
Scotland	Scotland_1	Unknown
	Scotland_2	Unknown
	Scotland_3	Unknown
	Soctland_4	Unknown
Wales	Wales_1	Unknown

Symbol	Laboratory
▼	East_1
▼	East_2
▼	East_Midlands_1
▼	East_Midlands_2
▼	London_1
▼	London_2
▼	London_3
▼	London_4
▼	North West_1
▼	North West_2
▼	North West_3
▼	Scotland_1
▼	Scotland_2
▼	Scotland_3
▼	Scotland_4
▼	Wales_1
▼	West Midlands_1
▼	West Midlands_2

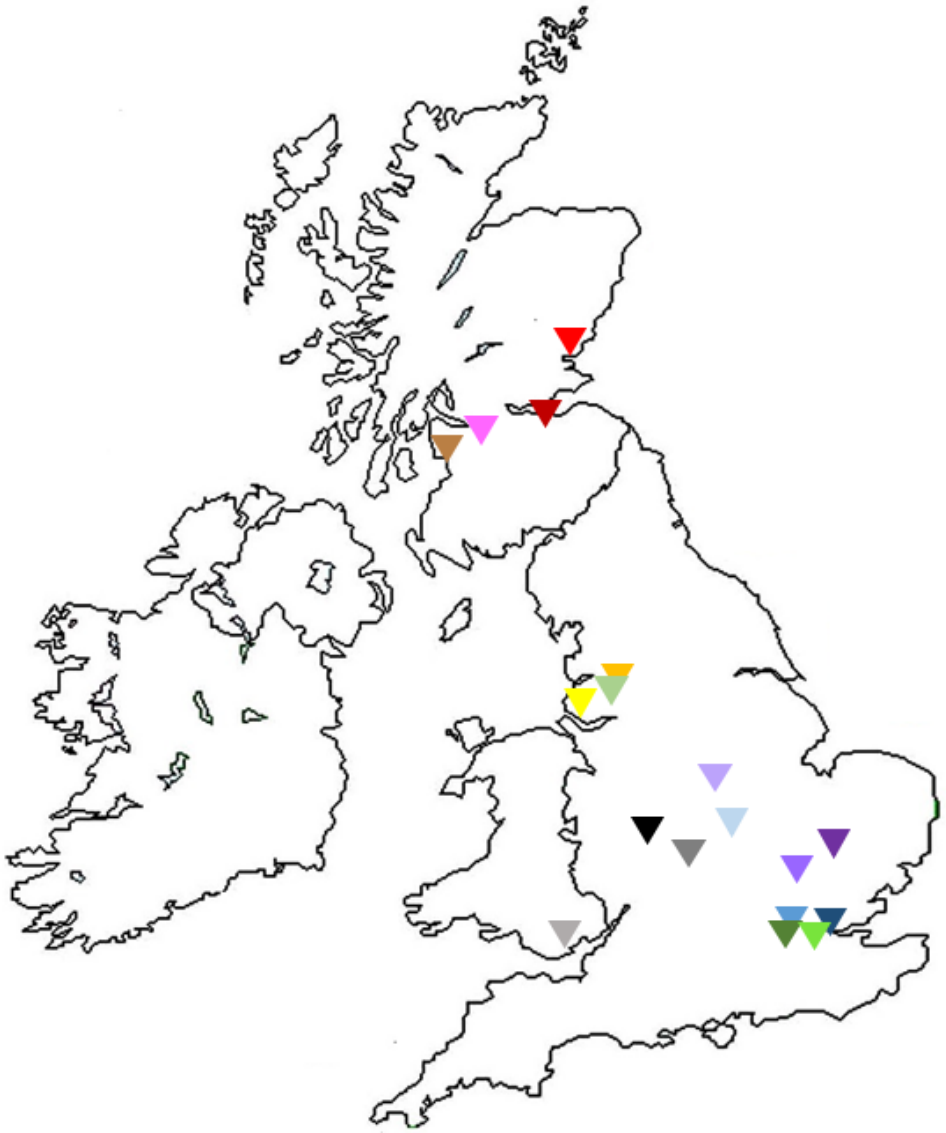


Figure 6.3: Geographic distribution of the 18 laboratories participating in the prospective surveillance study for 16S RMTases.

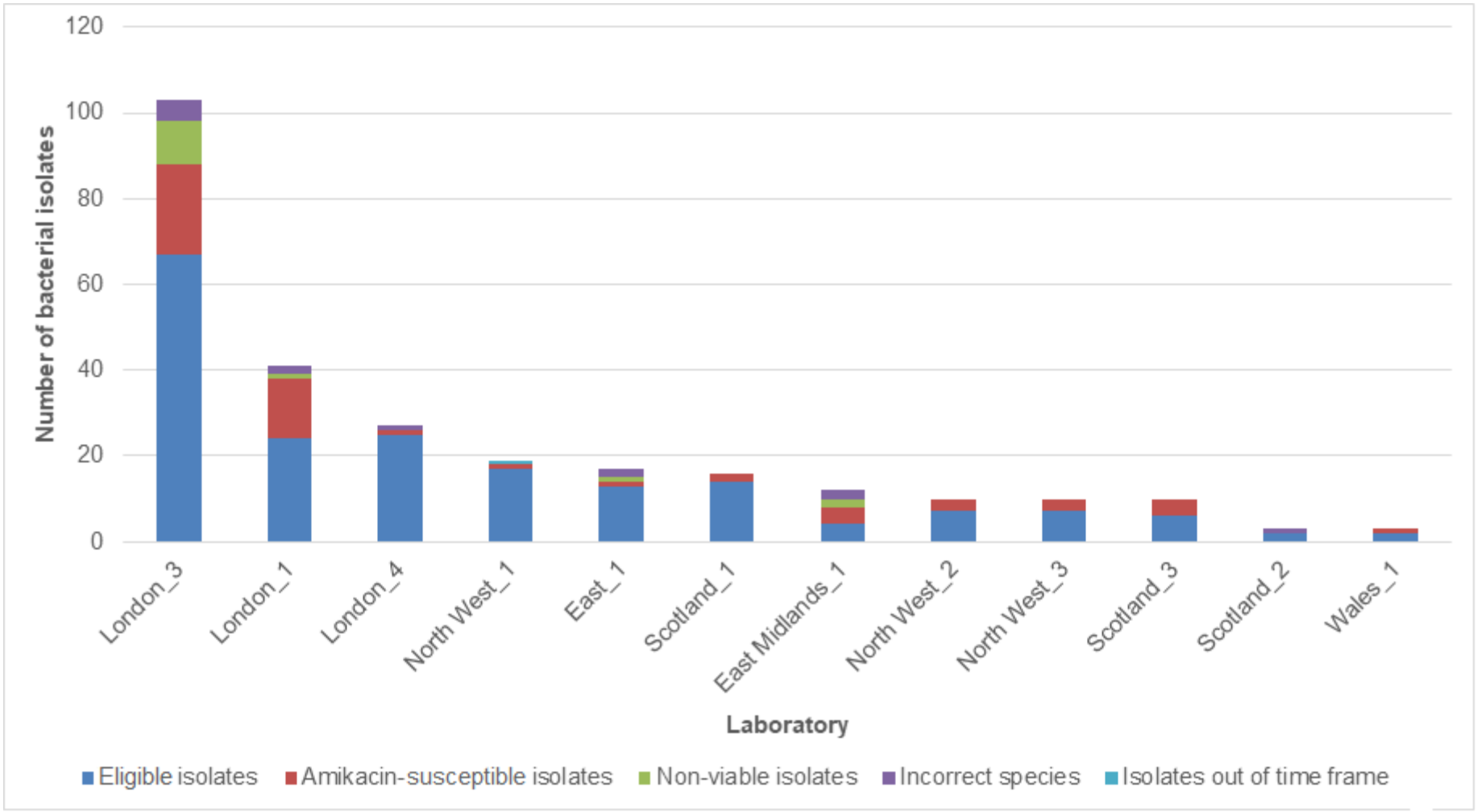


Figure 6.4: Total number of bacterial isolates sent from participating laboratories including discarded isolates.

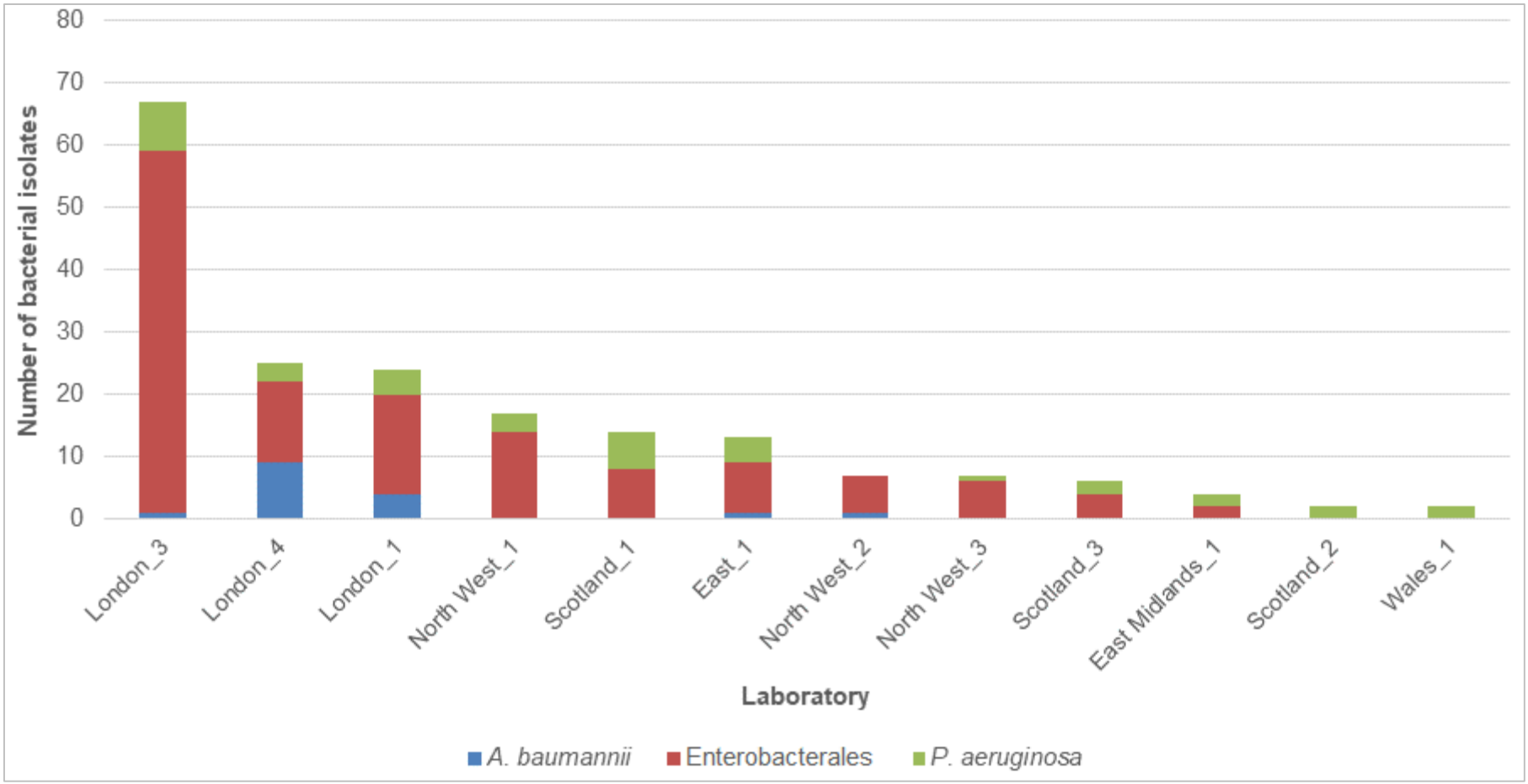


Figure 6.5: Total number of eligible isolates by species (*A. baumannii*, Enterobacterales and *P. aeruginosa*) sent by participating hospital laboratories, between 1st May to 31st October 2016, that were screened for 16S RMTases.

The most common isolation site was 'screening swab' (59.6%, 112/188), followed by tissue and fluid (16.5%, 31/188), urine (11.7%, 22/188), respiratory (7.4%, 14/188), blood and line tip (4.3%, 8/188) and faecal (0.5%, 1/188).

6.4.3 Detection of high-level amikacin resistance and 16S RMTase genes

Out of the 188 isolates, 84 (44.7%) isolates exhibited high-level amikacin resistance (MICs >256 mg/L) where 79 (94.0%) isolates were positive for 16S RMTase genes. Isolates that exhibited high-level amikacin resistance but were 16S RMTase PCR-negative were investigated further in Section 6.4.8. Isolates (n=104) that were amikacin-resistant (MIC >16 mg/L) but not high-level amikacin-resistant (MIC >256 mg/L) were all negative for 16S RMTase genes. The most common 16S RMTase gene detected was *armA* (54.4%, 43/79), followed by *rmtB* (17.7%, 14/79), *rmtF* (13.9%, 11/79), *rmtC* (12.7%, 10/79) and *armA* + *rmtF* (1.3%, 1/79). No *rmtA*, *rmtD*, *rmtE*, *rmtG*, *rmtH* or *npmA* genes were identified.

16S RMTase genes were mainly found in *K. pneumoniae* (51.9%, 41/79) (Figure 6.6), with *armA* (56.1%, 23/41) and *rmtF* (29.3%, 12/41) the most common. *rmtB* and *rmtC* were more common in *E. coli* (53.8%, 7/13) and *C. freundii* (66.7%, 6/9), respectively.

The 16S RMTase-producing isolates were sent by eight laboratories from the regions London (68.4%, 54/79), the North West of England (11.4%, 9/79), the East of England (10.1%, 8/79), Scotland (8.9%, 7/79) and the East Midlands (1.3%, 1/79) (Figure 6.7). None of the isolates from Wales or the West Midlands were found to produce a 16S RMTase. London submitted isolates with the widest variety of 16S RMTase genes compared with the other UK regions.

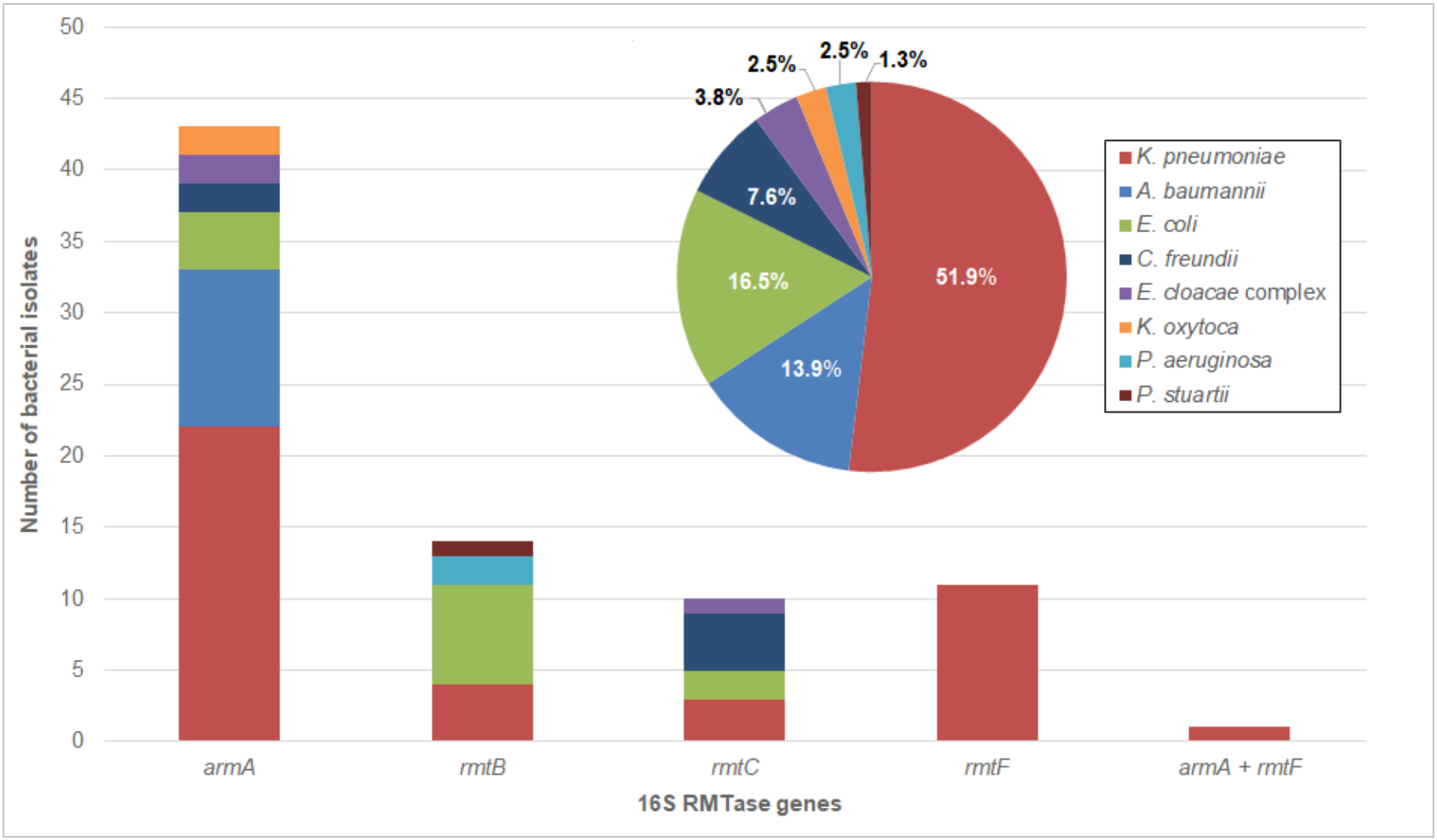


Figure 6.6: Total number of 16S RMTase genes found in pan-aminoglycoside-resistant Gram-negative bacteria (n=79) from UK laboratories from May 1st to October 31st 2016. The pie chart shows the total percentage of 16S RMTase-positive species.

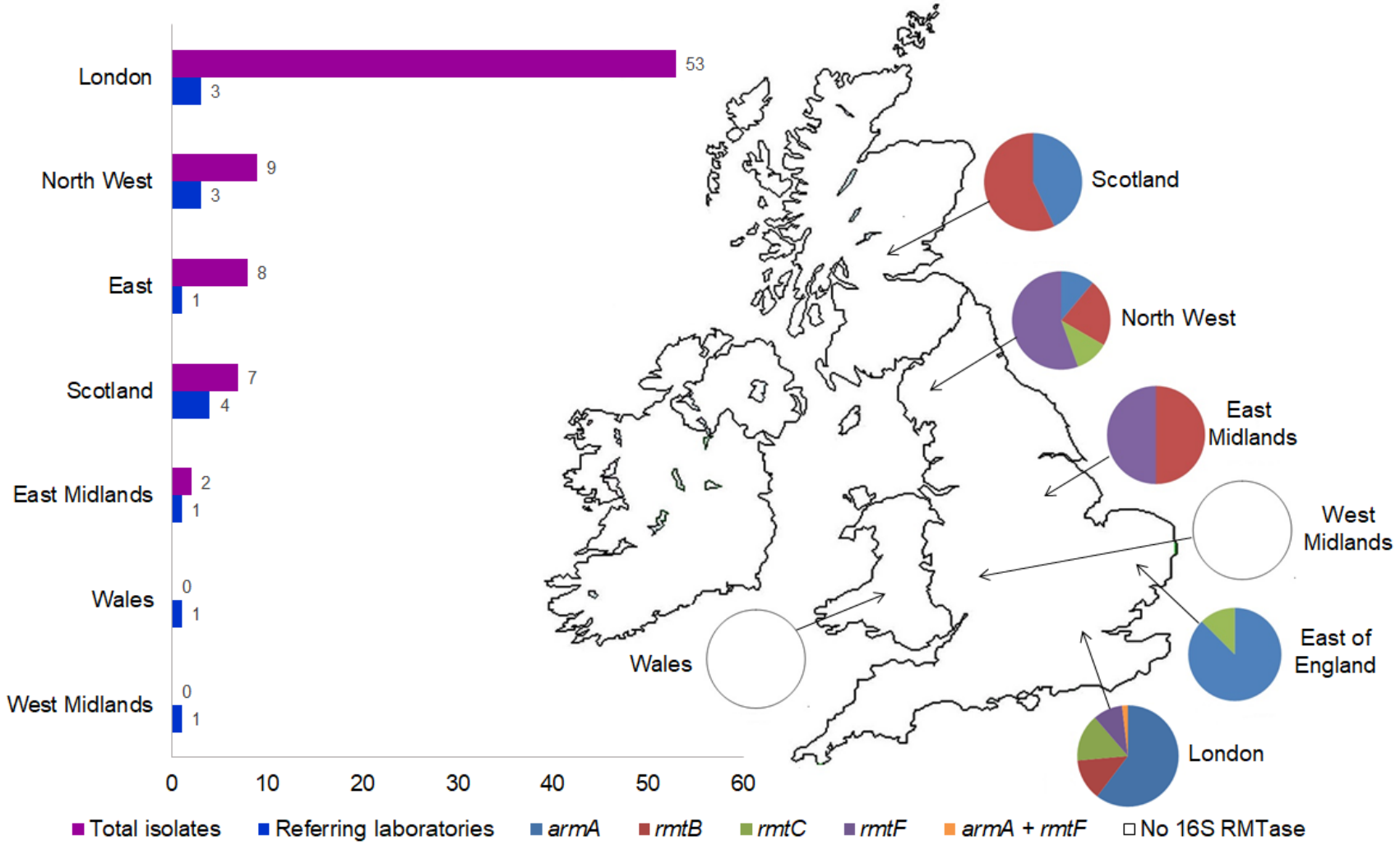


Figure 6.7: The geographical distribution of 16S RMTase-positive Gram-negative bacterial isolates (n=79) in the UK from 1st May to October 31st. The total number of 16S RMTase-positive isolates per region, as well as the number of diagnostic laboratories they came from, can be found on the left-hand side. On the right-hand side is the geographical distribution of 16S RMTase-positive isolates. The pie charts indicate diversity of 16S RMTase genes identified from each region according to the legend above.

6.4.4 Antibiotic susceptibility

MIC data for the 16S RMTase-positive and -negative *A. baumannii*, Enterobacterales and *P. aeruginosa* isolates can be found in Tables 6.3, 6.4 and 6.5, respectively.

All 16S RMTase-positive *A. baumannii* isolates were resistant to imipenem, meropenem and ciprofloxacin but no isolates were resistant to colistin (Table 6.3). All 16S RMTase-negative *A. baumannii* isolates were resistant to imipenem, meropenem and ciprofloxacin but no isolates were resistant to colistin. No EUCAST clinical breakpoints were available for ceftazidime so resistance could not be calculated.

A total of 98.5% (65/66) 16S RMTase-positive Enterobacterales isolates were resistant to cefotaxime as well as ceftazidime and 93.9% (62/66) and 92.4% (61/66) were resistant to ciprofloxacin and ertapenem, respectively (Table 6.4). Additionally, over 62.1% (41/66) and 54.5% (36/66) isolates were resistant to meropenem and imipenem, respectively, but lower levels of resistance were found for colistin (18.2%, 12/66) and tigecycline (16.7%, 11/66). Over 75% of 16S RMTase-negative isolates were resistant to cefotaxime as well as ceftazidime and 85.5% (59/69) were resistant to ciprofloxacin. A total of 65.2% (45/69) 16S RMTase-negative isolates were resistant to ertapenem and 26.1% (18/69) as well as 24.6% (17/69) isolates were resistant to meropenem and imipenem, respectively. However, only 17.4% (12/69) and 8.7% (6/69) isolates were resistant to colistin and tigecycline, respectively.

All 16S RMTase-positive *P. aeruginosa* isolates were resistant imipenem, meropenem, ceftazidime and ciprofloxacin but no isolates exhibited resistance to colistin (Table 6.5). In contrast, 65.7% (23/35) and 51.4% (18/35) of 16S RMTase-negative *P. aeruginosa* isolates were resistant to ciprofloxacin and imipenem, respectively. A total of 37.1% (13/35) and 34.3% (12/35) were resistant to meropenem and ceftazidime, respectively, but only 8.6% (3/35) isolates were resistant to colistin.

MIC values for the other antibiotics tested can be found in Tables 9, 10 and 11 in Appendix 1.

Table 6.3: MIC values for 16S RMTase-positive (n=11) and -negative (n=5) *A. baumannii* isolates submitted by hospital laboratories during the study period. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)											%R
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	
imipenem (0.06-128)	≤2/>8	Positive								1	5	4	1	100
		Negative									1	3	1	100
meropenem (0.06-32)	≤2/>8	Positive									11[a]			100
		Negative									5[a]			100
ceftazidime (0.125-256)	-	Positive											11	-
		Negative										1	4	-
ciprofloxacin (0.125-8)	≤1/>1	Positive							11[a]					100
		Negative							5[a]					100
colistin (0.5-32)	≤2/>2	Positive			8[b]	3								0
		Negative			1[b]	4								0

[a] = MIC greater than or equal to indicated value; [b] = MIC less than or equal to indicated value. R = resistance. Dark blue indicates resistance and no colour indicates susceptibility.

Table 6.4: MIC values for 16S RMTase-positive (n=66) and -negative (n=69) Enterobacterales isolates submitted by hospital laboratories during the study period. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTases	MIC (mg/L)											%R
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	
ertapenem (0.125-16)	≤0.5/>1	Positive	2	1	1	1	2	3	6	50[a]				92.4
		Negative	11	2	8	3	7	9	3	26[a]				65.2
imipenem (0.06-128)	≤2/>8	Positive	1	2	4	2	6	2	13	10	8	7	11	54.5
		Negative	4	12	12	4	6	8	6	6	6	2	3	24.6
meropenem (0.06-32)	≤2/>8	Positive	4	2	1	3	4	3	8	12	29[a]			62.1
		Negative	23	7	6	5	1	2	7	11	7[a]			26.1
cefotaxime (0.125-256)	≤1/>2	Positive		1							3	3	59	98.5
		Negative	2	1	1	2	3	2	1	2	4	6	45	87.0
ceftazidime (0.125-256)	≤1/>4	Positive		1					1		1	2	61	98.5
		Negative	4	1	3	2	4	3	8	3	4	5	32	75.4
ciprofloxacin (0.125-8)	≤0.25/>0.5	Positive	3		1	1		6	55[a]					93.9
		Negative	6		4	1		4	54[a]					85.5
colistin (0.5-32)	≤2/>2	Positive			38[b]	14	2	4	2	2	4[a]			18.2
		Negative			48[b]	8	1	1	1	1	9[a]			17.4
tigecycline (0.25-16)	≤1/>2	Positive		14[b]	10	13	18	9	1	1[a]				16.7
		Negative		17[b]	22	12	12	3	1	2[a]				8.7

[a] = MIC greater than or equal to indicated value; [b] = MIC less than or equal to indicated value. R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 6.5: MIC values for 16S RMTase-positive (n=2) and -negative (n=35) *P. aeruginosa* isolates submitted by hospital laboratories during the study period. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTases	MIC (mg/L)											%R
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	
imipenem (0.06-128)	≤4/>8	Positive											2	100
		Negative		1		1	5	5	5	4	5	3	6	51.4
meropenem (0.06-32)	≤2/>8	Positive									2[a]			100
		Negative		4	6	4	2	3	3	3	10[a]			37.1
ceftazidime (0.125-256)	≤8/>8	Positive											2	100
		Negative			2	5	10	3	3	1	3	5	3	34.3
ciprofloxacin (0.125-8)	≤0.5/>0.5	Positive							2[a]					100
		Negative	3	3	6	1	2	5	15[a]					65.7
colistin (0.5-32)	≤2/>2	Positive				1	1							0
		Negative			5[b]	20	7		3					8.6

[a] = MIC greater than or equal to indicated value; [b] = MIC less than or equal to indicated value. R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

6.4.5 Analysis of carbapenemase genes

A total of 87.3% (69/79) 16S RMTase-producing isolates were positive for one or more carbapenemase genes (Table 6.6), where the association of 16S RMTase genes with carbapenemase genes was found to be statistically significant (p <0.05). *bla*$_{NDM}$ (66.7%, 46/69) was the most common carbapenemase gene identified and its association with 16S RMTase genes was found to be statistically significant (p <0.05). *bla*$_{NDM}$ was commonly associated with *rmtC* (100%, 10/10), *rmtB* (78.6%, 11/14), *armA* (51.2%, 22/43) and *rmtF* (27.3%, 3/11). *bla*$_{NDM}$ was closely associated with *E. coli* (84.6%, 11/13), followed by *K. pneumoniae* (63.4%, 26/41). The carbapenemase gene combination *bla*$_{OXA-23-like}$ + *bla*$_{OXA-51-like}$ was specific to *A. baumannii*, and was identified in all isolates (100%, 11/11) but one isolate also harboured *bla*$_{NDM}$. *bla*$_{OXA-48-like}$ genes were identified in 26.1% (18/69) isolates, particularly *K. pneumoniae* (34.1%, 14/41) and were closely associated with *rmtF* (81.8%, 9/11), followed by *armA* (16.3%, 7/43) and *armA* + *rmtF* (100%, 1/1). The combination of *bla*$_{NDM}$ + *bla*$_{OXA-48-like}$ was only found in 16S RMTase-producing bacteria, particularly *K. pneumoniae* (85.7%, 6/7) and was mainly associated with *armA* (57.1%, 4/7). *bla*$_{VIM}$ was found in the single *rmtB*-positive *P. stuartii* isolate.

Analysis of the WGS data of the 16S RMTase-producing isolates (Table 6.7) identified that the most common *bla*$_{NDM}$ variant was *bla*$_{NDM-1}$ (73.9%, 34/46) followed by *bla*$_{NDM-5}$ (21.7%, 10/46), *bla*$_{NDM-7}$ (2.2%, 1/46) and *bla*$_{NDM-11}$ (2.2%, 1/46). *bla*$_{NDM-1}$ was the most common variant associated with all 16S RMTase genes apart from *rmtB*, which was more closely associated with *bla*$_{NDM-5}$ (50.0%, 7/14) compared with *bla*$_{NDM-1}$ (21.4%, 3/14). The *bla*$_{OXA-48-like}$ gene variants *bla*$_{OXA-181}$, *bla*$_{OXA-48}$ and *bla*$_{OXA-232}$ were identified in 38.9% (7/18), 33.3% (6/18) and 27.8% (5/18) isolates, respectively. *bla*$_{OXA-48-like}$ genes were more frequently associated with *rmtF*-positive isolates than *bla*$_{NDM}$ as 81.8% (9/11) isolates harboured *bla*$_{OXA-48-like}$ genes compared with 27.3% (3/11) *bla*$_{NDM}$-positive isolates, with *bla*$_{OXA-181}$ the most common variant (45.5%, 5/11). The most common combination of *bla*$_{OXA-23-like}$ and *bla*$_{OXA-51-like}$ genes in *armA*-

positive *A. baumannii* isolates was *bla*$_{OXA-23}$ + *bla*$_{OXA-66}$ (81.8%, 9/11), followed by *bla*$_{OXA-23}$ + *bla*$_{OXA-64}$ (9.1%, 1/11) and *bla*$_{OXA-23}$ + *bla*$_{OXA-203}$ + *bla*$_{NDM-1}$ (9.1%, 1/11).

Analysis of the 16S RMTase-negative isolates (Table 6.6) showed that 36.7% (40/109) isolates harboured carbapenemase genes, with *bla*$_{OXA-48-like}$ (47.5%, 19/40) the most common. The occurrence of *bla*$_{OXA-48-like}$ genes in 16S RMTase-negative isolates was higher than amongst 16S RMTase-positive isolates (26.1%, 18/69) and was found to be statistically significant (p <0.05). However, the occurrence of *bla*$_{NDM}$ (17.5%, 7/40) in 16S RMTase-negative isolates was lower than amongst 16S RMTase-positive isolates (65.2%, 45/69). Additionally, *bla*$_{GES-5}$ (2.5%, 1/40) was identified in a 16S RMTase-negative isolate but not in the 16S RMTase-positive isolates.

Table 6.6: Total number of carbapenemase genes found in 16S RMTase-positive (n=79) and -negative (n=109) bacteria.

16S RMTase gene	Species	Carbapenemase gene(s)										
		Negative	*bla*$_{NDM}$	*bla*$_{OXA-48-like}$	*bla*$_{VIM}$	*bla*$_{KPC}$	*bla*$_{GES-5}$	*bla*$_{NDM}$ + *bla*$_{OXA-48-like}$	*bla*$_{OXA-23-like}$ + *bla*$_{OXA-51-like}$	*bla*$_{NDM}$ + *bla*$_{OXA-23-like}$ + *bla*$_{OXA-51-like}$	*bla*$_{XA-40-like}$ + *bla*$_{OXA-51-like}$	Total
Positive	*K. pneumoniae*	6	20	8	-	1	-	6	-	-	-	41
	E. coli	2	10	-	-	-	-	1	-	-	-	13
	A. baumannii	-	-	-	-	-	-	-	10	1	-	11
	C. freundii	-	4	2	-	-	-	-	-	-	-	6
	E. cloacae complex	2	1	-	-	-	-	-	-	-	-	3
	K. oxytoca	-	1	1	-	-	-	-	-	-	-	2
	P. aeruginosa	-	2	-	-	-	-	-	-	-	-	2
	P. stuartii	-	-	-	1	-	-	-	-	-	-	1
Negative	*P. aeruginosa*	33	-	-	2	-	-	-	-	-	-	35
	E. coli	20	3	2	1	-	1	-	-	-	-	27
	K. pneumoniae	11	3	9	-	2	-	-	-	-	-	25
	E. cloacae complex	1	-	4	1	1	-	-	-	-	-	7
	A. baumannii	-	-	-	-	-	-	-	3	1	1	5
	C. freundii	-	1	2	-	-	-	-	-	-	-	3
	S. marcescens	3	-	-	-	-	-	-	-	-	-	3
	K. oxytoca	1	-	-	1	-	-	-	-	-	-	2
	C. amalonaticus	-	-	1	-	-	-	-	-	-	-	1
	M. morganii	-	-	1	-	-	-	-	-	-	-	1
Total		79	45	30	6	4	1	7	13	2	1	188

Table 6.7: Carbapenemase gene variants found in 16S RMTase-positive isolates (n=79) via WGS.

Carbapenemase gene(s)	16S RMTase gene(s)					Total
	armA	*rmtB*	*rmtC*	*rmtF*	*armA* + *rmtF*	
Negative	6	2	-	2	-	10
***bla*$_{NDM-1}$**	17	3	9	-	-	29
***bla*$_{OXA-23}$ + *bla*$_{OXA-66}$**	9	-	-	-	-	9
***bla*$_{NDM-5}$**	-	7	-	-	-	7
***bla*$_{OXA-48}$**	4	-	-	1	-	5
***bla*$_{OXA-181}$**	-	-	-	2	1	3
***bla*$_{OXA-232}$**	-	-	-	3	-	3
***bla*$_{NDM-5}$ + *bla*$_{OXA-181}$**	-	-	-	3	-	3
***bla*$_{NDM-1}$ + *bla*$_{OXA-232}$**	2	-	-	-	-	2
***bla*$_{KPC-2}$**	1	-	-	-	-	1
***bla*$_{NDM-1}$ + *bla*$_{OXA-48}$**	1	-	-	-	-	1
***bla*$_{NDM-1}$ + *bla*$_{OXA-181}$**	-	-	1	-	-	1
***bla*$_{NDM-7}$**	-	1	-	-	-	1
***bla*$_{NDM-11}$**	1	-	-	-	-	1
***bla*$_{NDM-1}$ + *bla*$_{OXA-23}$ + *bla*$_{OXA-203}$**	1	-	-	-	-	1
***bla*$_{OXA-23}$ + *bla*$_{OXA-64}$**	1	-	-	-	-	1
***bla*$_{VIM-1}$**	-	1	-	-	-	1
Total	43	14	10	11	1	79

6.4.6 Typing of 16S RMTase-producing bacterial isolates

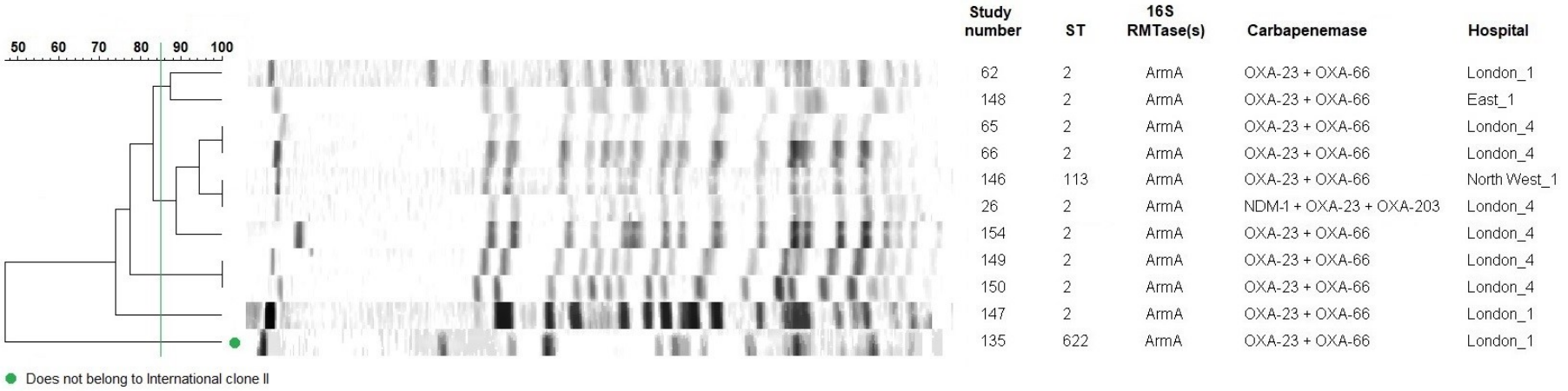
6.4.6.1 PFGE analysis

PFGE results for 16S RMTase-producing *A. baumannii* isolates identified five distinct PFGE patterns using a cut-off value of 85% similarity (Figure 6.8). Three pairs of PFGE patterns shared 100% genetic similarity, where two pairs of PFGE patterns represented isolates from London_4 and the third pair represented isolates from London_4 and North West_1. All were from different patients. A total of 90.9% (10/11) isolates belonged to International Clone II.

PFGE results for 16S RMTase-producing *E. coli* isolates identified 11 distinct PFGE patterns using a cut-off value of 85% similarity (Figure 6.9). No isolates shared identical PFGE patterns but two ST405 isolates from London_1, which were *armA* and $bla_{\text{NDM-1}}$-positive, shared 97.5% similarity.

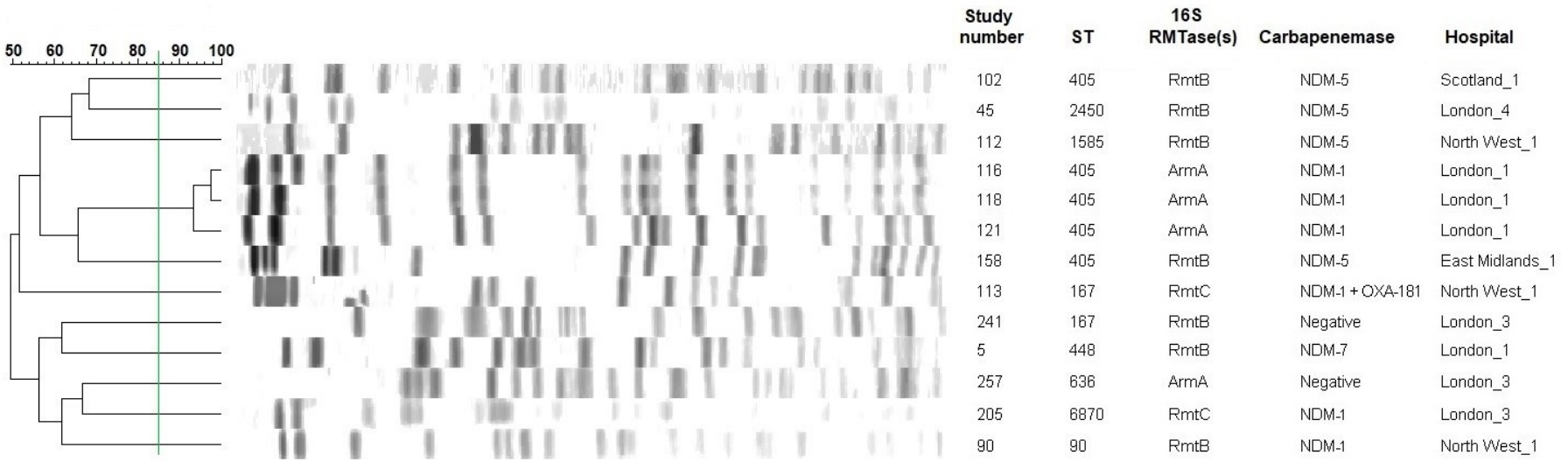
A cut-off value of 85% similarity identified five PFGE profiles for 16S RMTase- producing *C. freundii* isolates where no identical PFGE patterns were found (Figure 6.10). However, two ST18 isolates from London_3, which were *rmtC* and $bla_{\text{NDM-1}}$ positive, had PFGE patterns with 92.0% similarity.

Additionally, two PFGE profiles were identified in 16S RMTase-producing *E. cloacae* complex isolates, where one profile was shared by two ST265 isolates, which belonged to the same patient but were isolated from different anatomical sites (Figure 6.11). PFGE data were only available for 50.0% (1/2) 16S RMTase-producing *K. oxytoca* isolates as one isolate was untypeable due to DNA lysis so no comparison could be made.



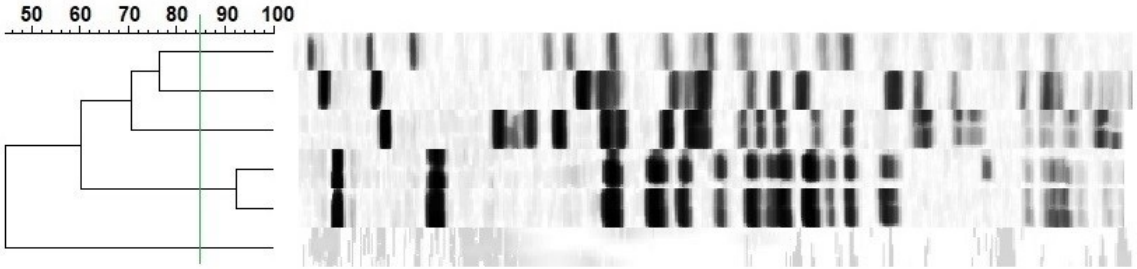
Study number	ST	16S RMTase(s)	Carbapenemase	Hospital
62	2	ArmA	OXA-23 + OXA-66	London_1
148	2	ArmA	OXA-23 + OXA-66	East_1
65	2	ArmA	OXA-23 + OXA-66	London_4
66	2	ArmA	OXA-23 + OXA-66	London_4
146	113	ArmA	OXA-23 + OXA-66	North West_1
26	2	ArmA	NDM-1 + OXA-23 + OXA-203	London_4
154	2	ArmA	OXA-23 + OXA-66	London_4
149	2	ArmA	OXA-23 + OXA-66	London_4
150	2	ArmA	OXA-23 + OXA-66	London_4
147	2	ArmA	OXA-23 + OXA-66	London_1
135	622	ArmA	OXA-23 + OXA-66	London_1

Figure 6.8: PFGE results for 16S RMTase-producing *A. baumannii* isolates (n=11) submitted by UK hospital laboratories between May 1st to October 31st 2016. Green circles indicate isolates that do not belong to International Clone II. The 85% cut-off value is signified by the green line. The scale represents percentage of genetic similarity.



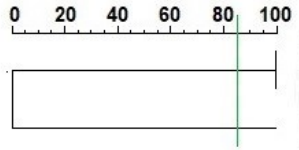
Study number	ST	16S RMTase(s)	Carbapenemase	Hospital
102	405	RmtB	NDM-5	Scotland_1
45	2450	RmtB	NDM-5	London_4
112	1585	RmtB	NDM-5	North West_1
116	405	ArmA	NDM-1	London_1
118	405	ArmA	NDM-1	London_1
121	405	ArmA	NDM-1	London_1
158	405	RmtB	NDM-5	East Midlands_1
113	167	RmtC	NDM-1 + OXA-181	North West_1
241	167	RmtB	Negative	London_3
5	448	RmtB	NDM-7	London_1
257	636	ArmA	Negative	London_3
205	6870	RmtC	NDM-1	London_3
90	90	RmtB	NDM-1	North West_1

Figure 6.9: PFGE results for 16S RMTase-producing *E. coli* isolates (n=13) submitted by UK hospital laboratories between May 1st to October 31st 2016. The 85% cut-off value is signified by the green line. The scale represents percentage of genetic similarity.



Study number	ST	16S RMTase(s)	Carbapenemase	Hospital
120	22	RmtC	NDM-1	East_1
254	11	ArmA	OXA-48	London_3
189	111	ArmA	OXA-48	London_3
204	18	RmtC	NDM-1	London_3
200	18	RmtC	NDM-1	London_3
264	170	RmtC	NDM-1	London_3

Figure 6.10: PFGE results for 16S RMTase-producing *C. freundii* isolates (n=6) submitted by UK hospital laboratories between May 1st to October 31st 2016. The 85% cut-off value is signified by the green line. The scale represents percentage of genetic similarity.





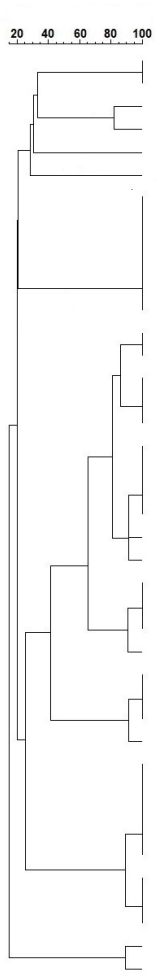
Study number	ST	16S RMTase(s)	Carbapenemase	Hospital
139	265	ArmA	Negative	Scotland_3
82	265	ArmA	Negative	Scotland_3
184	275	RmtC	NDM-1	London_3

Figure 6.11: PFGE results for *E. cloacae* complex isolates (n=3) submitted by UK hospital laboratories between May 1st to October 31st 2016. The 85% cut-off value is signified by the green line. The scale represents percentage of genetic similarity.

6.4.6.2 VNTR analysis

VNTR analysis of 16S RMTase-positive *K. pneumoniae* isolates (Figure 6.12) identified 18 VNTR profiles with nine clusters of isolates sharing identical VNTR profiles. Two profiles were associated with specific laboratories: 6,3,4,0,1,1,4,1,1 with East_1 and 1,3,1,5,1,2,4,1,4,2,4 with London_3 isolates, where the former VNTR profile were all *armA*- and bla_{NDM-1}-positive but those sharing the latter harboured varied 16S RMTase and carbapenemase genes. The profile 6,3,4,0,1,1,3,1,1 was found in two *armA*-positive isolates from two London laboratories where one was bla_{NDM-1}-positive and the other was carbapenemase negative. Additionally, the profiles 6,3,4,0,1,1,4,1,5,2,3 and 3,3,3,0,1,1,4,1,5,2,7 were found only among *armA*-positive isolates. The profile 6,3,4,0,1,1,4,1,5,2,3 was associated with two bla_{NDM-1}-positive isolates isolated from East_1 as well as two bla_{NDM-1} + $bla_{OXA-232}$-positive isolates isolated from Northwest_1 and London_1. Three isolates with the profile 3,3,3,0,1,1,4,1,5,2,7 were isolated from London and harboured various carbapenemases (bla_{KPC} [n=1], bla_{NDM-1} [n=1] and bla_{OXA-48} [n=1]).

Only a single profile (12,4,6,5,3,1,10,9,12) was identified in the two ST773 *P. aeruginosa* isolates, which were *rmtB*- and bla_{NDM-1}-positive and isolated from two laboratories in London (London_1 and London_3) (data not shown).



A	E	H	J	K	D	N1	N2	52	45	60	Study number	ST	16S RMTase(s)	Carbapenemase	Hospital
4	4	4	5	2	1	1	2	4	2	3	256	307	RmtC	NDM-1	London_3
4	4	4	5	2	1	1	2	4	2	3	138	307	ArmA	Negative	London_1
0	5	3	5	2	2	3	3	3	2	5	106	16	ArmA	NDM-11	Scotland_1
4	5	3	5	2	2	3	3	3	2	7	67	336	RmtF	Negative	London_4
3	1	5	2	0	2	3	3	4	2	3	93	383	RmtF	OXA-48	North West_1
4	4	2	1	2	2	2	4	3	2	4	190	1248	RmtB	NDM-5	London_3
3	6	5	1	2	1	3	2	4	2	3	8	231	RmtF	OXA-232	London_1
3	6	5	1	2	1	3	2	4	2	3	68	231	RmtF	Negative	London_4
3	6	5	1	2	1	3	2	4	2	3	133	231	RmtF	OXA-232	London_1
3	6	5	1	2	1	3	2	4	2	3	212	231	ArmA + RmtF	OXA-181	London_3
3	6	5	1	2	1	3	2	4	2	3	20	231	RmtB	Negative	Scotland_3
3	6	5	1	2	1	3	2	4	2	3	85	231	RmtF	OXA-232	North West_1
6	3	4	0	1	1	3	1	1			239	14	ArmA	Negative	London_3
6	3	4	0	1	1	3	1	1			126	14	ArmA	NDM-1	London_1
6	3	4	0	1	1	4	1	1			80	14	ArmA	NDM-1	East_1
6	3	4	0	1	1	4	1	1			123	78	ArmA	NDM-1	East_1
6	3	4	0	1	1	4	1	1			124	78	ArmA	NDM-1	East_1
6	3	4	0	1	1	4	1	5	2	3	128	78	ArmA	NDM-1	East_1
6	3	4	0	1	1	4	1	5	2	3	162	14	ArmA	NDM-1 + OXA-232	North West_1
6	3	4	0	1	1	4	1	5	2	3	125	78	ArmA	NDM-1	East_1
6	3	4	0	1	1	4	1	5	2	3	31	14	ArmA	NDM-1 + OXA-232	London_1
6	3	4	0	1	1	5	1	5	2	3	79	78	ArmA	NDM-1	East_1
6	3	4	0	1	1	3	1	5	2	3	222	14	ArmA	NDM-1	London_3
3	3	3	0	1	1	4	1	5	2	7	129	15	ArmA	KPC-2	London_4
3	3	3	0	1	1	4	1	5	2	7	251	15	ArmA	OXA-48	London_3
3	3	3	0	1	1	4	1	5	2	7	201	15	ArmA	NDM-1	London_3
3	3	3	0	1	1	4	2	5	2	7	242	15	ArmA	NDM-1	London_3
1	3	1	5	1	2	4	1	4	2	4	182	29	RmtC	NDM-1	London_3
1	3	1	5	1	2	4	1	4	2	4	233	29	ArmA	Negative	London_3
1	3	1	5	1	2	4	1	4	2	4	180	29	RmtC	NDM-1	London_3
1	4	1	5	1	2	4	1	4	2	4	253	29	ArmA	NDM-1	London_3
5	3	5	14	14	2	4	2	5	3	6	161	147	RmtF	NDM-5 + OXA-181	North West_1
5	3	5	14	14	2	4	2	5	3	6	108	147	RmtF	NDM-5 + OXA-181	North West_1
5	3	5	14	14	2	4	2	5	3	6	157	147	RmtF	OXA-181	East Midlands_1
5	3	5	14	14	2	4	2	5	3	6	224	147	ArmA	NDM-1	London_3
5	3	5	14	14	2	4	2	5	3	6	250	147	ArmA	NDM-1 + OXA-48	London_3
5	3	5	14	14	2	4	2	1			266	147	ArmA	NDM-1	London_3
5	3	5	14	14	2	4	2	1			51	147	RmtF	OXA-181	London_1
5	3	5	14	14	2	4	2	1			110	147	RmtF	NDM-5 + OXA-181	North West_1
0	0	0	1	0	1	2	0	3			103	138	RmtB	NDM-5	Scotland_1
0	0	0	1	1	1	2	0	3			19	138	RmtB	NDM-5	Scotland_1

Figure 6.12: VNTR analysis of 16S RMTase-producing *K. pneumoniae* isolates (n=41) submitted by UK hospital laboratories between May 1st to October 31st 2016. STs were inferred from WGS data or from VNTR profiles via AMRHAI's ST/VNTR library. Gaps in the VNTR profile indicate loci that failed to amplify. The scale represents percentage of genetic similarity.

6.4.6.3 MLST

MLST data were available for the 16S RMTase-producing isolates including *K. pneumoniae* (n=41), *E. coli* (n=13), *A. baumannii* (n=11), *C. freundii* (n=6), *E. cloacae* complex (n=3), *K. oxytoca* (n=2) and *P. aeruginosa* (n=2). *P. stuartii* (n=1) was not typed using MLST as no MLST scheme was available for this species.

Thirteen STs were identified among the 41 *K. pneumoniae* isolates positive for 16S RMTase genes (Figure 6.13). The most common *K. pneumoniae* STs identified were ST147 (19.5%, 8/41), followed by ST14 (14.6%, 6/41) and ST231 (14.6%, 6/41) (Table 6.8). *K. pneumoniae* ST147 was mainly associated with *rmtF* (62.5%, 5/8) along with $bla_{\text{NDM-5}}$ + $bla_{\text{OXA-181}}$ (60.0%, 3/5). VNTR analysis observed variation within ST147 isolates as two different VNTR profiles (5,3,5,14,14,2,4,2,5,3,6 [n=5] and 5,3,5,14,14,2,4,2,1,-,- [n=3]) were identified. *K. pneumoniae* ST14 isolates were positive for *armA* and 83.3% (5/6) were positive for $bla_{\text{NDM-1}}$. VNTR analysis observed variation within ST14 isolates as four different VNTR profiles (6,3,4,0,1,1,3,1,1,-,- [n=2], 6,3,4,0,1,1,4,1,5,2,3 [n=2], 6,3,4,0,1,1,4,1,1,-,- [n=1] and 6,3,4,0,1,1,3,1,5,2,3 [n=1]) were identified (Figure 6.12). *K. pneumoniae* ST231 isolates were mainly associated with *rmtF* (83.3%, 5/6) as well as $bla_{\text{OXA-232}}$ (50.0%, 3/6). No variation was found within ST231 as only a single VNTR profile was identified (3,6,5,1,2,1,3,2,4,2,3). The association between ST and carriage of a 16S RMTase gene can be seen in Figure 6.13.

The most common *E. coli* ST (Table 6.9) was ST405 (38.5%, 5/13). *E. coli* ST405 was associated with *armA* (60.0%, 3/5) and *rmtB* (40.0%, 2/5). The association between ST and carriage of an 16S RMTase gene can be seen in Figure 6.14.

The most common *C. freundii* ST (Table 6.10) identified was ST18 (33.3%, 2/6), which was only associated with *rmtC* and $bla_{\text{NDM-1.}}$ The association between ST and carriage of an 16S RMTase gene can be seen in Figure 6.15.

Table 6.8: The most common STs found in 16S RMTase-producing *K. pneumoniae* isolates (n=41) as well as their association with carbapenemases and geographical distribution.

ST (no. of isolates)	16S RMTase (no. of isolates)	Carbapenemase (no. of isolates)	Laboratory (no. of isolates)
147 (8)	ArmA (3)	NDM-1 (2)	London_3 (2)
		NDM-1 + OXA-48 (1)	London_3 (1)
	RmtF (5)	NDM-5 + OXA-181 (3)	North West_1 (3)
		OXA-181 (2)	East Midlands_1 (1) and London_1 (1)
14 (6)	ArmA (6)	NDM (5)	London_1 (2), East_1 (1), London_3 (1) and North West_1 (1)
		Negative (1)	London_3 (1)
231 (6)	ArmA + RmtF (1)	OXA-181 (1)	London_3 (1)
	RmtB (1)	Negative (1)	Scotland_3 (1)
	RmtF (4)	OXA-232 (3)	London_1 (2) and North West_1 (1)
		Negative (1)	London_4 (1)

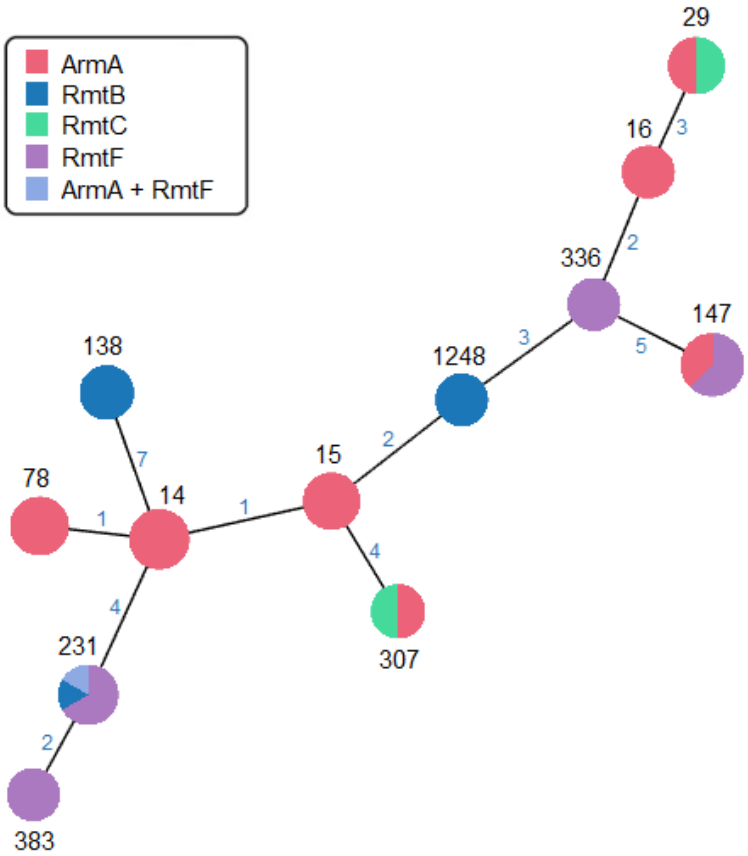


Figure 6.13: eBURST diagram of 16S RMTase-producing *K. pneumoniae* isolates (n=41) with WGS data showing the specific associations between STs and 16S RMTases. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of SLVs between STs (black font).

Table 6.9: The most common STs found in 16S RMTase-producing *E. coli* isolates (n=13) as well as their association with carbapenemases and geographical distribution.

ST (no. of isolates)	16S RMTase (no. of isolates)	Carbapenemase (no. of isolates)	Laboratory (no. of isolates)
405 (5)	ArmA (3)	NDM-1 (3)	London_1 (3)
	RmtB (2)	NDM-5 (2)	East Midlands_1 (1) and Scotland_1 (1)
167 (2)	RmtB (1)	NDM-1 (1)	London_3 (1)
	RmtC (1)	NDM-1 + OXA-181 (1)	North West_1 (1)
90 (1)	RmtB (1)	NDM-1 (1)	North West_1 (1)
448 (1)	RmtB (1)	NDM-7 (1)	London_1 (1)
636 (1)	ArmA (1)	Negative	London_3 (1)
1585 (1)	RmtB (1)	NDM-5 (1)	North West_1 (1)
2450 (1)	RmtB (1)	NDM-5 (1)	London_4 (1)
6870 (1)	RmtC (1)	NDM-1 (1)	London_3 (1)

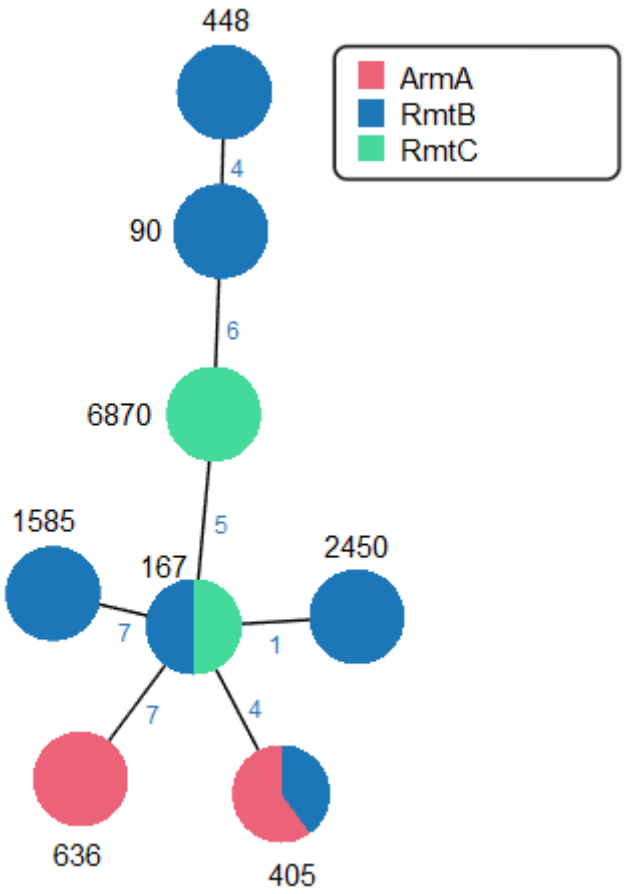


Figure 6.14: eBURST diagram of 16S RMTase-producing *E. coli* isolates (n=13) with WGS data showing the specific associations between STs and 16S RMTases. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of SLVs between STs (black font).

Table 6.10: The most common STs found in 16S RMTase-producing *C. freundii* isolates (n=6) as well as their association with carbapenemases and geographical distribution.

ST (no. of isolates)	16S RMTase (no. of isolates)	Carbapenemase (no. of isolates)	Laboratory (no. of isolates)
18 (2)	RmtC (2)	NDM-1 (2)	London_3 (2)
11 (1)	ArmA (1)	OXA-48 (1)	London_3 (1)
22 (1)	RmtC (1)	NDM-1 (1)	East_1 (1)
111 (1)	ArmA (1)	OXA-48 (1)	London_3 (1)
170 (1)	RmtC (1)	NDM-1 (1)	London_3 (1)

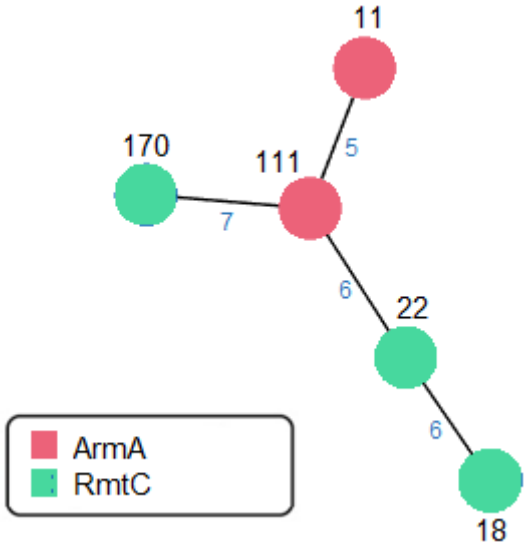


Figure 6.15: eBURST diagram of 16S RMTase-producing *C. freundii* isolates (n=6) with WGS data showing the specific associations between STs and 16S RMTases. The size of the nodes represents the number of bacterial isolates and values in blue represent the number of SLVs between STs (black font).

Two *E. cloacae* complex STs were identified among the three 16S RMTase-producing isolates including ST265 (66.7%, 2/3), which was positive for *armA* and was carbapenemase-negative and referred from one laboratory (Scotland_3). The other isolate belonged to ST275, which was positive for *rmtC* and *bla*$_{NDM-1}$ (100%, 1/1) and was referred by London_3 (data not shown).

The *armA*-positive *A. baumannii* isolates belonged to three STs, which were ST2 (81.8%, 9/11), ST113 (9.1%, 1/11) and ST622 (9.1%, 1/11). *A. baumannii* ST2 isolates were referred from three laboratories: London_4 (66.7%, 6/9), London_1 (22.2%, 2/9) and East_1 (11.1%, 1/9). Most *A. baumannii* ST2 isolates were positive for *bla*$_{OXA-23}$ + *bla*$_{OXA-66}$, but one isolate was positive for *bla*$_{NDM-1}$ + *bla*$_{OXA-23}$ + *bla*$_{OXA-203}$.

MLST identified that both *rmtB*- and *bla*$_{NDM-1}$-positive *P. aeruginosa* isolates belonged to ST773, which were referred from two laboratories (London_1 and London_3). No variation was found within this ST as both isolates shared the same VNTR profile (12,4,6,5,3,1,10,9,12). One *K. oxytoca* belonged to ST84 and the other belonged to an unknown ST.

6.4.7 Detection of antibiotic resistance genes in 16S RMTase-producing bacteria using Illumina Hiseq sequencing.

WGS of the 16S RMTase-producing isolates confirmed the presence of 16S RMTase genes and identified the variant *rmtB4*, which was found in the two *rmtB*-positive *P. aeruginosa* isolates only. No other variants of 16S RMTase genes were identified in *A. baumannii*, Enterobacterales or *P. aeruginosa* and there were no discrepancies between the 16S RMTase genes detected by PCR and those by WGS.

6.4.7.1 Analysis of ESBL genes

ESBL genes were identified in 78.5% (62/79) isolates (Tables 6.11), where 35.5% (22/62) isolates harboured multiple ESBL genes. ESBL genes belonging to the *bla*$_{CTX-M}$ family were the most common as they were identified in 88.7% (55/62) isolates, including 100% (3/3) *E. cloacae* complex, 85.4% (35/41) *K. pneumoniae*, 84.6% (11/13) *E. coli*, 66.7% (4/6) *C. freundii*

and 18.2% (2/11) *A. baumannii* isolates but were not found in *K. oxytoca*, *P. stuartii* or *P. aeruginosa* isolates. The most common variant was $bla_{\text{CTX-M-15}}$, which was found in 64.6% (51/79) isolates and was closely associated with *rmtF* (90.9%, 10/11), followed by 90.0% (9/10) *rmtC*, 58.1% (25/43) *armA* and 42.9% (6/14) *rmtB*-positive isolates as well as the single *armA* + *rmtF*-positive isolate. Within the most commonly identified STs, $bla_{\text{CTX-M-15}}$ was identified in 100% (2/2) *C. freundii* ST18, 100% (2/2) *E. cloacae* complex ST265, 100% (5/5) *E. coli* ST405 and 100% (8/8) *K. pneumoniae* ST147 isolates. One *K. pneumoniae* ST147 harboured $bla_{\text{CTX-M-14B}}$ and $bla_{\text{CTX-M-15}}$.

The second most common ESBL gene family was bla_{SHV}, which was identified in 42.0% (26/62) isolates including 100% (1/1) *P. stuartii*, 56.1% (23/41) *K. pneumoniae*, 50.0% (1/2) *K. oxytoca* and 16.7% (1/6) *C. freundii* isolates. These genes were not identified in *A. baumannii*, *E. cloacae* complex, *E. coli* or *P. aeruginosa* isolates. The most common variant was $bla_{\text{SHV-39}}$ (38.5%, 10/26), which was mainly associated with *rmtF* (54.5%, 6/11), followed by *armA* (9.3%, 4/43). Within the most commonly identified STs, $bla_{\text{SHV-39}}$ was identified in 87.5% (7/8) *K. pneumoniae* ST147 and 16.7% (1/6) *K. pneumoniae* ST231 isolates.

6.4.7.2 Analysis of PMQR genes and chromosomal mutations conferring fluoroquinolone resistance

Chromosomal mutations in the QRDRs of the genes *gyrA* and *parC* (Table 6.12) were found in 67.1% (53/79) isolates including *E. coli* (100%, 13/13), *E. cloacae* complex (100%, 3/3), *K. pneumoniae* (78.0%, 32/41), *C. freundii* (66.7%, 4/6) and *K. oxytoca* (50.0%, 1/2) isolates. Mutations were not identified in *A. baumannii*, *P. stuartii* and *P. aeruginosa* isolates but this may be due to ResFinder and ARG-ANNOT not having mutations specific to these species in its database.

Table 6.11: ESBL genes identified in 16S RMTase-producing isolates (n=79) with WGS data.

ESBL gene	16S RMTase gene(s)					Total
	armA	***rmtB***	***rmtC***	***rmtF***	***armA* + *rmtF***	
***bla*$_{\text{CTX-M-15}}$**	25	6	9	10	1	51
***bla*$_{\text{CTX-M-14B}}$**	1	-	-	1	-	2
***bla*$_{\text{CTX-M-33}}$**	1	-	-	-	-	1
***bla*$_{\text{CTX-M-42}}$**	1					1
***bla*$_{\text{CTX-M-64}}$**	-	1	-	-	-	1
***bla*$_{\text{PER-7}}$**	2	-	-	-	-	2
***bla*$_{\text{SHV-5}}$**	-	1	-	-	-	1
***bla*$_{\text{SHV-12}}$**	3	-	-	2	1	6
***bla*$_{\text{SHV-39}}$**	4	-	-	6	-	10
***bla*$_{\text{SHV-100}}$**	5	1	1	2	-	9
***bla*$_{\text{TEM-168}}$**	1	-	-	-	-	1
***bla*$_{\text{VEB-1}}$**	-	2	-	-	-	2
Total	43	11	10	21	2	87

Table 6.12: Substitutions in QRDRs of GyrA and ParC found in 16S RMTase-producing isolates (n=79) with WGS data.

Species	*armA*	*rmtB*	*rmtC*	*rmtF*	*armA* + *rmtF*	Total	GyrA position	ParC position
C. freundii	-	--	4	-	-	4		S80I
E. cloacae complex	-	-	1	-	-	1		S57T
	2	-	-	-	-	2	S83I	S80I S57T
E. coli	1	-	-	-	-	1	S83L	S80I
	3	6	2	-	-	11	S83L D87N	S80I
	-	1	-	-	-	1	S83L D87N	S80I E84G
K. oxytoca	1	-	-	-	-	1	S83I	
K. pneumoniae	7	-	1	9	-	17		S80I
	1	-	-	-	-	1	D87G	
	9	-	-	-	1	10	D87G	S80I
	1	-	-	1	-	2	D87N	S80I
	1	-	-	-	-	1	D87N	E84K
	-	1	-	-	-	1	D87Y	S80I
Total	26	8	8	10	1	53		

A total of 58.5% (31/53) isolates had mutations in *gyrA*. Substitutions at codons 83 and 87 in GyrA were identified in 30.2% (16/53) and 50.9% (27/53) isolates, respectively. Twenty-two (41.5%) isolates had no mutation in *gyrA*. A total of 96.2% (51/53) isolates had mutations in *parC* including 93.5% (29/31) isolates with *gyrA* mutations. Substitutions at codons 80, 57 and 84 of ParC were identified in 96.1% (49/51), 5.9% (3/51) and 3.9% (2/51) isolates, respectively. Two (3.9%) isolates had no mutation in *parC*. Twenty-two (43.1%) isolates were found to have mutations in *parC* only, where 53.1% (17/32) *K. pneumoniae* and 100% (4/4) *C. freundii* isolates had substitutions at S80I and one *E. cloacae* complex isolate had a substitution at S57T. These isolates included 100% (2/2) *C. freundii* ST18, 100% (1/1) *C. freundii* ST22, 100% (1/1) *C. freundii* ST170, 100% (1/1) *E. cloacae* complex ST275, 100% (8/8) *K. pneumoniae* ST147, 100% (2/2) *K. pneumoniae* ST307, 75.0% (3/4) *K. pneumoniae* ST15 and 66.7% (4/6) *K. pneumoniae* ST231 isolates.

The most common combination of GyrA and ParC substitutions was S83L + D87N and S80I (20.8%, 11/53), which was only found in *E. coli* (84.6%, 11/13) and in all *E. coli* ST405 (100%, 5/5) and ST167 (100%, 2/2) isolates as well the single ST90, ST1585, ST2450 and ST6870 isolates. Another frequently identified combination was D87G and S80I, which was only found in *K. pneumoniae* (31.3%, 10/32), particularly in *K. pneumoniae* ST78 (100%, 5/5) and ST14 (66.7%, 4/6) as well as a single ST231 (16.7%, 1/6) isolate. The largest combination of substitutions in GyrA and ParC was S83L + D87N and S80I + E84G, which was only identified in *E. coli* (7.7%, 1/13), which belonged to ST448 (100%, 1/1).

PMQR genes were found in 79.7% (63/79) isolates (Table 6.13). PMQR determinants were found in 100% (6/6) *C. freundii*, 100% (3/3) *E. cloacae* complex, 100% (41/41) *K. pneumoniae*, 100% (2/2) *P. aeruginosa*, 76.9% (10/13) *E. coli* and 50.0% (1/2) *K. oxytoca* isolates. No PMQR determinants were found in *A. baumannii* or *P. stuartii* isolates.

Table 6.13: Total number of PMQR genes found in 16S RMTase-producing isolates (n=79) with WGS data.

PMQR gene	16S RMTase genes(s)					Total
	armA	***rmtB***	***rmtC***	***rmtF***	***armA* + *rmtF***	
aac(6')-Ib-cr	29	4	9	11	1	54
qnrB1	11	2	7	5	-	25
qnrB9	-	-	1	-	-	1
qnrB35	1	-	-	-	-	1
qnrB38	1	-	-	-	-	1
qnrS1	5	-	1	4	-	10
qnrVC1	-	2	-	-	-	2
qepA1	-	1	-	-	-	1
oqxA	22	4	3	11	1	41
oqxB	22	4	3	11	1	41
Total	91	17	24	42	3	177

The most common PMQR determinant gene identified was *aac(6')-Ib-cr* (85.7%, 54/63). This gene was identified in 100% (3/3) *E. cloacae* complex, 90.0% (9/10) *E. coli*, 87.8% (36/41) *K. pneumoniae*, 83.3% (5/6) *C. freundii* and the single *K. oxytoca* isolate, but was not identified in *P. aeruginosa* isolates (100%, 2/2). It was closely associated with *rmtF* (100%, 11/11) followed by *rmtC* (90.0%, 9/10), *armA* (67.4%, 29/43), *rmtB* (28.6%, 4/14) and *armA* + *rmtF* (100%, 1/1). Out of the most commonly identified STs, *aac(6')-Ib-cr* was found in 100% (2/2) *C. freundii* ST18, 100% (2/2) *E. cloacae* complex ST265, 87.5% (7/8) *K. pneumoniae* ST147 and 60.0% (3/5) *E. coli* ST405 isolates.

A total of 63.5% (40/63) isolates harboured *qnr* genes, including 100% (6/6) *C. freundii*, 100% (1/1) *K. oxytoca*, 100% (2/2) *P. aeruginosa*, 70.7% (29/41) *K. pneumoniae*, 33.3% (1/3) *E. cloacae* complex and 10.0% (1/10) *E. coli* isolates. These genes were not present in *A. baumannii* or *P. stuartii* isolates. The most common variant was *qnrB1* (62.5%, 25/40), which was closely associated with *rmtC* (70.0%, 7/10), followed by *rmtF* (45.5%, 5/11), *armA* (25.6%, 11/43) and *rmtB* (14.3%, 2/14). Out of the most common STs identified, *qnrB1* was identified in 100% (2/2) *C. freundii* ST18 and 62.5% (5/8) *K. pneumoniae* ST147 isolates.

Other identified PMQR determinants included genes for efflux pumps QepA and OqxAB. The gene *qepA1* was found in one *rmtB*-positive *E. coli* isolate belonging to ST167. Genes *oqxA* and *oqxB* are intrinsic in *K. pneumoniae* and, consistent with this, were found in 100% (41/41) *K. pneumoniae* isolates.

6.4.8 Analysis of PCR-negative pan-aminoglycoside-resistant bacteria

Five 16S RMTase-negative isolates, consisting of *P. aeruginosa* (40.0%, 2/5), *C. freundii* (20.0%, 1/5), *E. coli* (20.0%, 1/5) and *K. pneumoniae* (20.0%, 1/5), were analysed further to determine if they harboured novel 16S RMTase genes.

All five isolates grew on all agar plates containing 256 mg/L amikacin, gentamicin and tobramycin. These isolates were sent for WGS, where species was confirmed and MLST

identified the species belonged to *C. freundii* ST113, *E. coli* ST450, *K. pneumoniae* ST11, *P. aeruginosa* ST223 and ST357 (Table 6.14).

The most common AMEs identified were *aac(6')-Ib-cr* (80.0%, 4/5), which was found in all isolates except the *P. aeruginosa* ST233 isolate. Additionally, *aac(3)-IIa* was identified in all three Enterobacterales isolates. All of the isolates had genes conferring resistance to all three aminoglycosides. No previously described 16S RMTase genes were identified in these isolates (i.e. there were no 16S RMTase PCR false-negatives).

In order to see if any isolates harboured novel 16S RMTase genes, amino acid sequences from individual contigs from each isolate were analysed using Pfam 31.0 to see if any shared identity with the ribosomal RNA methyltransferase family FmrO (Pfam ID: PF07091) or Methyltransf_4 family (Pfam ID: PF02390). None of the amino acid sequences from the contigs matched to either of the protein families.

Table 6.14: Genes encoding AMEs identified in 16S RMTase-negative isolates (n=5) that demonstrate high-level resistance to amikacin, gentamicin and tobramycin.

Species (individual isolates)	ST	AME genes					
		***aac(6')-Ib-cr*[ac]**	***aac(3)-IIa*[bc]**	***aph(3')-VIb*[a]**	***aac(3)-Id*[b]**	***aac(6')-Ib*[ac]**	***aph(3')-VIa*[a]**
C. freundii	113	1	1	1	-	-	-
E. coli	405	1	1	-	-	-	-
K. pneumoniae	11	1	1	1	-	1	-
P. aeruginosa	233	-	-	1	1	-	-
P. aeruginosa	357	1	-	-	-	-	1
Total		4	3	3	1	1	1

Enzymes confer resistance to [a] = amikacin; [b] = gentamicin and [c] = tobramycin.

6.4.9 Questionnaires

6.4.9.1 Patients with 16S RMTase-producing isolates

16S RMTase-producing isolates originated from 65 patients, where 57 (87.7%) patients had single isolates and eight (12.3%) patients had multiple isolates. Amongst patients with multiple isolates, seven (87.5%) had bacterial isolates belonging to different species and one (12.5%) patient had isolates obtained from different anatomical sites. Most patients were inpatients (89.2%, 58/65), although patient origin was unknown for two (3.1%) patients. The average age was 67 years, ranging from 23 years to 89 years and most patients were male (70.8%, 46/65).

Only 30 (46.2%) patients had questionnaires fully completed; forms for 35 (53.8%) patients lacked information on either travel history (36.9%, 24/65), overseas medical contact (36.9%, 24/65) or previous use of antibiotics (20.0%, 13/65).

Fifty-eight (89.2%) patients were UK residents; seven (10.8%) patients lived outside the UK in Kuwait (28.6%, 2/7) or Pakistan (28.6%, 2/7), and single patients lived in Egypt, Greece and India.

Twenty-two (33.8%) patients had previously travelled within two years prior to inclusion in the study, whereas 19 (29.2%) patients had no history of travel and 24 (36.9%) patients had an unknown travel history as no information was provided. The most frequently visited countries were Kuwait (18.2%, 4/22) and India (18.2%, 4/22) (Table 6.15) and the most frequently visited continent was Asia (59.1%, 13/22). The geographical distribution of 16S RMTase-producing bacteria obtained from patients with a travel history can be seen in Figure 6.16. Most patients who had a travel history had received medical treatment abroad (81.8%, 18/22), with treatment in intensive care (22.2%, 4/18) and cardiology (16.7%, 3/18) the most common (Table 6.16). India was the country where most patients (22.2%, 4/18) received medical treatment, followed by Kuwait (16.7%, 3/18), Pakistan (16.7%, 3/18), Russia (11.1%, 2/18) and single patients received medical treatment in Crete, Egypt, France, Thailand, Philippines and Nigeria.

Table 6.15: Countries visited by patients with 16S RMTase-positive (n=22) and -negative (n=10) bacterial isolates within two years prior to inclusion in the study.

Country	Patients with 16S RMTase-positive bacteria (n=22)	Patients with 16S RMTase-negative bacteria (n=10)
India	4 (18.2%)	1 (10.0%)
Kuwait	4 (18.2%)	-
Pakistan	3 (13.6%)	-
Egypt	2 (9.1%)	1 (10.0%)
Russia	2 (9.1%)	-
Turkey	-	2 (20.0%)
Cambodia	-	1 (10.0%)
Crete	1 (4.5%)	-
Croatia	-	1 (10.0%)
France	1 (4.5%)	-
Greece	1 (4.5%)	-
Lebanon	-	1 (10.0%)
Nigeria	1 (4.5%)	-
Philippines	1 (4.5%)	-
Romania	-	1 (10.0%)
Spain	-	1 (10.0%)
Thailand	1 (4.5%)	-
Australia, Asia, North America, South America, Europe and New Zealand	-	1 (1.0%)
Unknown	1 (4.5%)	-

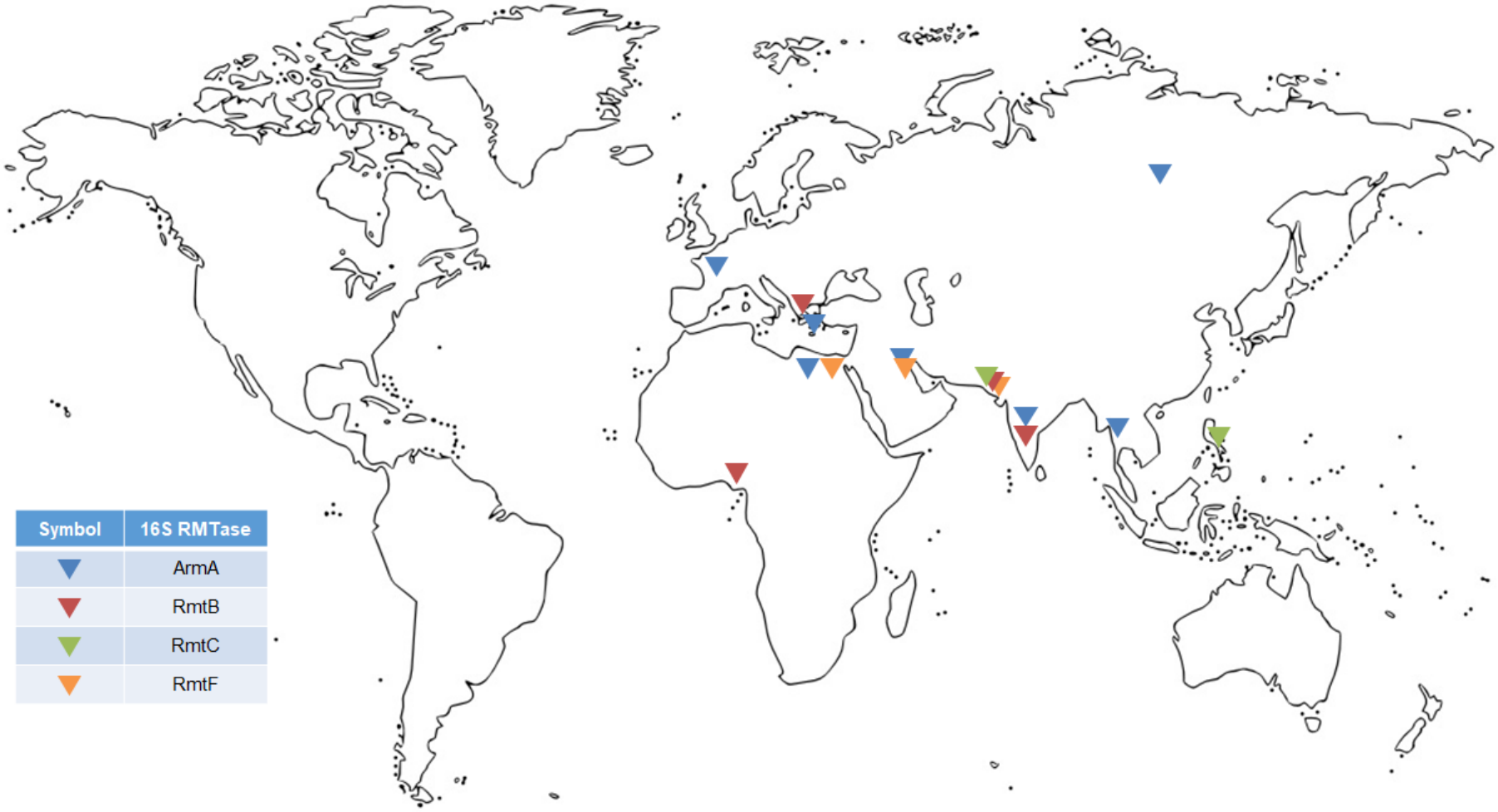


Figure 6.16: Countries visited by patients with 16S RMTase-producing bacterial isolates (n=22) in a two-year period prior to their inclusion in the surveillance study.

Most patients (67.7%, 44/65) had received antibiotic treatment within two years prior to inclusion in the study but seven (10.8%) patients had no history of antibiotic treatment within this time period; antibiotic use was unknown for 14 (21.5%) patients. The antibiotics piperacillin-tazobactam, meropenem and vancomycin were the most consumed as they were prescribed to 20 (45.5%), 14 (31.8%) and 12 (27.3%) patients, respectively (Table 6.16).

6.4.9.2 Patients with 16S RMTase-negative isolates

16S RMTase-negative isolates originated from 101 patients, where 99 (98.0%) patients had single isolates and two (2.0%) patients had multiple isolates, which belonged to different species. Most patients were inpatients (77.2%, 78/101). The average age was 60 years, ranging from 0.9 years to 93 years and most patients were male (54.5%, 55/101).

Only 30 (29.7%) patients had their questionnaires fully completed; 71 (70.3%) patients were missing information on either UK residency (2.8%, 2/71), travel history (100%, 71/71), overseas medical contact (95.8%, 68/71) or previous use of antibiotics (29.6%, 21/71).

Most patients with 16S RMTase-negative isolates lived in the UK (97.0%, 98/101) but two (2.0, 2/101) patients had an unknown country of residence and one patient (1.0%, 1/101) lived in Lebanon.

A total of ten (9.9%) patients had previously travelled within two years prior to inclusion in the study, whereas 20 (19.8%) patients had no history of travel and 71 (70.3%) patients had an unknown travel history as no information was provided. The most frequently visited country was Turkey (20.0%, 2/10). Most patients who had a travel history had received medical treatment abroad (70.0%, 7/10), with surgical treatment (57.1%, 4/7) the most common. The type of medical treatment was unknown for one patient. The most common country where medical treatment was received was Turkey (28.6%, 2/7).

Most patients (68.3%, 69/101) had received antibiotic treatment within two years prior to inclusion in the study with piperacillin-tazobactam and meropenem the most prescribed as they were consumed by 26.7% (27/101) and 20.8% (21/101) patients, respectively.

6.4.10 Identification of risk factors for the acquisition of 16S RMTase-producing bacteria.

Single variable analysis of the data obtained from 166 patients identified that the variables age, gender, patient type, UK residence, medical treatment overseas and aminoglycoside use were statistically significant (p <0.05), indicating that they may be involved in risk of acquiring 16S RMTase-producing bacteria (Table 6.16).

Analysis of the odds ratios identified that patients had twice the odds of acquiring 16S RMTase-producing bacteria if they were male, over 65 years of age, had been prescribed polymyxins or if they had travelled abroad, particularly to Asia or Africa (Table 6.16). Patients had three times the odds of acquiring 16S RMTase-producing bacteria if they had received medical treatment abroad and inpatients had five times the odds than outpatients (Table 6.16). In contrast, patients that were UK residents had ten times lower odds of acquiring 16S RMTase-producing bacteria than non-UK residents, and those prescribed aminoglycosides had three times lower odds of acquiring 16S RMTase-producing bacteria than patients not prescribed aminoglycosides (Table 6.16).

Multivariable analysis was performed on 65.1% (108/166) patients as those with missing questionnaire data were excluded from the analysis (Table 6.17). Multivariable analysis using the variables age, gender, patient type, UK residency, aminoglycoside use and polymyxin use identified that only the variables UK residency and aminoglycoside use were statistically significant (p <0.05), meaning they may be involved in the acquisition of 16S RMTase-producing bacteria. Odds ratios were identical to the odds ratios from the single-variable analysis apart from those for outpatients, which increased from 0.2 (95% CI 0.05-0.8) to 0.8 (0.1-4.6), as well as polymyxin use, which increased from 2.2 (0.7-6.9) to 2.8 (0.7-10.5). Odds ratios and 95% CIs for GP patients and UK residency could not be calculated due to the loss of samples in the analysis.

Table 6.16: Odds ratios, 95% CIs and *p* values obtained from single variable analysis of questionnaire data using the likelihood-ratio test. NE: not estimated.

Variable		16S RMTase		Odds ratio (95% CI)	*p* value
		Positive (n=65)	Negative (n=101)		
Age (min, median, max)		**23,67, 89**	**0.9, 60, 93**	1.0 (1.0-1.0)	0.008
Age	**≤1-17**	**0**	**4**	NE	0.09
	18-45	**7**	**20**	Baseline 1	
	46-65	**24**	**37**	1.7 (0.6-5.0)	
	66-85	**30**	**36**	2.5 (0.9-7.0)	
	86+	**4**	**4**	2.1 (0.4-11.6)	
Gender	**Male**	**46**	**55**	2.1 (1.1-4.2)	0.03
	Female	**19**	**46**		
Patient type	**Inpatient**	**58**	**78**	Baseline 1	0.03
	Outpatient	**3**	**17**	0.2 (0.05-0.8)	
	GP	**2**	**6**	0.6 (0.9-3.9)	
UK Residency	**Yes**	**58**	**98**	0.09 (0.01-0.8)	0.008
	No	**7**	**1**		
Previous travel history	**Yes**	**22**	**10**	2.3 (0.9-6.2)	0.09
	No	**19**	**20**		
Travel to Asia	**Yes**	**13**	**6**	2.3 (0.7-7.0)	0.2
	No	**29**	**30**		
Travel to Europe	**Yes**	**5**	**4**	0.9 (0.2-4.1)	0.9
	No	**37**	**31**		
Travel to Africa	**Yes**	**3**	**1**	2.7 (0.3-28.6)	0.4
	No	**39**	**34**		
Medical treatment abroad	**Yes**	**18**	**7**	3.0 (1.1-8.5)	0.03
	No	**23**	**27**		
Treatment type abroad	**Surgical**	**7**	**4**	Baseline 1	0.08
	Renal	**3**	**1**	1.5 (0.07-3.1)	
	ITU	**4**	**0**	NE	
	Cardiology	**3**	**0**	NE	
	Other	**1**	**1**	0.3 (0.003-38.4)	

Table 6.16 continued

Variable		16S RMTase		Odds ratio (95% CI)	*p* value
		Positive (n=65)	Negative (n=101)		
Previous antibiotic use	Yes	44	69	1.0 (0.4-2.8)	1.0
	No	7	11		
Penicillins	Yes	28	37	1.4 (0.6-3.0)	0.4
	No	16	29		
Carbapenems	Yes	14	22	0.9 (0.4-2.1)	0.9
	No	30	44		
Aminoglycosides	Yes	10	35	0.3 (0.1-0.6)	0.001
	No	34	31		
Glycopeptides	Yes	18	20	1.6 (0.7-4.1)	0.5
	No	26	46		
Cephalosporins	Yes	6	6	1.6 (0.5-5.3)	0.5
	No	38	60		
Macrolides	Yes	3	4	1.1 (0.2-5.3)	0.9
	No	41	62		
Oxazolidinones	Yes	3	4	1.1 (0.2-5.3)	0.9
	No	41	62		
Polymyxins	Yes	8	6	2.2 (0.7-6.9)	0.2
	No	36	60		
Quinolones	Yes	12	16	1.2 (0.5-2.8)	0.7
	No	32	50		
Tetracyclines	Yes	7	6	1.9 (0.6-6.1)	0.3
	No	37	60		
Other[a]	Yes	5	6	1.3 (0.4-4.5)	0.3
	No	39	60		

[a] = Aztreonam, chloramphenicol, clindamycin, co-trimoxazole, daptomycin, fusidic acid, fosfomycin, isoniazid, lincosamide, lipopeptide, monobactam, nitrofurantoin, pyrazinamide and trimethoprim.

Table 6.17: Odds ratios, 95% CIs and *p* values obtained from multivariable analysis of questionnaire data using the likelihood-ratio test. NE: not estimated.

Variable		16S RMTase		Odds ratio (95% CI)	*p* value
		Positive (n=42)	Negative (n=66)		
Age (min, median, max)		**23, 64, 89**	**0.9, 63, 92**	1.0 (1.0-1.0)	0.3
Gender	**Male**	**29**	**37**	2.0 (0.8-5.1)	0.1
	Female	**13**	**29**		
Patient type	**Inpatient**	**40**	**57**	Baseline 1	0.6
	Outpatient	**2**	**8**	0.8 (0.1-4.6)	
	GP	**0**	**1**	NE	
UK Residency	**Yes**	**37**	**5**	NE	0.003
	No	**66**	**0**		
Aminoglycoside use	**Yes**	**10**	**32**	0.3 (0.1-0.7)	0.006
	No	**32**	**31**		
Polymyxin use	**Yes**	**8**	**6**	2.8 (0.7-10.5)	0.1
	No	**34**	**60**		

6.4.11 Calculating the period prevalence of 16S RMTases in the UK

The period prevalence of 16S RMTase-producing isolates in the UK was 0.1% (79/71,063) (Table 6.18), where London was found to have the highest prevalence (0.2%, 53/22,056) followed by the North West of England (0.1%, 9/8,141), the East of England (0.05%, 8/16,256), Scotland (0.06%, 7/11,005) and the East Midlands (0.06%, 2/3,120). The laboratory with the highest prevalence of 16S RMTase-producing isolates was London_3 (0.4%, 26/6,959). No 16S RMTase-producing isolates were isolated from the West Midlands and Wales over the study period.

The prevalence rates of 16S RMTase genes amongst *A. baumannii* (Table 6.19), Enterobacterales (Table 6.20) and *P. aeruginosa* (Table 6.21) in the UK were 3.3% (11/338), 0.1% (66/56,172) and 0.01% (2/14,553), respectively.

Table 6.18: Total prevalence of 16S RMTases per laboratory and within the UK.

Laboratory	Total isolates over the study period	Isolates with 16S RMTase genes	Prevalence rate
East_1	16,526	8	0.05%
East Midlands_1	3,120	2	0.06%
London_1	10,235	16	0.2%
London_3	6,959	26	0.4%
London_4	4,862	11	0.2%
North West_1	3,696	9	0.2%
North West_2	2,297	-	-
North West_3	2,148	-	-
West Midlands_2	7,155	-	-
Scotland_1	1,657	4	0.2%
Scotland_2	3,354	-	-
Scotland_3	3,462	3	0.09%
Scotland_4	2,532	-	-
Wales_1	3,060	-	-
Total	71,063	79	0.1%

Table 6.19: Prevalence of 16S RMTase genes in *A. baumannii*.

Laboratory	Total isolates over the study period	Isolates with 16S RMTase genes	Prevalence rate
East_1	22	1	4.5%
East Midlands_1	10	-	-
London_1	36	3	8.3%
London_3	135	-	-
London_4	57	7	12.3%
North West_1	30	-	-
North West_2	3	-	-
North West_3	0	-	-
West Midlands_2	3	-	-
Scotland_1	1	-	-
Scotland_2	0	-	-
Scotland_3	0	-	-
Scotland_4	18	-	-
Wales_1	23	-	-
Total	338	11	3.3%

Table 6.20: Prevalence of 16S RMTase genes in Enterobacterales.

Laboratory	Total isolates over the study period	Isolates with 16S RMTase genes	Prevalence rate
East_1	14,823	7	0.05%
East Midlands_1	1,860	2	0.1%
London_1	8,675	12	0.1%
London_3	4,298	25	0.6%
London_4	3,598	4	0.1%
North West_1	2,465	9	0.4%
North West_2	2,273	-	-
North West_3	1,690	-	-
West Midlands_2	6,632	-	-
Scotland_1	1,312	4	0.3%
Scotland_2	2,060	-	-
Scotland_3	2,341	3	0.1%
Scotland_4	1,750	-	-
Wales_1	2,395	-	-
Total	56,172	66	0.1%

Table 6.21: Prevalence of 16S RMTase genes in *P. aeruginosa*.

Laboratory	Total isolates over the study period	Isolates with 16S RMTase genes	Prevalence rate
East_1	1,681	-	-
East Midlands_1	1,250	-	-
London_1	1,524	1	0.07%
London_3	2,526	1	0.04%
London_4	1,207	-	-
North West_1	1,201	-	-
North West_2	21	-	-
North West_3	458	-	-
West Midlands_2	520	-	-
Scotland_1	344	-	-
Scotland_2	1,294	-	-
Scotland_3	1,121	-	-
Scotland_4	764	-	-
Wales_1	642	-	-
Total	14,553	2	0.01%

6.5 Discussion

In this study, bacterial isolates sent by UK hospitals between 1st May to 31st October 2016 were screened for 16S RMTases in order to calculate the period prevalence of 16S RMTase-producing isolates in the UK and potential risk factors for the acquisition of 16S RMTase-producing bacteria were identified.

armA (55.7%) was the most common 16S RMTase gene identified, followed by *rmtB* (17.7%), *rmtF* (15.2%), and *rmtC* (12.7%). These findings support the literature where *armA* and *rmtB* have been reported as two of the most commonly identified 16S RMTase genes globally [127,149]. The two-gene combination, *armA* + *rmtF*, was identified in a single *K. pneumoniae* isolate and has previously been reported in *E. cloacae* complex in South Africa [212] and *K. pneumoniae* in Australia [185]. In contrast to the strain collection from the AMRHAI Reference Unit (see Chapter 4), where various two-gene combinations were identified, *armA* + *rmtF* was the only combination identified in this study. *armA* + *rmtF* was a two-gene combination frequently identified in the AMRHAI Reference Unit (32.7%, 17/52), which was mainly identified in *K. pneumoniae* ST231 (75.0%, 6/8), as in this study. Therefore, this indicates that this combination is circulating in the UK, particularly in *K. pneumoniae* ST231.

The five high-level amikacin-resistant isolates that lacked 16S RMTase genes were found to harbour combinations of AMEs that conferred high-level amikacin resistance, which was also seen in 16S RMTase-negative isolates from the AMRHAI Reference Unit (see Chapter 4). Therefore, 16S RMTases are not the only mechanisms causing high-level aminoglycoside resistance in the UK.

K. pneumoniae was the most common species associated with 16S RMTase genes (51.9%, 41/79). This is likely due to *K. pneumoniae* being one of the most common species associated with infections in the hospital setting, as *K. pneumoniae* is known for being virulent and capable of acquiring and disseminating antibiotic resistance genes [424,475]. *K. pneumoniae* was also the only species to be associated with *rmtF*, although *rmtF* has been reported

elsewhere in *C. freundii* [206,207], *C. koseri* [207], *E. cloacae* complex [185,212], *E. coli* [206,291], *P. mirabilis* [207] and *P. aeruginosa* [144,204,277] in the literature. Most *rmtF* genes (93.9%, 155/165) were also identified in *K. pneumoniae* isolates from the AMRHAI Reference Unit (see Chapter 4) and the single *rmtC* + *rmtF* isolate from the BSAC Resistance Surveillance Project (see Chapter 5) belonged to *K. pneumoniae*, confirming that *rmtF* is mainly identified in this species.

Several 'high-risk' bacterial clones were found to harbour 16S RMTase genes in this prevalence study, including *A. baumannii* International Clone II, *E. coli* ST405, *K. pneumoniae* ST147 and *P. aeruginosa* ST773. These clones were also some of the most common STs identified in 16S RMTase-producing bacteria from the AMRHAI Reference Unit (see Chapter 4), but in contrast to this study, *K. pneumoniae* ST14 and *P. aeruginosa* ST357 were the most common STs. *K. pneumoniae* ST147 is a 'high-risk' bacterial clone known to carry carbapenemases such as bla_{NDM} as well as $bla_{OXA\text{-}48\text{-}like}$ carbapenemases [467,476,477] and was found to be mainly associated with *rmtF* (62.5%, 5/8) and $bla_{OXA\text{-}181}$ (62.5%, 5/8) in this study. The association of this clone with *rmtF* has previously been reported in Pakistan [478], the United Arab Emirates (UAE) [479] and the USA [211]. *P. aeruginosa* ST773 is an international 'high-risk' clone known to carry bla_{VIM} carbapenemase genes and has been reported in India, Iran and the UK [387,480,481]. The identification of the same 'high-risk' clones in these studies indicates that they are commonly associated with 16S RMTase genes, which gives 16S RMTase genes the opportunity to spread throughout the UK alongside other antibiotic resistance genes in virulent bacterial strains.

As seen in the bacterial isolates from the AMRHAI Reference Unit, PFGE and VNTR analysis demonstrated that bacterial strains carrying 16S RMTase genes within the UK were diverse. Therefore, it supports the finding that clonal expansion is not the sole reason for the spread of 16S RMTase genes in the UK.

This study showed that 16S RMTase genes have a close association with carbapenemase genes, as 87.3% (69/79) 16S RMTase-positive isolates harboured carbapenemase genes

compared with 38.5% (42/109) 16S RMTase-negative isolates. As this difference was found to be statistically significant (p <0.05), one could infer that 16S RMTase genes are spreading in the UK due to their association with carbapenemase genes, albeit it not an exclusive association. This high-level association with carbapenemase genes was also found in 16S RMTase-positive bacterial isolates from the AMRHAI Reference Unit (94.3%, 1,237/1,312), which was higher than 16S RMTase-negative isolates (65.9%, 174/264). Therefore, this supports the finding in this study that 16S RMTase genes appear to be associated with carbapenemase genes. This high-level association with carbapenemase genes is higher than other countries such as Greece (50.0%, 3/6) [471] and Angola (45.5%, 10/22) [227] but is lower than a study by Rahmen *et al*. [291] in India who found an association of 100% (57/57). Regions such as London and the North West of England have a large population of people from the Indian subcontinent, which could explain the prevalence of carbapenemases [451].

Analysis of WGS data demonstrated that other acquired antibiotic resistance genes were closely associated with 16S RMTase genes, including *aac(6')-Ib-cr* (68.4%, 54/79), $bla_{\text{CTX-M-15}}$ (64.6%, 51/79) and *qnrB1* (31.6%, 25/79), which were also the most common genes identified in bacterial isolates that had undergone WGS from the AMRHAI Reference Unit (see Chapter 4) and the BSAC Resistance Surveillance Project (see Chapter 5). This demonstrates that these genes are commonly associated with 16S RMTase genes and may be linked on MGEs such as plasmids, as previously described in the literature [161,162,205,221,267,315,431]. This will be investigated further in Chapter 7.

Antibiotic susceptibility testing showed that 16S RMTase-producing bacteria exhibited high-level resistance to carbapenems, 3rd generation cephalosporins and fluoroquinolones. As found in isolates from the AMRHAI Reference Unit (see Chapter 4) and the BSAC Resistance Surveillance Project (see Chapter 5), lower levels of resistance were observed for tigecycline (16.7%, 11/66) and colistin (15.2%, 12/79). This demonstrates that the most commonly used antibiotics for Gram-negative infections (aminoglycosides, β-lactams and fluoroquinolones [58,59]) will most likely be ineffective and last line antibiotics such as tigecycline and colistin,

as well as newer agents that are currently in phase III clinical trials, such as cefiderocol [377], will be the most effective in treating 16S RMTase-producing bacteria.

Analysis of patient demographic data showed that most patients with a travel history who had 16S RMTase-producing bacteria had travelled to India and Kuwait, which are known reservoirs of carbapenemase genes [451-453]. However, 24 (36.9%) patients had an unknown travel history as no information was provided so these data are unreliable. However, the majority of patients who had a history of travel (81.8%, 18/22) had received medical treatment, where India was the country where most patients (22.2%, 4/18) received medical treatment, followed by Kuwait (16.7%, 3/18) and Pakistan (16.7%, 3/18). As patients who had received medical treatment abroad were found to have three times the odds of acquiring 16S RMTase-producing bacteria through statistical analysis, this indicates that patients who had previously received treatment abroad will need to be screened for 16S RMTases, as well as carbapenemases, to determine in they need to undergo isolation.

Statistical analysis of the questionnaire data identified several factors that could increase the odds of acquiring an 16S RMTase-producing bacterial isolate, which included being male, being over 65 years of age, being an inpatient, travelling abroad particularly to Asia and Africa and receiving medical treatment abroad as well as being prescribed polymyxins. These factors may directly influence the odds of acquiring 16S RMTase-producing bacteria, or they may simply be surrogate markers of at risk populations. The elderly (>65 years of age) are more prone to infections due to a lowered immune system and the presence of comorbidities making them vulnerable to hospitalisation [482]; males have a lower life expectancy than females and are more prone to illnesses such as infection [483]; inpatients are hospitalised due to having serious illnesses and are at risk of hospital-acquired infections due to having procedures such as the insertion of central venous lines or urinary catheters [484] and parts of Asia and Africa have areas with limited access to clean water, hygiene and sanitation as well as poor control over the distribution of antibiotics [485], meaning antibiotic resistance is easily spread and makes patients receiving medical treatment abroad vulnerable to MDR infections. Polymyxins

are last-line antibiotics used to treat MDR bacteria [448-450] and were shown in this study to be one of the only classes of antibiotics that may be useful in treating 16S RMTase-producing bacteria. Therefore, the apparent association between polymyxin exposure and acquisition of 16S RMTase-producing bacteria was surprising, although not significant; the result should be interpreted with caution as 20.5% (34/166) patients had missing data regarding previous antibiotic use, and polymyxin use may be a marker of suspected exposure to carbapenemase-producing bacteria. Furthermore, the timing of polymyxin administration is unknown, as information regarding antibiotic use within 24 months of admission was provided rather than antibiotic use at the time of admission. Thus, polymyxins may not have been administered when patients were infected by 16S RMTase-producing bacteria and may not be involved in their acquisition at all. Surprisingly, patients that had received aminoglycosides in the preceding 24 months had lower odds of acquiring 16S RMTase-producing bacteria. This result rebuts the theory that aminoglycoside use is driving the spread of aminoglycoside resistance caused by 16S RMTases.

Single-variable analysis identified age, gender, patient type, UK residency, medical treatment abroad and aminoglycoside use to be significantly associated with acquisition of an 16S RMTase-producing isolate, but multivariable analysis only identified UK residency and aminoglycoside use to be statistically significant. While the protective effect of UK residency is scientifically plausible, the protection conferred by prior aminoglycoside prescription is harder to explain and may simply be a marker of antimicrobial stewardship efforts in the UK. However, the multivariable analysis was only conducted on 65.1% of the patient data due to the multivariable analysis excluding patients without full data, therefore, these results need to be interpreted with caution. To our knowledge, this is the first study to identify potential risk factors for the acquisition of 16S RMTase-producing bacteria, but future studies need to be conducted, ideally with a larger patient cohort where all patient data are available, in order to identify potential risk factors more reliably.

The period prevalence of 16S RMTase-producing bacteria in the UK from 1st May to 31st October 2016 was 0.1% (79/71,063). This was lower than studies investigating the prevalence of 16S RMTase genes in Gram-negative bacteria conducted in China (26.0%, 193/741) [157] and Bulgaria (1.2%, 20/1,649) [299] but higher than a study conducted in Japan (0.03% (26/87,626) [176]. As EARS-Net data shows the prevalence of aminoglycoside resistance in the UK in 2016 was between <1% to 10% [486] in *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates from blood and cerebrospinal fluid, this indicates that 16S RMTases are responsible for only a small proportion of aminoglycoside resistance and other mechanisms such as AMEs are more likely involved. However, the prevalence rate identified does not represent the whole of the UK as the North East and the South West of England were not included as well as Northern Ireland due to the inability of recruiting these regions into the study. As only 14 laboratories in the UK participated in this study, the true prevalence of 16S RMTase-producing bacteria in the UK could differ from the rate reported in this study. Yet, this prevalence rate is the first estimation of the prevalence of 16S RMTase-producing bacteria in the UK and can be used a basis for future studies.

There were several limitations to this study, as insufficient clinical details were collected, which may have provided greater insight into the types of infection 16S RMTase-producing bacteria are associated with, and whether the 16S RMTase-producing isolates represented colonisation rather than clinical infections. Although several isolates were submitted from primary care sources, the number of bacterial infections arising in the community compared with hospital settings is unknown as was history of recent hospital exposure, therefore it is unknown how many 16S RMTase-producing isolates were hospital-acquired. Additionally, there was no information on the outcomes of the patients meaning that the role of 16S RMTase-producing bacteria in morbidity of patients is not known. However satisfactory completion of surveillance questionnaires is a major limitation to such studies and compliance is likely to be greatest when participants do not need to have recourse to clinical notes. A more

intensive study at a Trust with high numbers of 16S RMTase-positive isolates may be more useful.

In conclusion, this study was able to determine the period prevalence rate of 16S RMTase-producing bacteria in the UK between May 1st to October 31st 2016, which was 0.1%. 16S RMTase genes appeared to have a strong association with *bla*$_{NDM}$ carbapenemase genes suggesting they are emerging in the UK through co-selection. The following chapter will investigate if this close association is due to 16S RMTase and carbapenemase genes being located on the same plasmid.

Chapter 7: Analysis of the genetic environments and MGEs associated with 16S RMTase genes.

7.1 Introduction

MGEs are genetic structures that can move between bacterial genomes and have been found to be associated with 16S RMTase genes, potentially enabling spread of these resistance genes between bacteria through horizontal transfer. MGEs include transposable elements such as Tn*1548*, Tn*2* and IS*Ecp1*, which are associated with *armA* [165,153,162,163], *rmtB* [135,169,221,315,332] and *rmtC* [201,311,339], respectively, while *rmtF* has been found associated with IS*CR5* [143] and IS*91* [206]. Additionally, 16S RMTase genes have been found on plasmids, which have potentially enabled them to spread alongside other antibiotic resistance genes, such as those that encode carbapenemases, ESBLs and PMQR genes [127,149]. This generates MDR bacteria and enables the spread of these antibiotic resistance genes through co-selection [127,149].

Analysis of Gram-negative bacterial strain collections from the AMRHAI Reference Unit, the BSAC Resistance Surveillance Project's Bacteraemia Programme and the six-month prospective surveillance study involving UK laboratories indicated that 16S RMTase genes may be genetically linked to carbapenemase genes, in addition to other antibiotic resistance genes such as *aac(6')-Ib-cr*, $bla_{\text{CTX-M-15}}$ and *qnrB1*. Therefore, this study was carried out to identify if these genes are associated with 16S RMTase genes either in their genetic environments or on plasmids using WGS data from these three studies. Additionally, this study was conducted to identify which transposable elements and plasmids are associated with 16S RMTase genes and could contribute to their spread throughout the UK.

7.2 Aims

The aims of this study were to: i) characterise the genetic environments of genes that encode 16S RMTases to identify associated antibiotic resistance genes and transposable elements, and, ii) characterise plasmids carrying 16S RMTase genes and identify associated antibiotic resistance genes.

7.2 Methods

7.2.1 Bacterial isolates

16S RMTase-producing bacterial isolates with WGS data from the AMRHAI Reference Unit (comprising of 449 Enterobacterales, 19 *P. aeruginosa* and the *rmtE3*-positive *A. baumannii* isolate), the prospective surveillance study (comprising of 66 Enterobacterales, 11 *A. baumannii* and two *P. aeruginosa* isolates) as well the BSAC Resistance Surveillance Project's Bacteraemia Programme (consisting of three *K. pneumoniae* and one *A. baumannii* isolate) were analysed to identify the genetic environments of the 16S RMTase genes.

7.2.2 Analysis of genetic environment

The genetic environment of the 16S RMTase genes was confirmed using Artemis 16.0.0 (Wellcome Trust Sanger Institute, Cambridge, UK) to identify and annotate 16S RMTase gene-containing contigs. The annotated 16S RMTase gene-containing contigs were analysed with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the genetic environment and identify related DNA sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Mauve (http://darlinglab.org/mauve/mauve.html) was used to visually confirm the alignment of 16S RMTase-containing contigs (and surrounding contigs) to the published DNA sequences from GenBank [487]. Genetic environments of 16S RMTase genes were visualised using EasyFig version 2.2.2 (http://mjsull.github.io/Easyfig/) [488].

7.2.3 Analysis of plasmids harbouring 16S RMTase genes

7.2.3.1 Bacterial isolates

All 16S RMTase-producing isolates (n=79) from the prospective surveillance study (Table 7.1) as well as a subset of 55 isolates from the AMRHAI Reference Unit (Table 7.2) were selected for the extraction and characterisation of 16S RMTase-containing plasmids. The 55 isolates from the AMRHAI Reference Unit were selected by analysing the WGS data, where isolates were chosen due to having novel gene combinations or due to having the most frequently identified gene combinations, which signified the possible carriage of identical plasmids.

Additionally, the *rmtE3*-positive *A. baumannii* isolate was included to identify the genetic location of the *rmtE3* gene.

7.2.3.2 Extraction of plasmid DNA

To isolate plasmids contributing to high-level amikacin resistance plasmid DNA was extracted from bacterial isolates and then electroporated into *E. coli* TOP10 cells as described in Section 2.7. Transformants containing plasmids with 16S RMTase genes underwent DNA extraction and WGS as described in Section 2.6.

7.2.3.3 Analysis of antibiotic resistance genes and plasmid Inc groups

Purity of the WGS data was checked using KmerFinder 3.0 (https://cge.cbs.dtu.dk/services/KmerFinder/) [412-414] and antibiotic resistance genes were identified using ResFinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/) [39] and the ARG-ANNOT database [38] via the read mapping-based tool SRST2 (https://github.com/katholt/srst2) [40]. Plasmid replicon typing was carried out to identify Inc groups using PlasmidFinder 2.0 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) [489].

7.2.3.4 Identification of TA system genes

FASTA sequences of Type I-VI TA system genes (Table 7.3) were obtained from TADB 2.0 (http://bioinfo-mml.sjtu.edu.cn/TADB2/download.html) [490] and were aligned to FASTA sequences encoding the plasmids using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The absence of TA system genes, or the presence of TA system genes that were missing from the TADB 2.0 database, were confirmed by comparing the plasmid DNA to published plasmid sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) following BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 7.1: 79 Gram-negative bacterial isolates from the prospective surveillance study selected for the extraction and characterisation of plasmids. Isolates are grouped based on combinations of 16S RMTase, carbapenemase, ESBL and PMQR genes.

Number of isolates	Species	STs (no. of isolates)	16S RMTase, carbapenemase, ESBL and PMQR genes
armA			
9	*A. baumannii*	2 (9)	*armA, bla*$_{OXA-23}$ and *bla*$_{OXA-66}$
7	*K. pneumoniae*	78 (3), 14 (2) and 29 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib-cr* and *qnrB1*
	E. coli	405 (1)	
3	*E. cloacae* complex	265 (2)	*armA*, *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*
	E. coli	636 (1)	
2	*E. coli*	405 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*
	K. pneumoniae	Unknown	
1	*A. baumannii*	113 (1)	*armA, bla*$_{OXA-23}$*, bla*$_{OXA-66}$ and *bla*$_{PER-7}$
1	*A. baumannii*	622 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{OXA-23}$, *bla*$_{OXA-203}$ and *bla*$_{PER-7}$
1	*C. freundii*	11 (1)	*armA*, *bla*$_{OXA-48}$, *aac(6')-Ib-cr* and *qnrB35*
1	*C. freundii*	111 (1)	*armA*, *bla*$_{OXA-48}$, *bla*$_{SHV-12}$, *aac(6')-Ib-cr* and *qnrB38*
1	*E. coli*	405 (1)	*armA, bla*$_{NDM-1}$ and *bla*$_{CTX-M-15}$
1	*K. oxytoca*	84 (1)	*armA* and *bla*$_{NDM-1}$
1	*K. oxytoca*	Unknown	*armA*, *bla*$_{OXA-48}$, *bla*$_{SHV-12}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	14 (1)	*armA, bla*$_{CTX-M-15}$, *bla*$_{SHV-100}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{OXA-232}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-100}$ and *qnrB1*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{OXA-232}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	15 (1)	*armA, bla*$_{OXA-48}$, *bla*$_{SHV-100}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	15 (1)	*armA, bla*$_{KPC-2}$, *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	15 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	15 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-39}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	16 (1)	*armA*, *bla*$_{NDM-11}$, *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	29 (1)	*armA*, *bla*$_{CTX-M-15}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	78 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-39}$, *bla*$_{TEM-168}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	147 (1)	*armA, bla*$_{NDM-1}$*, bla*$_{CTX-M-15}$ and *qnrS1*
1	*K. pneumoniae*	147 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	307 (1)	*armA*, *bla*$_{CTX-M-33}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr*, *qnrB1* and *qnrS1*
1	*K. pneumoniae*	147 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *bla*$_{CTX-M-14b}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrS1*

Table 7.1 continued

Number of isolates	Species	STs (no. of isolates)	16S RMTase, carbapenemase, ESBL and PMQR genes
rmtB			
2	*E. coli*	405 (1) and 1585 (1)	*rmtB*, *bla*$_{NDM-5}$, *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*
2	*K. pneumoniae*	138 (2)	*rmtB* and *bla*$_{NDM-5}$
2	*P. aeruginosa*	773 (2)	*rmtB*, *bla*$_{NDM-1}$ and *qnrVC1*
1	*E. coli*	90 (1)	*rmtB*, *bla*$_{NDM-1}$ and *bla*$_{CTX-M-15}$
1	*E. coli*	167 (1)	*rmtB, bla*$_{CTX-M-64}$ and *qepA1*
1	*E. coli*	405 (1)	*rmtB*, *bla*$_{NDM-5}$ and *bla*$_{CTX-M-15}$
1	*E. coli*	448 (1)	*rmtB*, *bla*$_{NDM-7}$, *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*
1	*E. coli*	2450 (1)	*rmtB*, *bla*$_{NDM-5}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	231 (1)	*rmtB*, *bla*$_{CTX-M-15}$, *bla*$_{SHV-100}$, *bla*$_{VEB-1}$ and *qnrB1*
1	*K. pneumoniae*	1248 (1)	*rmtB*, *bla*$_{NDM-5}$ and *qnrB1*
1	*P. stuartii*	-	*rmtB*, *bla*$_{VIM-1}$, *bla*$_{SHV-5}$ and *bla*$_{VEB-1}$
rmtC			
6	*Citrobacter* sp.	18 (2) and 22 (1)	*rmtC*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib-cr* and *qnrB1*
	K. pneumoniae	29 (1) and 89 (1)	
	E. cloacae complex	275 (1)	
1	*C. freundii*	170 (1)	*rmtC*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib-cr* and *qnrB9*
1	*E. coli*	167 (1)	*rmtC*, *bla*$_{NDM-1}$, *bla*$_{OXA-181}$, *bla*$_{CTX-M-15}$, *aac(6')-Ib-cr* and *qnrS1*
1	*E. coli*	6870 (1)	*rmtC*, *bla*$_{NDM-1}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	307 (1)	*rmtC*, *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-100}$, aac*(6')-Ib-cr* and *qnrB1*
rmtF			
2	*K. pneumoniae*	147 (2)	*rmtF, bla*$_{OXA-181}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrB1*
2	*K. pneumoniae*	147 (2)	*rmtF*, *bla*$_{NDM-5}$, *bla*$_{OXA-181}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrB1*
2	*K. pneumoniae*	231 (2)	*rmtF*, *bla*$_{OXA-232}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	147 (1)	*rmtF*, *bla*$_{NDM-5}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	231 (1)	*rmtF*, *bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	231 (1)	*rmtF*, *bla*$_{OXA-232}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	336 (1)	*rmtF*, *bla*$_{CTX-M-15}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	383 (1)	*rmtF*, *bla*$_{OXA-48}$, *bla*$_{CTX-M-14b}$ and *aac(6')-Ib-cr*
Two 16S RMTase genes			
1	*K. pneumoniae*	231 (1)	*armA* + *rmtF*, *bla*$_{OXA-181}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$ and *aac(6')-Ib-cr*

Table 7.2: Subset of 55 Gram-negative bacterial isolates from the AMRHAI Reference Unit selected for the extraction and characterisation of plasmids. Isolates are grouped based on combinations of 16S RMTase, carbapenemase, ESBL and PMQR genes.

Number of isolates	Species	STs (no. of isolates)	16S RMTase, carbapenemase, ESBL and PMQR genes
armA			
3	*K. pneumoniae*	14 (2) and 307 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr* and *qnrB1*
2	*K. pneumoniae*	14 (2)	*armA*, *bla*$_{NDM-1}$, *bla*$_{SHV-53}$, *aac(6')-Ib-cr* and *qnrB1*
2	*K. pneumoniae*	147 (1) and 268 (1)	*armA*, *bla*$_{NDM-1}$, *aac(6')-Ib-cr* and *qnrS1*
1	*E. coli*	405 (1)	*armA*, *bla*$_{OXA-181}$ and *bla*$_{CTX-M-15}$
1	*E. coli*	540 (1)	*armA*, *bla*$_{NDM-1}$ and *bla*$_{CTX-M-15}$
1	*K. pneumoniae*	1 (1)	*armA*, *bla*$_{NDM-1}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	11 (1)	*armA*, *bla*$_{NDM-1}$, *aac(6')-Ib-cr* and *qnrB9*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{OXA-48}$, *bla*$_{SHV-100}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{OXA-232}$, *bla*$_{SHV-100}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{OXA-232}$, *bla*$_{SHV-100}$ and *qnrS1*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{OXA-232}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	14 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{VIM-4}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	15 (1)	*armA*, *bla*$_{NDM-1}$, *bla*$_{SHV-53}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	37 (1)	*armA*, *bla*$_{GES-5}$ and *qnrB4*
1	*K. pneumoniae*	101 (1)	*armA*, *bla*$_{KPC-3}$ and *bla*$_{SHV-39}$
1	*K. pneumoniae*	152 (1)	*armA* and *bla*$_{KPC-2}$
1	*K. pneumoniae*	515 (1)	*armA*, *bla*$_{NDM-1}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	859 (1)	*armA*, *bla*$_{NDM-5}$, *aac(6')-Ib-cr* and *qnrS1*
rmtB			
3	*E. coli*	46 (1), 167 (1) and 405 (1)	*rmtB*, *bla*$_{NDM-5}$ and *aac(6')-Ib-cr*
2	*E. coli*	167 (1) and 405 (1)	*rmtB* and *bla*$_{NDM-5}$
1	*E. cloacae* complex	78 (1)	*rmtB* and *bla*$_{NDM-1}$
1	*E. coli*	410 (1)	*rmtB*, *bla*$_{NDM-4}$ and *qnrB7*
1	*E. coli*	410 (1)	*rmtB*, *bla*$_{NDM-5}$, *bla*$_{OXA-181}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	147 (1)	*rmtB*, *bla*$_{KPC-2}$ and *bla*$_{SHV-11}$
1	*K. pneumoniae*	1587 (1)	*rmtB*, *bla*$_{NDM-1}$, *bla*$_{OXA-232}$, *aac(6')-Ib-cr* and *qepA1*

Table 7.2 continued:

Number of isolates	Species	STs (no. of isolates)	16S RMTase, carbapenemase, ESBL and PMQR genes
		rmtC	
1	*E. coli*	147 (1)	*rmtC*, *bla*$_{NDM-1}$ and *aac(6')-Ib-cr*
2	*K. pneumoniae*	131 (1)	rmtC, *bla*$_{NDM-1}$ and *aac(6')-Ib-cr*
	E. cloacae complex	11 (1)	*rmtC*, *bla*$_{NDM-1}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	78 (1)	*rmtC*, *bla*$_{NDM-1}$ and *qnrS1*
		rmtF	
1	*K. pneumoniae*	147 (1)	*rmtF*, *bla*$_{OXA-232}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	16 (1)	*rmtF*, *bla*$_{NDM-1}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	11 (1)	*rmtF*, *bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	336 (1)	*rmtF*, *bla*$_{OXA-232}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	147 (1)	*rmtF*, *bla*$_{OXA-48}$, *bla*$_{SHV-100}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	395 (1)	*rmtF*, *bla*$_{OXA-181}$ and *aac(6')-Ib-cr*
1	*K. pneumoniae*	101 (1)	*rmtF*, *bla*$_{OXA-181}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	11 (1)	*rmtF*, *bla*$_{NDM-1}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr* and *qnrB1*
1	*K. pneumoniae*	231 (1)	*rmtF*, *bla*$_{NDM-1}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	437 (1)	*rmtF*, *bla*$_{OXA-232}$, *aac(6')-Ib-cr* and *qnrB1*
2	*K. pneumoniae*	231 (2)	*rmtF*, *bla*$_{OXA-232}$, *bla*$_{SHV-39}$, *aac(6')-Ib-cr* and *qnrS1*
		Two 16S RMTase genes	
1	*E. coli*	448 (1)	*armA* + *rmtB*, *bla*$_{NDM-1}$ and *aac(6')-Ib-cr*
1	*E. coli*	1702 (1)	*rmtB* + *rmtF*, *bla*$_{NDM-5}$, *aac(6')-Ib-cr* and *qnrS1*
1	*E. coli*	4108 (1)	*rmtB* + *rmtF*, *bla*$_{NDM-7}$, *bla*$_{SFO-1}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	231 (1)	*armA* + *rmtF*, *bla*$_{OXA-181}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr* and *qnrS1*
1	*K. pneumoniae*	231 (1)	*armA* + *rmtF*, *bla*$_{NDM-1}$, *bla*$_{OXA-181}$, *bla*$_{SHV-100}$, *aac(6')-Ib-cr* and *qnrB1*
1	*P. stuartii*	-	*armA* + *rmtC*, *bla*$_{NDM-1}$, *aac(6')-Ib-cr* and *qnrD*

Table 7.3: Type I-VI TA system genes obtained from TADB 2.0 [490].

Class of TA system	TA system genes
Type I	*bsrG*/*SR4*, *cjpT*/*cjrA*, *dinQ*/*agrB*, *hok*/*sok*, *ibs*/*sib*, *ldrD*/*rdlD*, *pndA*/*pndB*, *ralR*/*ralA*, *RNAI*/*RNAII*, *shoB*/*ohsC*, *sprG*/*sprF*, *srnB*/*srnC*, *symE*/*symR*, *tisB*/*istR*, *txpA*/*ratA*, *yonT*/*as-yonT* and *zorO*/*orzO*
Type II	*brnT*/*brnA*, *ccdB*/*ccdA*, *hicB*/*hicA*, *higB*/*higA*, *hipA*/*hipB*, *mazF*/*mazE*, *mosT*/*mosA*, *mqsR*/*mqsA*, *parE*/*parD*, *relE*/*relB*, *SO_3166*/*SO_3165*, *vapC*/*vapB*, *yafO*/*yafN*, *yoeB*/*yefM* and *yhaV*/*prlF*
Type III	*abiQ*/*antiQ*, *cptN*/*cptI*, *tenpN*/*tenpI* and *toxN*/*toxI*
Type IV	*abiEII*/*abiEI*, *abiGII*/*abiGI*, *cbtA*/*cbeA* and *cptA*/*cptB*
Type V	*ghoT*/*ghoS*
Type VI	*socB*/*socA*

7.2.3.5 Identification of the size of plasmids

The size of plasmids in successful transformants was predicted by removing contigs encoding the *E. coli* TOP10 genome as well as contigs (~150 bp) that were identified as duplicates according to BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis. The lengths of the final contigs were combined to predict the size of the plasmid.

7.2.3.6 Plasmid database

A database of plasmid DNA sequences harbouring 16S RMTase genes from 27th December 2002 to 10th April 2019 was created by collecting accession numbers of published 16S RMTase-positive plasmids from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). This included 134 *rmtB* plasmids, 127 *armA* plasmids, 72 *rmtC* plasmids and 17 *rmtF* plasmids. FASTA sequences of the published plasmids were collected using Batch Entrez (https://www.ncbi.nlm.nih.gov/sites/batchentrez), and were used for the mapping of WGS reads from this study using SRST2 (https://github.com/katholt/srst2) [40] to identify plasmids sharing a minimum of 60.0% coverage to ensure results for plasmids could be obtained due to the unlikelihood of all plasmids sharing >90.0% coverage with published plasmids owing to the plasticity of plasmids.

Alignments to the plasmids were confirmed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), where individual contigs were analysed and compared with published sequences. Plasmids that had <60.0% coverage to reference plasmids were also investigated using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if they shared identity with published plasmids, including those that lack 16S RMTase genes. Additionally, alignments were visually confirmed using Mauve (http://darlinglab.org/mauve/mauve.html) [487]. Comparison of plasmid DNA to published plasmid sequences from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) was visualised using BLAST Ring Image Generator (BRIG) [491].

Only plasmids with ≥90.0% identity are reported in order to specifically identify which published plasmids are circulating in the UK. The results of the remaining plasmids can be found in Tables 12-18 in Appendix 1.

7.3 Results

7.3.1 Analysis of the genetic environments of 16S RMTase genes

7.3.1.1 Enterobacterales

7.3.1.1.1 *armA*

Analysis of single contigs harbouring *armA* identified that *armA* was associated with Tn*1548*, which is a composite transposon that contains *armA* flanked by transposases; a class 1 integron that contains gene cassettes that are mostly antibiotic resistance genes (or genes encoding virulence factors or biochemical functions) as well as an additional variable region that harbours the macrolide resistance genes *mphE* and *msrE.* Complete Tn*1548* structures were not identified in most isolates (84.8%, 217/256) due to contigs containing *armA* not covering the full length of Tn*1548* but additional contigs appeared to harbour the rest of Tn*1548* in 82.9% (180/217) isolates, suggesting that it was present in these isolates but could not be confirmed (Figure 7.1). Thirty-seven (14.5%) isolates appeared to have truncated Tn*1548* transposons as *mphE and msrE* or *sul1* (which confers sulphonamide resistance) and *qacEΔ1* (which confers resistance to quaternary ammonium compounds) were absent or gene cassettes could not be identified.

Only 8.2% (21/256) *armA*-containing contigs were found to have the whole Tn*1548* transposon on a single contig, which had an identical genetic environment to *K. pneumoniae* strain AR_0153 plasmid unnamed1 (accession number: CP028929.1), ranging from 173,880-190,913 bp and harboured the gene cassettes *aadA2* (encoding aminoglycoside resistance) and *dfrA12* (encoding trimethoprim resistance, Figure 7.1A). Most of these isolates were *K. pneumoniae* (90.5%, 19/21), ST14 (68.4%, 13/19) and were isolated from London (76.2%, 16/21) (Table 7.4). Fifty-eight (22.7%) *armA*-containing contigs had an identical genetic

environment from 173,880-186,310 bp, where *armA* was associated with *mphE* and *msrE* but lacked the class 1 integron components *sul1* and *qacEΔ1* as well as the gene cassettes due to the contig being fragmented (Figure 7.1C). This genetic environment was mainly found in *K. pneumoniae* (94.8%, 55/58), particularly ST14 (80.0%, 44/55), and was mostly found in London (89.7%, 52/58) (Table 7.4). Forty-three (16.8%) *armA*-containing contigs also harboured the gene cassettes *aadA2* and *dfrA12* but were either missing *repB*, the DNA-binding protein and a hypothetical protein (100%, 43/43) (Figure 7.1G, J, O and R); IS*Kpn21* (90.7% 39/43) (Figure 7.1J, O and R); *mphE* and *msrE* (20.9%, 9/43) (Figure 7.1O and R) and IS*Ec29* (4.7%, 2/43) (Figure 7.1R). Sixteen other forms of this genetic environment were identified in 30.9% (79/256) *armA*-containing contigs, where the gene cassettes *aadA2* and *dfrA12* were missing (100%, 79/79) as well as *repB* and the DNA-binding protein (75.9%, 60/79); *qacEΔ1* (68.4%, 54/79); *sul1* (59.4%, 47/79); IS*Kpn21* (59.5%, 47/79); IS*91* (36.7%, 29/79); *mphE* and *msrE* (20.3%, 16/79) and IS*Ec29* (8.9%, 7/79). *armA* was found alone on a contig in one (1.3%) isolate (Figure 7.1).

Eighteen (7.0%) *armA*-containing contigs harboured a Tn*1548* transposon that had an identical genetic environment to *K. pneumoniae* strain ST101:960186733 plasmid p19-10_01 (accession number: CP023488.1), which included *mphE*, *msrE*, *hypA* (encoding a hydrogenase maturation nickel metallochaperone [492]), *ampR* (which encodes a LysR-family transcriptional regulator that regulates the expression of bla_{DHA-1} [493]), bla_{DHA-1} (conferring cephalosporin resistance), *trpF* (a phosphoribosylanthranilate isomerase, which is involved in amino acid biosynthesis [494]), ble_{MBL} (conferring bleomycin resistance) and bla_{NDM-1} (Figure 7.2A). This genetic environment was mainly identified in *K. pneumoniae* (44.4%, 8/18) and *E. coli* (38.9%, 7/18) and was found in a range of STs but was mostly identified from London (50.0%, 9/18) (Table 7.4). A single *armA*-containing contig (0.4%, 1/256) belonging to *K. oxytoca* ST44 from Yorkshire and the Humber (Table 7.4) also had the gene cassettes *hypA*, *ampR*, bla_{DHA-1}, *trpF*, ble_{MBL} and bla_{NDM-1} but was missing *repB*, the DNA-binding protein, *mphE* and *msrE* (0.4%, 1/256) (Figure 7.2I) and a *K. pneumoniae* ST37 isolate from London was

found to have all genes in Tn*1548* on a single contig apart from *trpF*, *ble*$_{MBL}$, *bla*$_{NDM-1}$ and IS*30* (Figure 7.2B). Six other forms of this genetic environment (Figure 7.2C-H), were found in 13.3% (34/256) *armA*-containing contigs where the gene cassettes *hypA*, *ampR*, *bla*$_{DHA-1}$, *trpF*, *ble*$_{MBL}$ and *bla*$_{NDM-1}$ were missing along with *qacEΔ1* (85.3%, 29/34); *sul1* (76.5%, 26/34); IS*91* (67.6%, 23/34); IS*5* (47.1%, 16/34) and a hypothetical protein (5.9%, 2/34).

Additionally, one *armA*-containing contig from *K. pneumoniae* ST152 from Yorkshire and the Humber had an identical genetic environment with *K. pneumoniae* strain AR_0153 plasmid unnamed1 (accession number: CP028929.1), from 177,883-186,310 bp. This included *mphE*, *msrE* and *armA*. However, downstream of *armA* the contig shared identity to *E. coli* strain N15-01078 plasmid pNDM15-1078 (accession number: CP012902.1) from 72,867-81,255 bp, which included *sul1* and *qacEΔ1* as well as the gene cassettes *aadA1*, *bla*$_{OXA-10}$ (conferring β-lactam resistance), *cmlA* (conferring chloramphenicol resistance) and *arr-2* (conferring rifampicin resistance) (Figure 7.1W).

It could not be determined if the *armA* genes were on chromosomes or plasmids due to DNA only encoding Tn*1548* being present on the contigs. However, nine *armA* genes were later confirmed to be present on plasmids (see Section 7.3.2).

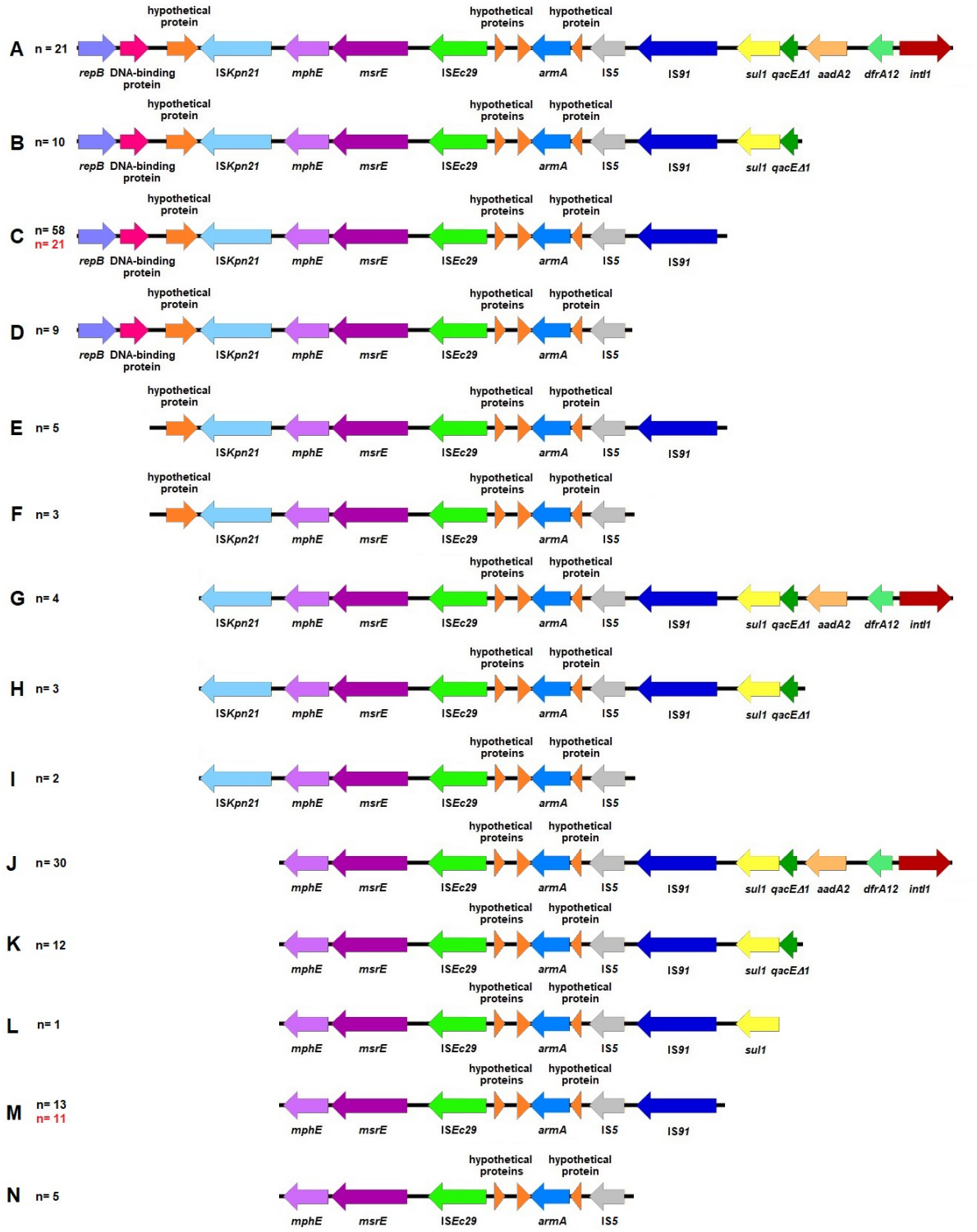


Figure 7.1: Genetic environments of *armA* identified on single contigs in Enterobacterales that shared identity with *K. pneumoniae* strain AR_0153 plasmid unnamed1 (accession number: CP028929.1). A: 173,880-190,913 bp; B: 173,880-187,895 bp; C: 173,880-186,310 bp; D: 173,880-183,592 bp; E: 175,577-186,310 bp; F: 175,577-183,592 bp; G: 176,238-190,913 bp; H: 176,238-187,895 bp; I: 176,238-183,592 bp; J: 177,883-190,913 bp; K: 177,883-187,895 bp; L: 177,883-187,554 bp; M: 177,883-186,310 bp and N: 177,883-183,592 bp. The number of isolates can be found on the left-hand side but those in red signify how many isolates had truncated Tn*1548* transposons, where the missing sections of Tn*1548* were not found on separate contigs.

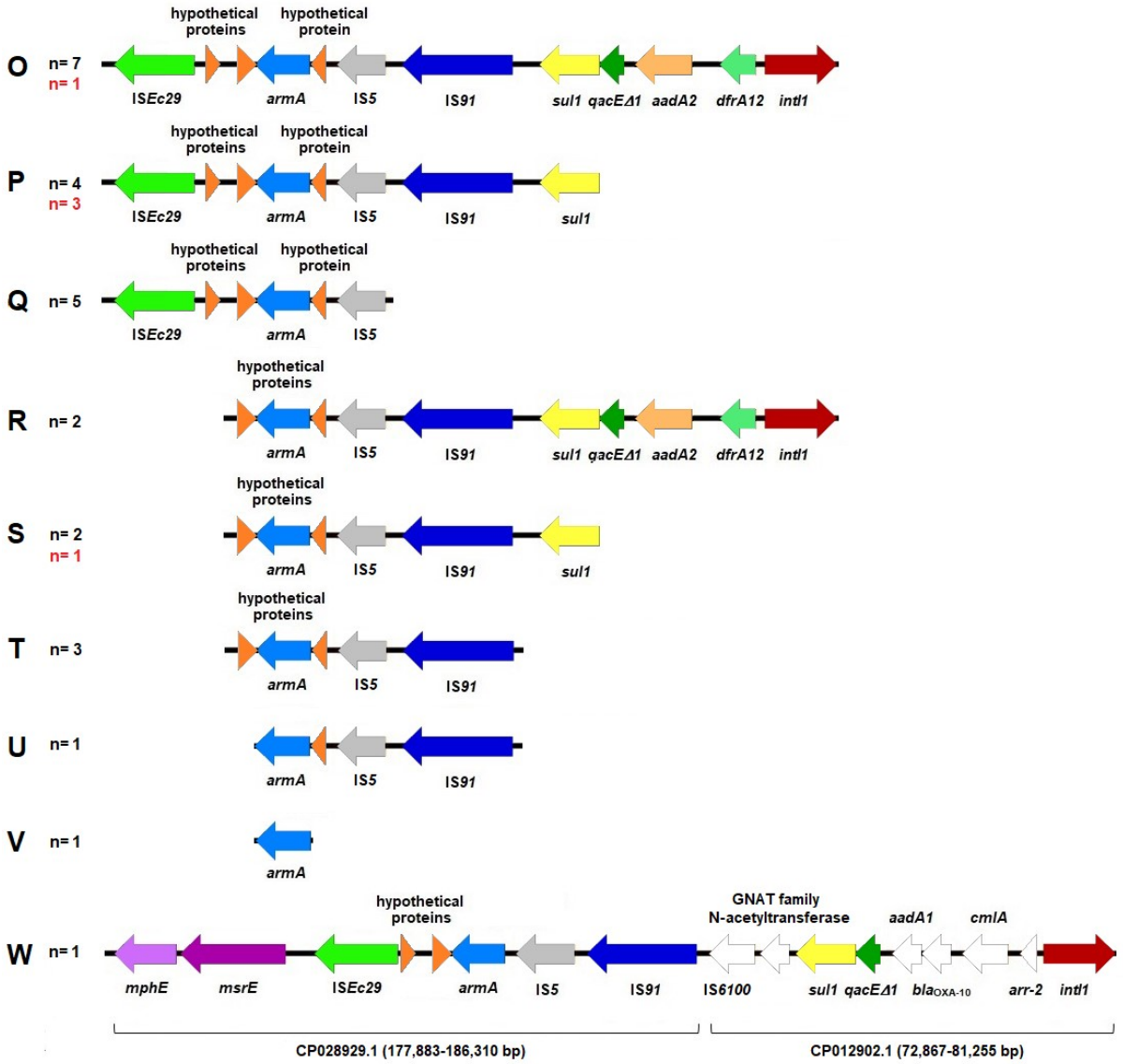


Figure 7.1 continued. O: 180,697-190,913 bp; P: 180,697-187,554 bp; Q: 180,697-183,592 bp; R: 182,335-190,913 bp; S: 182,335-187,554 bp; T: 182,335-186,310 bp; U: 182,597-186,310 bp; V: 182,597-183,370 bp and W: CP028929.1 (177,883-186,310 bp) and CP012902.1 (72,867-81,255 bp). Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side but those in red signify how many isolates had truncated Tn*1548* transposons, where the missing sections of Tn*1548* were not found on separate contigs.

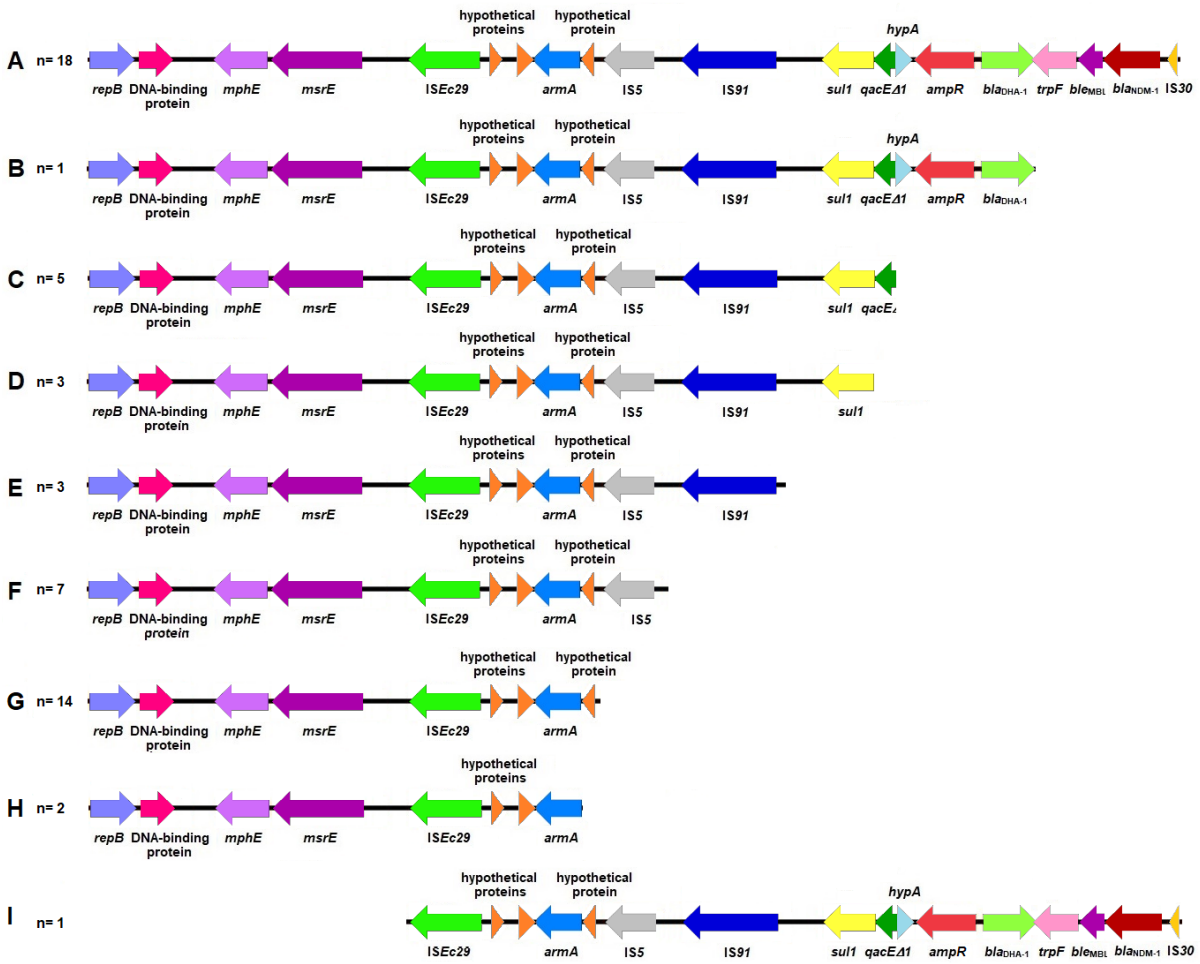


Figure 7.2: Genetic environments of *armA* identified on single contigs in Enterobacterales that shared identity with *K. pneumoniae* strain ST101:960186733 plasmid p19-10_01 (accession number: CP023488.1). A: 137,966-153,826 bp; B: 139,989-153,826 bp; C: 142,031-153,826 bp; D: 142,360-153,826 bp; E: 143,604-153,826 bp; F: 145,379-153,826 bp; G: 146,322-153,826 bp; H: 146,544-153,826 bp and I: 137,966-149,217 bp. The number of isolates can be found on the left-hand side.

Table 7.4: Genetic environment of *armA*-containing contigs identified in Enterobacterales with their associated species, STs and geographical distribution.

***armA* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
repB-DNA-binding protein-hypothetical protein-IS*Kpn21*-*mphE*-*msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*-*sul1*-*qacE*Δ*1*-*aadA2*-*dfrA12*-*intI1*	CP028929.1 (173,880-190,913)	21	*K. pneumoniae* (19)	14 (13), 78 (4) and 231 (2)	London (14), East (4) and North West (1)
			E. coli (2)	38 (1) and 405 (1)	London (2)
repB-DNA-binding protein-hypothetical protein-IS*Kpn21*-*mphE*-*msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*-*sul1*-*qacE*Δ*1*	CP028929.1 (173,880-187,895)	10	*K. pneumoniae* (10)	14 (10)	London (9) and North West (1)
repB-DNA-binding protein-hypothetical protein-IS*Kpn21*-*mphE*-*msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*	CP028929.1 (173,880-186,310)	58	*K. pneumoniae* (55)	14 (44), 231 (6), 15 (4) and unknown (1)	London (51), North West (3) and South West (1)
			P. stuartii (2)	-	East (2)
			E. coli (1)	156 (1)	London (1)
repB-DNA-binding protein-hypothetical protein-IS*Kpn21*-*mphE*-*msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*	CP028929.1 (173,880-183,592)	9	*K. pneumoniae* (8)	14 (6), 78 (1) and 231 (1)	London (7) and East (1)
			E. coli (1)	405 (1)	London (1)
hypothetical protein-IS*Kpn21*-*mphE*-*msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*	CP028929.1 (175,577-186,310)	5	*K. pneumoniae* (5)	14 (3), 147 (1) and 231 (1)	London (3), East (1) and South West (1)

Table 7.4 continued

***armA* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
hypothetical protein-IS*Kpn21-mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*	CP028929.1 (175,577-183,592)	3	*K. pneumoniae* (3)	1 (3)	London (3)
IS*Kpn21-mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1-qacE*Δ*1-aadA2-dfrA12-intI1*	CP028929.1 (176,238-190,913)	4	*K. pneumoniae* (4)	11 (1), 147 (1), 307 (1) and 515 (1)	London (4)
IS*Kpn21-mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1-qacE*Δ*1*	CP028929.1 (176,238-187,895)	3	*K. pneumoniae* (3)	14 (3)	London (1), West Midlands (1) and Yorkshire and the Humber (1)
IS*Kpn21-mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*	CP028929.1 (176,238-183,592)	2	*K. pneumoniae* (2)	14 (1) and 448 (1)	East (1) and London (1)
mphE-msrE-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1-qacE*Δ*1-aadA2-dfrA12-intI1*	CP028929.1 (177,883-190,913)	30	*K. pneumoniae* (24)	14 (9), 11 (3), 515 (3), 15 (2), 147 (1), 231 (1), 273 (1), 395 (1), 1680 (1) and unknown (2)	London (19), West Midlands (3), East Midlands (1) and South East (1)
			E. cloacae complex (4)	265 (2), 200 (1) and 231 (1)	London (2) and Scotland (2)
			E. coli (1)	401 (1)	London (1)
			K. oxytoca (1)	36 (1)	London (1)

Table 7.4 continued

***armA* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
mphE-msrE-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1-qacE*Δ*1*	CP028929.1 (177,883-187,895)	12	*K. pneumoniae* (9)	14 (7), 147 (1) and unknown (1)	London (6), West Midlands (2) and East (1)
			C. freundii (2)	91 (1) and 284 (1)	London (2)
			K. ornithinolytica (1)	-	London (1)
mphE-msrE-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1*	CP028929.1 (177,883-187,554)	1	*K. pneumoniae* (1)	14 (1)	East (1)
mphE-msrE-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*	CP028929.1 (177,883-186,310)	13	*K. pneumoniae* (7)	1 (1), 15 (1), 35 (1), 231 (1),1813 (1), 3052 (1) and unknown (1)	London (5), East (1) and North East (1)
			C. freundii (3)	11 (2) and 111 (1)	London (3)
			E. coli (1)	636 (1)	London (1)
			K. oxytoca (1)	144 (1)	London (1)
			P. mirabilis (1)	-	East (1)
mphE-msrE-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*	CP028929.1 (177,883-183,592)	5	*K. pneumoniae* (5)	14 (3), 15 (1) and 2990 (1)	London (4) and Yorkshire and the Humber (1)
IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1-qacE*Δ*1-aadA2-dfrA12-intI1*	CP028929.1 (180,697-190,913)	7	*K. pneumoniae* (6)	14 (4), 11 (1) and 231 (1)	London (5) and North East (1)
			E. coli (1)	10 (1)	London (1)

Table 7.4 continued

***armA* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*-*sul1*	CP028929.1 (180,697-187,554)	4	*K. pneumoniae* (4)	147 (2), 859 (1) and 2648 (1)	East (1), London (1), North West (1) and South East (1)
IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*	CP028929.1 (180,697-183,592)	5	*K. pneumoniae* (3)	14 (3)	London (3)
			E. coli (1)	405 (1)	London (1)
			P. mirabilis (1)	-	East Midlands (1)
hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*-*sul1*-*qacE*Δ*1*-*aadA2*-*dfrA12*-*intI1*	CP028929.1 (182,335-190,913)	2	*E. coli* (1)	405 (1)	London (1)
			K. pneumoniae (1)	14 (1)	London (1)
hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*-*sul1*	CP028929.1 (182,335-187,554)	2	*K. pneumoniae* (2)	147 (1) and 859 (1)	East (1) and London (1)
hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*	CP028929.1 (182,335-186,310)	3	*K. pneumoniae* (2)	14 (1) and 15 (1)	London (1) and South East (1)
			K. aerogenes (1)	4 (1)	Yorkshire and the Humber (1)
armA-hypothetical protein-IS*5*-IS*91*	CP028929.1 (182,597-186,310)	1	*C. freundii* (1)	399 (1)	North East (1)
armA	CP028929.1 (182,597-183,370)	1	*K. pneumoniae* (1)	14 (1)	West Midlands (1)
mphE-*msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-IS*5*-IS*91*-IS*6100*-putative acetyltransferase-*sul1*-*qacE*Δ*1*-*aadA1*-*bla*$_{OXA-10}$-*cmlA*-*arr-2*-*intI1*	CP028929.1 (177,883-186,310) and CP012902.1 (72,867-81,255)	1	*K. pneumoniae* (1)	152 (1)	Yorkshire and the Humber (1)

Table 7.4 continued

***armA* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
repB-DNA-binding protein-*mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1-qacE*Δ*1-hypA-ampR-bla*$_{DHA-1}$-*trpF-ble*$_{MBL}$-*bla*$_{NDM-1}$-IS*30*	CP023488.1 (137,966-153,826)	18	*K. pneumoniae* (8)	14 (3), 307 (3), 84 (1) and 392 (1)	London (5) and Yorkshire and the Humber (3)
			E. coli (7)	405 (3), 127 (1), 409 (1), 540 (1) and 1316 (1)	London (4), North East (2) and Yorkshire and the Humber (1)
			E. cloacae complex (2)	90 (2)	Yorkshire and the Humber (2)
			K. oxytoca (1)	44 (1)	Yorkshire and the Humber (1)
repB-DNA-binding protein-*mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1-qacE*Δ*1-hypA-ampR-bla*$_{DHA-1}$	CP023488.1 (139,989-153,826)	1	*K. pneumoniae* (1)	37 (1)	London (1)
repB-DNA-binding protein-*mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1-qacE*Δ*1*	CP023488.1 (142,031-153,826)	5	*K. pneumoniae* (3)	29 (2) and 14 (1)	London (2) and East (1)
			Citrobacter sp. (1)	-	South East (1)
			P. mirabilis (1)	-	London (1)
repB-DNA-binding protein-*mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91-sul1*	CP023488.1 (142,360-153,826)	3	*K. pneumoniae* (2)	23 (1) and 395 (1)	London (1) and South East (1)
			E. cloacae complex (1)	265 (1)	Scotland (1)

Table 7.4 continued

***armA* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
repB-DNA-binding protein-*mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*-IS*91*	CP023488.1 (143,604-153,826)	3	*E. cloacae* complex (2)	78 (1) and 566 (1)	West Midlands (1) and Yorkshire and the Humber (1)
			K. pneumoniae (1)	268 (1)	North East (1)
repB-DNA-binding protein-*mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*	CP023488.1 (145,379-153,826)	7	*K. pneumoniae* (3)	14 (2) and 16 (1)	Yorkshire and the Humber (2) and Scotland (1)
			E. coli (2)	409 (1) and 648 (1)	West Midlands (1) and Yorkshire and the Humber (1)
			P. stuartii (2)	-	East (1) and North West (1)
repB-DNA-binding protein-*mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein	CP023488.1 (146,322-153,826)	14	*K. pneumoniae* (14)	147 (6), 15 (3), 101 (2), 307 (1), 395 (1) and 1399 (1)	London (10), North West (2), South East (1) and West Midlands (1)
repB-DNA-binding protein-*mphE-msrE*-IS*Ec29*-hypothetical protein-hypothetical protein-*armA*	CP023488.1 (146,544-153,826)	2	*E. cloacae* complex (1)	274 (1)	Yorkshire and the Humber (1)
			K. pneumoniae (1)	392 (1)	London (1)
IS*Ec29*-hypothetical protein-hypothetical protein-*armA*-hypothetical protein-IS*5*- IS*91*-*sul1*-*qacEΔ1*-*hypA*- *ampR*-bla_{DHA-1}-*trpF*-ble_{MBL}-bla_{NDM-1}-IS*30*	CP023488.1 (137,966-149,217)	1	*K. oxytoca* (1)	44 (1)	Yorkshire and the Humber (1)

7.3.1.1.2 *rmtB*

Analysis of single contigs containing *rmtB* (Figure 7.3 and Table 7.5) identified that *rmtB* was associated with Tn*2* in 37.8% (31/82) isolates (Figure 7.3A and E). Eight (9.8%) isolates had truncated Tn*2* transposons, where the Tn*2* transposase was not found in seven isolates, even on a separate contig, and the other isolate had the Tn*2* transposase replaced by IS*26* (Figure 7.3L). Tn*2* was not found in 3.7% (3/82) isolates, even though bla_{TEM-1} was present. The remaining 48.8% (40/82) isolates had Tn*2* on separate contigs, suggesting that it was present in these isolates but could not be confirmed due to fragmentation of the contigs.

Thirty-one (37.8%) *rmtB*-containing contigs had Tn*2* present, where 96.8% (30/31) *rmtB*-containing contigs shared the same genetic environment as *E. coli* M309 plasmid pM309-NDM5 (accession number: AP018833.1) ranging from 32,778-36,607 bp (Figure 7.3A). This consisted of *groEL* (which encodes a chaperonin protein involved in protein folding [495]) and a putative Na^+/H^+ antiporter (which is a membrane protein involved in the exchange of Na^+ and H^+ [496]) associated with *rmtB* as well as Tn*2*. One (3.2%) *rmtB*-containing contig shared an identical genetic environment from 33,191-36,607 bp, where *rmtB* was associated with a putative Na^+/H^+ antiporter and Tn*2* but lacked *groEL* (Figure 7.3E). The *rmtB*-containing contigs associated with Tn*2* were most common in *E. coli* (87.1%, 27/31) and were not restricted to a specific UK region (Table 7.5). However, most (43.9%, 36/82) *rmtB*-containing contigs shared the same genetic environment from 32,778-34,575 bp (Figure 7.3D), which consisted of *groEL* and a putative Na^+/H^+ antiporter but Tn*2* was not present. This genetic environment was found in a range of species as well as STs and was not specific to any location in the UK. Six other forms of this genetic environment were present in 14.6% (12/82) *rmtB*-containing contigs (Figure 7.3B, C and F-I), where Tn*2* was truncated (50.0%, 6/12) or absent (50.0%, 6/12); *groEL* was missing in 41.7% (5/12) *rmtB*-containing contigs; the putative Na^+/H^+ antiporter was missing in 16.7% (2/12) *rmtB*-containing contigs and bla_{TEM-1} was missing in 16.7% (2/12) *rmtB*-containing contigs.

rmtB-containing contigs from two *E. coli* isolates belonging to ST101 and ST167 shared the same genetic environment as *E. coli* strain 3A11 plasmid pHN3A11 (accession number: JX997935.2) from 6,893-14,433 bp (Figure 7.3J), which harboured a truncated Tn*2* resolvase, or 6,893-13,858 bp (Figure 7.3K), which lacked the Tn*2* resolvase, respectively. *rmtB* was also associated with *groEL*, IS*CR3*, *dfr2* (encoding trimethoprim resistance) and *qepA* (encoding a quinolone efflux pump). Isolates harbouring *rmtB*-containing contigs with these genetic environments were isolated in the East Midlands and London (Table 7.5).

The *rmtB*-containing contig from one *K. pneumoniae* ST231 isolate from Scotland shared the same genetic environment as *K. pneumoniae* strain MSB1_8A-sc-2280397 plasmid unnamed2 (accession number: CP031802.1) from 28,195-46,699 bp (Table 7.5). *rmtB* was associated with a class 1 integron-like structure (as the *intI1* gene was absent from the class 1 integron, although present on a separate contig) harbouring gene cassettes including the ESBL gene bla_{VEB-1} as well as *aadA1*, *ant(2")-Ia*, bla_{OXA-10}, *cmlA5* and *arr-2* (Figure 7.3L). It was also associated with *floR2* (encoding chloramphenicol resistance) and *tet*(G) (encoding tetracycline resistance), which were downstream of the class 1 integron.

As no chromosome- or plasmid-specific genes were identified on the *rmtB*-containing contigs it is unknown if the *rmtB* genes were found on chromosomes or plasmids. However, 21 *rmtB* genes were later confirmed to be present on plasmids (see Section 7.3.2).

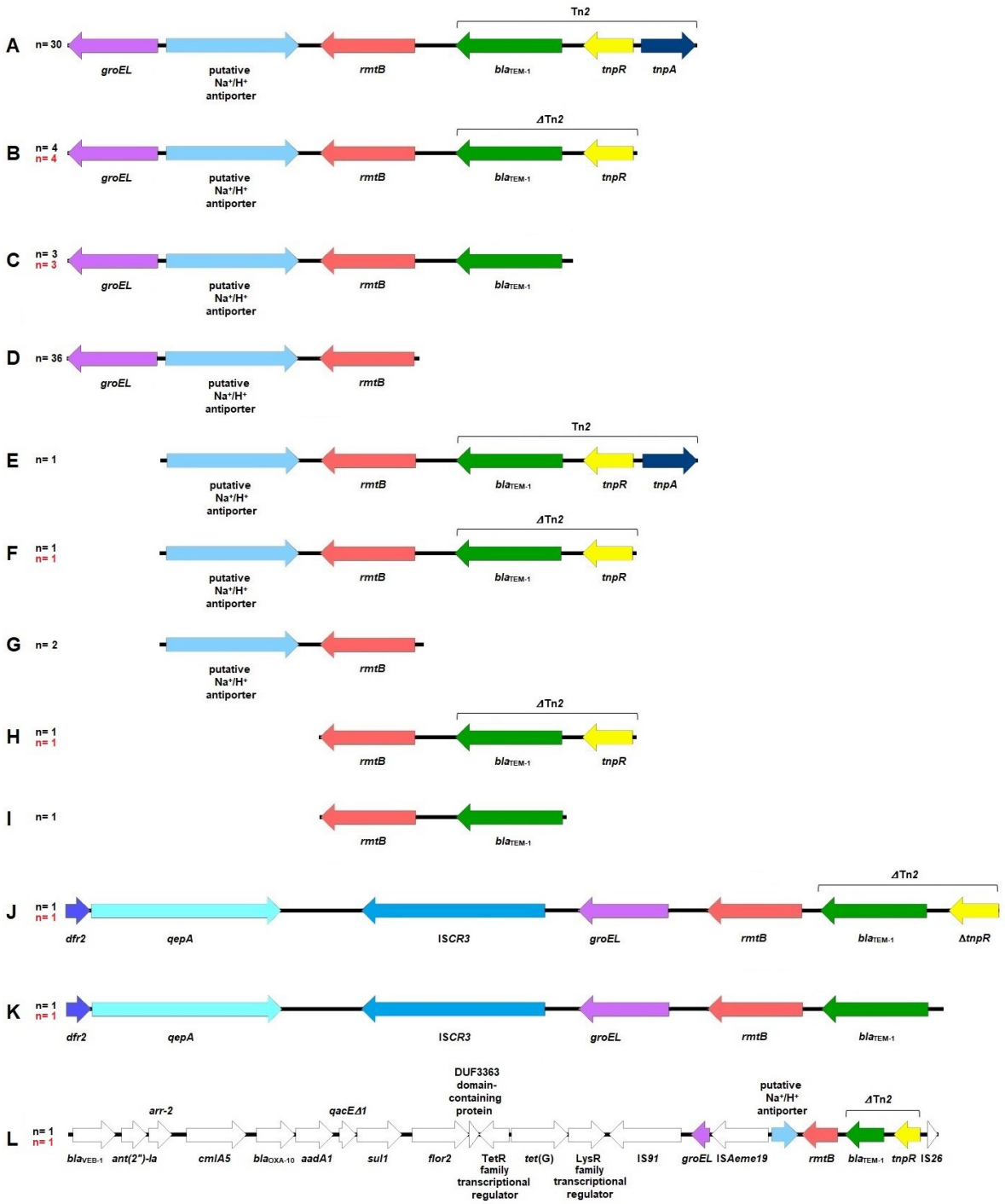


Figure 7.3: Genetic environments of *rmtB* identified on a single contig in Enterobacterales. A: AP018833.1 (32,778-36,607 bp); B: AP018833.1 (32,778-36,345 bp); C: AP018833.1 (32,778-35,605 bp); D: AP018833.1 (32,778-34,575 bp); E: AP018833.1 (33,191-36,607 bp); F: AP018833.1 (33,191-36,345 bp); G: AP018833.1 (33,191-34,575 bp); H: AP018833.1 (33,820-36,345 bp); I: AP018833.1 (33,820-35,605 bp); J: JX997935.2 (6,893-14,433 bp): K: JX997935.2 (6,893-13,858 bp) and L: CP031802.1 (28,195-46,699 bp). Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side but those in red signify how many isolates had truncated Tn*2* transposons, where the missing genes were not found on separate contigs.

Table 7.5: Genetic environments of *rmtB*-containing contigs identified in Enterobacterales with their associated species, STs and geographical distribution.

***rmtB* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
groEL-Na^+/H^+ antiporter-*rmtB*-bla_{TEM-1}-Tn*2* resolvase-Tn*2* transposase	AP018833.1 (32,778-36,607)	30	*E. coli* (26)	167 (9), 405 (7), 410 (3), 448 (3), 90 (1), 940 (1), 1702 (1) and 2450 (1)	London (13), West Midlands (4), East Midlands (3), North West (3), East (2) and South East (1)
			K. pneumoniae (4)	16 (1), 138 (1), 307 (1) and 1587 (1)	East (1), London (1), North East (1) and South West (1)
groEL-Na^+/H^+ antiporter-*rmtB*-bla_{TEM-1}-Tn*2* resolvase	AP018833.1 (32,778-36,345)	4	*E. coli* (4)	405 (2), 410 (1) and 648 (1)	East Midlands (1), London (2) and West Midlands (1)
groEL-Na^+/H^+ antiporter-*rmtB*-bla_{TEM-1}	AP018833.1 (32,778-35,605)	3	*E. coli* (1)	2659 (1)	London (1)
			K. pneumoniae (1)	138 (1)	London (1)
			P. stuartii (1)	-	London (1)
groEL-Na^+/H^+ antiporter-*rmtB*	AP018833.1 (32,778-34,575)	36	*E. coli* (28)	167 (8), 405 (4), 410 (5), 940 (3), 448 (2), 46 (1), 131 (1), 648 (1), 1585 (1), 1702 (1) and 5051 (1)	London (11), North West (6), East (4), East Midlands (2), South West (2), West Midlands (2) and Scotland (1)
			*K. pneumoniae (*4)	14 (1), 45 (1), 138 (1) and 1248 (1)	London (2), Scotland (1) and Yorkshire and the Humber (1)
			E. cloacae complex (2)	121 (2)	London (1) and West Midlands (1)
			C. freundii (1)	108 (1)	East (1)
			M. morganii (1)	-	West Midlands (1)

Table 7.5 continued

***rmtB* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
Na^+/H^+ antiporter-*rmtB*-*bla*$_{TEM-1}$-Tn*2* resolvase-Tn*2* transposase	AP018833.1 (33,191-36,607)	1	*E. coli* (1)	410 (1)	London (1)
Na^+/H^+ antiporter-*rmtB*-*bla*$_{TEM-1}$-Tn*2 resolvase*	AP018833.1 (33,191-36,345)	1	*K. pneumoniae (*1)	147 (1)	Scotland (1)
Na^+/H^+ antiporter-*rmtB*	AP018833.1 (33,191-34,575)	2	*K. pneumoniae (*1)	147 (1)	London (1)
			E. coli	410 (1)	West Midlands (1)
rmtB-*bla*$_{TEM-1}$-Tn*2 resolvase*	AP018833.1 (33,820-36,345)	1	*E. coli* (1)	4108 (1)	Yorkshire and the Humber (1)
rmtB-*bla*$_{TEM-1}$	AP018833.1 (33,820-35,605)	1	*E. cloacae* complex (1)	78 (1)	London (1)
dfr2-*qepA*-IS*CR3*-*rmtB*-*bla*$_{TEM-1}$-ΔTn*2*	JX997935.2 (6,893-14,433)	1	*E. coli* (1)	101 (1)	East Midlands (1)
dfr2-*qepA*-IS*CR3*-*rmtB*-*bla*$_{TEM-1}$	JX997935.2 (6,893-13,858)	1	*E. coli* (1)	167 (1)	London (1)
bla$_{VEB-1}$-*ant(2")-Ia*-*arr-2*-*cmlA5*-*bla*$_{OXA-10}$-*aadA1*-*qacEΔ1*-*sul1*-*floR2*-TetR family transcriptional regulator-*tet*(G)-LysR family transcriptional regulator-IS*91*-*groEL*-IS*Aeme19*-Na^+/H^+ antiporter-*rmtB*-*bla*$_{TEM-1}$-ΔTn*2*-IS*26*	CP031802.1 (28,195-46,699)	1	*K. pneumoniae* (1)	231 (1)	Scotland

7.3.1.1.3 *rmtC*

Analysis of single contigs harbouring *rmtC* identified that *rmtC* was associated with IS*Ecp1* in 67.4% (58/86) *rmtC*-containing contigs while the remaining 27.9% (24/86) *rmtC*-containing contigs harboured *rmtC* associated with an IS*91* element (Figure 7.4 and Table 7.6). Four *rmtC* genes were found alone on contigs, although three isolates had additional contigs that harboured IS*91* their association could not be confirmed. Nineteen (22.1%) *rmtC*-containing contigs had bla_{NDM-1} associated with *rmtC*, although it was found on separate contigs sharing the same genetic environment in 32.6% (28/86) isolates but the association could not be confirmed.

The most common genetic environment identified in 31.4% (27/86) *rmtC*-containing contigs was identical to *E. cloacae* strain ECL17464 plasmid pECL17464 (accession number: MH995508.1) from 103,157-108,558 bp, where *rmtC* was associated with IS*Ecp1*, endonuclease III, a DNA cytosine methyltransferase, *qacEΔ1* and the antibiotic resistance gene *sul1* (Figure 7.4C). However, 96.3% (26/27) *rmtC*-containing contigs were missing ~150 bp of the 3' end of IS*Ecp1* indicating it was truncated. This genetic environment was found in a range of species, STs and was not restricted to a specific region. Thirteen (15.1%) *rmtC*-containing contigs also shared the same genetic environment but from 103,157-111,333 bp, which also included IS*Kpn14*, bla_{NDM-1}, ble_{MBL} and *trpF* (Figure 7.4A). This was also found in multiple species, STs and regions. Four additional forms of this genetic environment were identified in 12.8% (11/86) *rmtC*-containing contigs (Figure 7.4B and D-F), where IS*Ecp1* was present along with endonuclease III (81.8%, 9/11), a DNA cytosine methyltransferase (72.7%, 8/11), *qacEΔ1* and *sul1* (27.3%, 3/11), IS*Kpn14* (27.3%, 3/11) or bla_{NDM-1}, ble_{MBL} and *trpF* (9.1%, 1/11). The 3' end of IS*Ecp1* was missing ~150 bp in all *rmtC*-containing contigs sharing the same genetic environment from 105,571-108,558 bp (n=5), which encoded a DNA cytosine methyltransferase, endonuclease III, *rmtC* as well as IS*Ecp1* (Figure 7.4D) and from 107,459-108,558 bp (n=2), which encoded *rmtC* and IS*Ecp1* (Figure 7.4F).

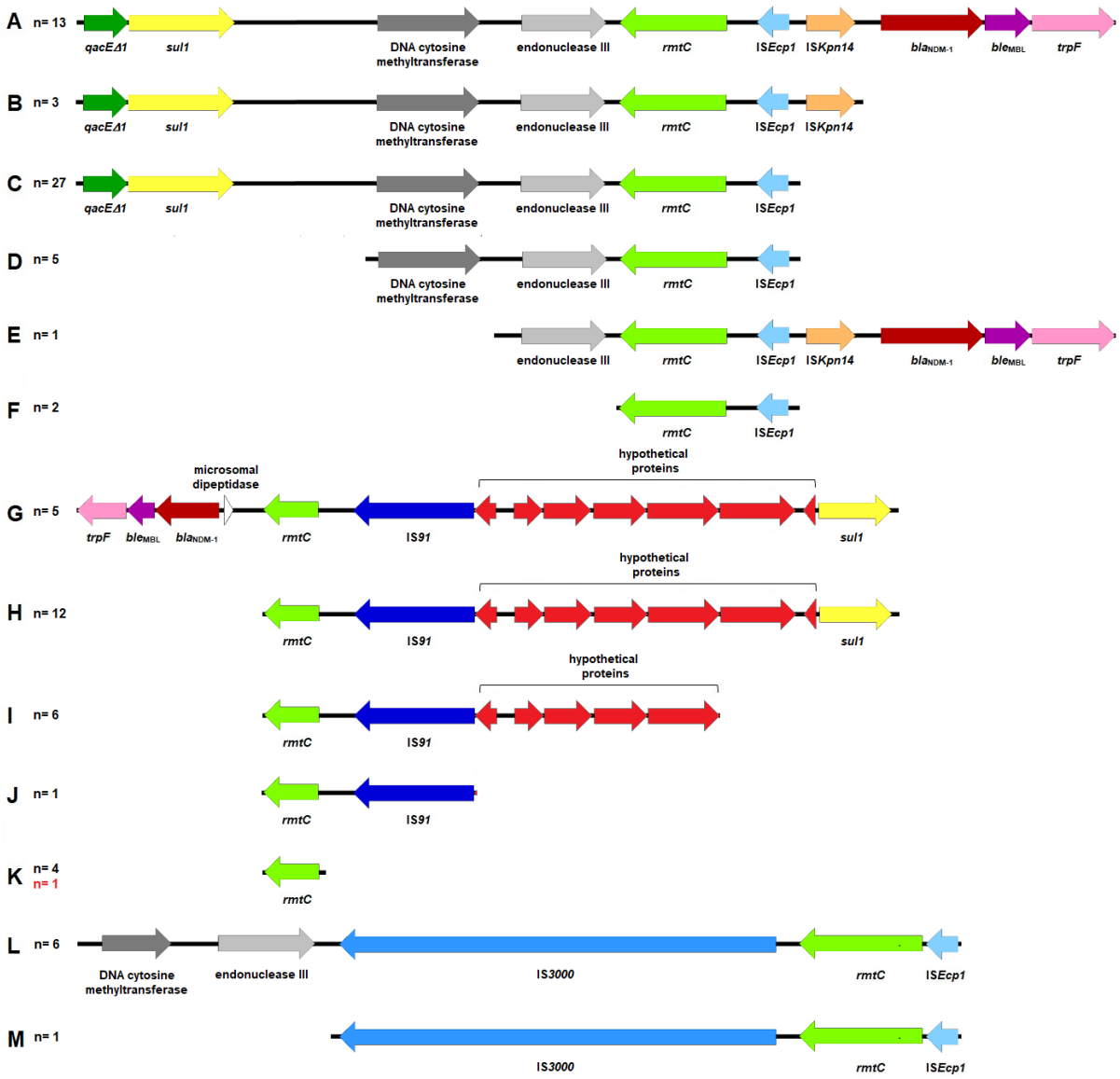


Figure 7.4: Genetic environments of *rmtC* identified on a single contig in Enterobacterales. A: MH995508.1 (103,157-111,333 bp); B: MH995508.1 (103,157-109,326 bp); C: MH995508.1 (103,157-108,558 bp); D: MH995508.1 (105,571-108,558 bp); E: MH995508.1 (106,693-111,333 bp); F: MH995508.1 (107,459-108,558 bp); G: MH909345.1 (13,510-23,901 bp); H: MH909345.1 (13,510-21,519 bp); I: MH909345.1 (16,624-21,519 bp); J: MH909345.1 (18,848-21,519 bp); K: MH909345.1 (20,830-21,519 bp); L: AP018832.1 (43,269-49,501 bp) and M: AP018832.1 (43,269-47,887 bp). Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side but those in red signify how many isolates had missing IS*Ecp1*/IS*91* elements associated with *rmtC*, which were not found on separate contigs.

Table 7.6: Genetic environments of *rmtC*-containing contigs identified in Enterobacterales with their associated species, STs and geographical distribution.

***rmtC* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
qacEΔ1-sul1-DNA cytosine methyltransferase-endonuclease III-*rmtC*-IS*Ecp1*-IS*Kpn14*-*bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF*	MH995508.1 (103,157-111,333)	13	*E. coli* (6)	167 (2), 131 (1), 410 (1), 448 (1) and 617 (1)	East (2), North East (1), North West (1), South West (1) and Yorkshire and the Humber (1)
			C. freundii (2)	18 (2)	London (2)
			K. pneumoniae (2)	11 (1) and 45 (1)	East (1) and London (1)
			P. stuartii (2)	-	East (2)
			E. cloacae complex (1)	90 (1)	West Midlands (1)
qacEΔ1-sul1-DNA cytosine methyltransferase-endonuclease III-*rmtC*-IS*Ecp1*-IS*Kpn14*	MH995508.1 (103,157-109,326)	3	*E. coli* (2)	405 (1) and 6870 (1)	East (1) and London (1)
			K. pneumoniae (1)	11 (1)	London (1)
qacEΔ1-sul1-DNA cytosine methyltransferase-endonuclease III-*rmtC*-IS*Ecp1*	MH995508.1 (103,157-108,558)	27	*K. pneumoniae* (12)	15 (3), 29 (3), 11 (2), 1 (1), 48 (1), 147 (1) and 231 (1)	East (4), London (2), West Midlands (2), North East (1), North West (1), Scotland (1) and Yorkshire and the Humber (1)
			E. coli (8)	405 (3), 10 (1), 69 (1), 131 (1), 361 (1) and 410 (1)	East (2), London (2), West Midlands (2), North West (1) and Yorkshire and the Humber (1)
			C. freundii (3)	18 (2) and 108 (1)	London (2) and South West (1)
			C. sedlakii (1)	Unknown (1)	London (1)
			E. cloacae complex (1)	171 (1)	East Midlands (1)
			P. rettgeri (1)	-	London (1)
			P. stuartii 1)	-	North West (1)

Table 7.6 continued

***rmtC* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
DNA cytosine methyltransferase-endonuclease III-*rmtC*-IS*Ecp1*	MH995508.1 (105,571-108,558)	5	*K. pneumoniae* (3)	15 (1), 147 (1) and 340 (1)	East Midlands (3)
			E. cloacae complex (1)	267 (1)	West Midlands (1)
			C. freundii (1)	112 (1)	London (1)
endonuclease III-*rmtC*-IS*Ecp1*-IS*Kpn14*-bla_{NDM-1}-ble_{MBL}-*trpF*	MH995508.1 (106,693-111,333)	1	*P. stuartii* (1)	-	East (1)
rmtC-IS*Ecp1*	MH995508.1 (107,459-108,558)	2	*E. coli* (1)	349 (1)	London (1)
			K. pneumoniae (1)	11 (1)	London (1)
sul1-hypothetical protein-hypothetical protein-hypothetical protein-hypothetical protein-hypothetical protein-hypothetical protein-IS*91*-*rmtC*-microsomal dipeptidase-bla_{NDM-1}-ble_{MBL}-*trpF*	MH909345.1 (13,510-23,901)	5	*E. cloacae* complex (2)	89 (1) and 171 (1)	London (1) and West Midlands (1)
			K. pneumoniae (2)	29 (1) and 89 (1)	London (2)
			C. freundii (1)	170 (1)	London (1)
sul1-hypothetical protein-hypothetical protein-hypothetical protein-hypothetical protein-hypothetical protein-hypothetical protein-IS*91*-*rmtC*	MH909345.1 (13,510-21,519)	12	*K. pneumoniae* (10)	147 (4), 152 (2), 14 (1), 15 (1), 265 (1) and 307 (1)	London (5), West Midlands (2), South East (1), South West (1) and Yorkshire and the Humber (1)
			C. freundii (1)	22 (1)	East (1)
			E. coli (1)	14 (1)	South West (1)
hypothetical protein-hypothetical protein-hypothetical protein-hypothetical protein-hypothetical protein-IS*91*-*rmtC*	MH909345.1 (16,624-21,519)	6	*K. pneumoniae* (6)	147 (3), 14 (1), 15 (1) and 152 (1)	London (3), East (1), North East (1) and Yorkshire and the Humber (1)

Table 7.6 continued

***rmtC* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
IS*91-rmtC*	MH909345.1 (18,848-21,519)	1	*K. pneumoniae* (1)	15 (1)	London (1)
rmtC	MH909345.1 (20,830-21,519)	4	*K. pneumoniae* (2)	15 (1) and 101 (1)	London (1) and South West (1)
			E. cloacae complex (1)	78 (1)	North West (1)
			E. coli (1)	167 (1)	West Midlands (1)
IS*Ecp1-rmtC*-IS*3000*-endonuclease III-DNA cytosine methyltransferase	AP018832.1 (43,269-49,501)	6	*K. pneumoniae* (6)	11 (5) and 1680 (1)	London (2), East (1), North East (1), Scotland (1) and West Midlands (1)
IS*Ecp1-rmtC*-IS*3000*	AP018832.1 (43,269-47,887)	1	*K. pneumoniae* (1)	11 (1)	East (1)

Twelve (14.0%) *rmtC*-containing contigs shared the same genetic environment as *K. pneumoniae* strain N201205880 plasmid p205880-NDM (accession number: MH909345.1) from 13,510-21,519 bp, where *rmtC* was associated with IS*91*, *sul1* and seven hypothetical proteins (Figure 7.4H). This genetic environment was found in a range of species, STs and was not restricted to a specific UK region. Five (5.8%) *rmtC*-containing contigs shared this genetic environment from 13,510-23,901 bp (Figure 7.4G), where *rmtC* was also associated with bla_{NDM-1}, ble_{MBL} and *trpF*. This genetic environment was found in multiple species and STs but was mainly identified in isolates from London (80.0%, 4/5) (Table 7.6). Three additional forms of this genetic environment were identified in 12.8% (11/86) *rmtC*-containing contigs (Figure 7.4I-K), where *rmtC* either was found alone (36.4%, 4/11) or with IS*91* (63.6%, 7/11) and hypothetical proteins (54.5%, 6/11).

Seven (8.1%) *rmtC*-containing contigs shared the same genetic environment as *E. hormaechei* subsp. *xiangfangensis* M308 plasmid pM308-NDM1 (accession number: AP018832.1). Six (85.7%) *rmtC*-containing contigs were identical to 43,269-49,501 bp, where *rmtC* was associated with IS*Ecp1* as well as IS*3000*, endonuclease III and a DNA cytosine methyltransferase (Figure 7.4L). The remaining *rmtC*-containing contig (14.3%) only had *rmtC* associated with IS*Ecp1* and IS*3000* as it was identical to 43,269-47,887 bp (Figure 7.4M). All isolates belonged to *K. pneumoniae* clonal complex 11, which included six *K. pneumoniae* ST11 isolates and one *K. pneumoniae* ST1680 isolate, but were found in the East of England, London, the North East of England, Scotland and the West Midlands (Table 7.6).

As no chromosome- or plasmid-specific genes were identified on the *rmtC*-containing contigs it is unknown if the *rmtC* genes were found on chromosomes or plasmids. However, one bacterial isolate had *rmtC* on a 793 kb contig, where 58,952-108,629 bp shared identity to *S. enterica* subsp. *enterica* serovar Stanley strain LS001 plasmid pHS36-NDM (accession number: KU726616.1) from 29,688-81,621 bp, which encoded *rmtC*. This region was flanked upstream by DNA that shared identity to *E. coli* plasmid pCMY-42 (accession number: KY463221.1) from 1-41,059 bp and downstream of *rmtC* the DNA shared identity to *E. coli*

strain Ecol_881 (accession number: CP019029.1) from 273,868-917,595 bp. Therefore, this *rmtC* gene may be located on a plasmid but was joined to chromosomal DNA during contig assembly, or the plasmid had been inserted into the chromosome. Twelve *rmtC* genes were later confirmed to be present on plasmids (see Section 7.3.2).

7.3.1.1.4 *rmtF*

Analysis of single *rmtF* contigs identified that most *rmtF* genes (88.5%, 100/113) were associated with IS*91* (Figures 7.5 and 7.6), where 49.0% (49/100) *rmtF* genes were found alone with IS*91* on contigs (Figure 7.5G). In the remaining isolates IS*91* was identified on separate contigs so the association of *rmtF* with IS*91* could not be confirmed.

Nineteen (16.8%) *rmtF*-containing contigs shared an identical genetic environment to *K. pneumoniae* strain AR_0138 plasmid tig00000001 (accession number: CP021758.1) from 17,147-21,490 bp, where *rmtF* was associated with IS*91*, two hypothetical proteins and *catB* (Figure 7.5E). This genetic environment was mainly found in *K. pneumoniae* (94.7%, 18/19) belonging to a range of STs and regions (Table 7.7). Seven other forms of this genetic environment were identified in 69.0% (78/113) *rmtF*-containing contigs (Figure 7.5A-D and F-H), where *rmtF* was either found alone on a contig (15.4%, 12/78) or associated with IS*91* (83.3%, 65/78), *aac(6')-Ib-cr* (6.4%, 5/78), *catB* (20.5%, 16/78), hypothetical proteins (20.5%, 16/78), a nucleoside triphosphate (NTP)-binding protein (3.8%, 3/78) or IS*6* (1.3%, 1/78).

Additionally, in 11.5% (13/113) *rmtF*-containing contigs *rmtF* genes were located in an identical genetic environment to *K. pneumoniae* strain AR_0075 plasmid unnamed3 (accession number: CP032188.1) from 19,671-27,905 bp, where *rmtF* was associated with IS*91*, *arr-2*, *aac(6')-Ib-cr*, a hypothetical protein, a NTP-binding protein, *ltrA* (which encodes a group II intron reverse transcriptase/maturase that is involved in intron mobility as well as RNA splicing [497]), a GCN5-related N-acetyltransferases (GNAT) family N-acetyltransferase (which is involved in the acetylation of amino groups [498]) and a glutathione S-transferase family protein (which is involved in cellular detoxification [499]) (Figure 7.6B). This genetic environment was mainly found in *K. pneumoniae* ST231 (76.9%, 10/13) and London (46.2%,

6/13) (Table 7.7). Three other forms of this genetic environment were identified in 2.7% (3/113) *rmtF*-containing contigs, where *arr-2* and *ltrA* were missing in 66.6% (2/3) isolates (Figure 7.6C-D); the NTP-binding protein was missing in 33.3% (1/3) *rmtF*-containing contigs (Figure 7.6D) and Tn*3*, *tnpA* and *intI1* was present in 33.3% (1/3) *rmtF*-containing contigs (Figure 7.6A).

In all *rmtF*-containing contigs sharing the same genetic environment as *K. pneumoniae* strain AR_0075 plasmid unnamed3 (accession number: CP032188.1), the *rmtF* gene was inserted between two truncated *rmtF* genes where one comprised of the 5' end of the *rmtF* gene and the other comprised of the 3' end of the *rmtF* gene. *rmtF* may have inserted itself into a previous copy of *rmtF*, therefore generating the *rmtF* gene with two adjacent truncated copies.

As no chromosome- or plasmid-specific genes were identified on the *rmtF*-containing contigs it is unknown if the *rmtF* genes were found on chromosomes or plasmids. However, 20 *rmtF* genes were later confirmed to be present on plasmids (see Section 7.3.2).

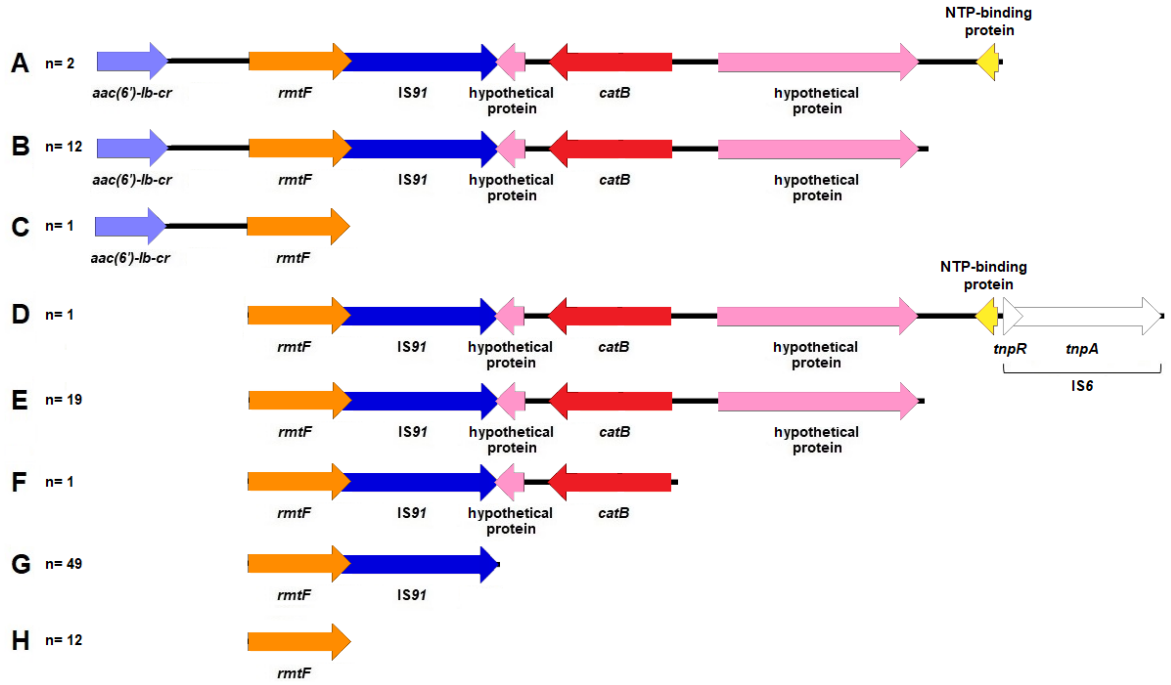


Figure 7.5: Genetic environments of *rmtF* identified on single contigs in Enterobacterales that shared identity with *K. pneumoniae* strain AR_0138 plasmid tig00000001 (accession number: CP021758.1). A: 16,182-21,909 bp; B: 16,182-21,490 bp; C: 16,182-17,926 bp; D: 17,147-22,761 bp; E: 17,147-21,490 bp; F: 17,147-20,207 bp; G: 17,147-19,315 bp and H: 17,147-17,926 bp. Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side.

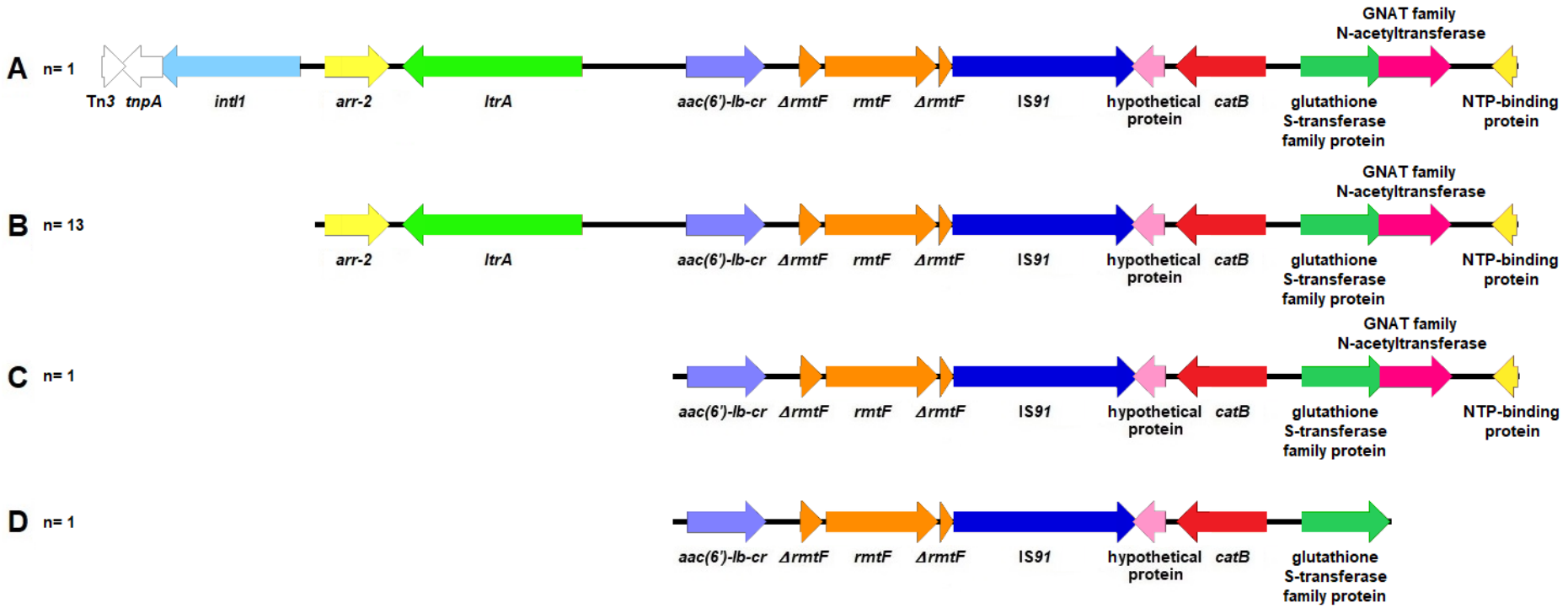


Figure 7.6: Genetic environments of *rmtF* identified on single contigs in Enterobacterales that shared identity with *K. pneumoniae* strain AR_0075 plasmid unnamed3 (accession number: CP032188.1). A: 18,149-27,905 bp; B: 19,671-27,905 bp; C: 22,178-27,905 bp and D: 22,178-27,055 bp. Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side.

Table 7.7: Genetic environments of *rmtF*-containing contigs identified in Enterobacterales with their associated species, STs and geographical distribution.

***rmtF* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
aac(6')-Ib-cr-rmtF-IS*91*-hypothetical protein-*catB*-hypothetical protein-NTP-binding protein	CP021758.1 (16,182-21,909)	2	*K. pneumoniae* (2)	147 (2)	East Midlands (1) and London (1)
aac(6')-Ib-cr-rmtF-IS*91*-hypothetical protein-*catB*-hypothetical protein	CP021758.1 (16,182-21,490)	12	*K. pneumoniae* (11)	101 (3), 147 (3), 231 (2), 16 (1), 336 (1) and 437 (1)	London (3), North West (2), West Midlands (2), East (1), East Midlands (1), North East (1) and South West (1)
			E. coli (1)	4108 (1)	London (1)
aac(6')-Ib-cr-rmtF	CP021758.1 (16,182-17,926)	1	*K. pneumoniae* (1)	231 (1)	London (1)
rmtF-IS*91*-hypothetical protein-*catB*-hypothetical protein-NTP-binding protein-resolvase-IS*6* family transposase	CP021758.1 (17,147-22,761)	1	*K. pneumoniae* (1)	231 (1)	London (1)
rmtF-IS*91*-hypothetical protein-*catB*-hypothetical protein	CP021758.1 (17,147-21,490)	19	*K. pneumoniae* (18)	231 (8), 395 (3), 336 (2), 437 (2), 11 (1), 15 (1) and 147 (1)	London (13), West Midlands (2), North East (1), North West (1) and South West (1)
			E. coli (1)	1702 (1)	West Midlands (1)
rmtF-IS*91*-hypothetical protein-*catB*	CP021758.1 (17,147-20,207)	1	*K. pneumoniae* (1)	147 (1)	Yorkshire and the Humber (1)
rmtF-IS*91*	CP021758.1 (17,147-19,315)	49	*K. pneumoniae* (49)	231 (17), 11 (9), 147 (7), 336 (6), 16 (4),437 (4), 383 (1) and 395 (1)	London (30), North West (8), Yorkshire and the Humber (3), East Midlands (2), West Midlands (2), East (1), North East (1), South East (1) and South West (1)

Table 7.7 continued

***rmtF* genetic environment**	**Accession number(s) (range, bp)**	**Number of isolates**	**Species (no. of isolates)**	**STs (no. of isolates)**	**Geographical distribution (no. of isolates)**
rmtF	CP021758.1 (17,147-17,926)	12	*K. pneumoniae* (12)	231 (9), 147 (2) and unknown (1)	London (7), West Midlands (2), North West (1), South East (1) and Yorkshire and the Humber (1)
Tn*3*-*tnpA*-*intI*-*arr-2*-*ltrA*-*aac(6')-Ib-cr*-Δ*rmtF*-*rmtF*-Δ*rmtF*-IS*91*-hypothetical protein-*catB*-glutathione S-transferase family protein-GNAT family N-acetyltransferase-NTP-binding protein	CP032188.1 (18,149-27,905)	1	*K. pneumoniae* (1)	231 (1)	London (1)
arr-2-*ltrA*-*aac(6')-Ib-cr*-Δ*rmtF*-*rmtF*-Δ*rmtF*-IS*91*-hypothetical protein-*catB*-glutathione S-transferase family protein-GNAT family N-acetyltransferase-NTP-binding protein	CP032188.1 (19,671-27,905)	13	*K. pneumoniae* (13)	231 (10), 147 (2) and 336 (1)	London (6), East (1), East Midlands (1), North East (1), North West (1), South West (1), West Midlands (1) and Yorkshire and the Humber (1)
aac(6')-Ib-cr-Δ*rmtF*-*rmtF*-Δ*rmtF*-IS*91*-hypothetical protein-*catB*-glutathione S-transferase family protein-GNAT family N-acetyltransferase-NTP-binding protein	CP032188.1 (22,178-27,905)	1	*K. pneumoniae* (1)	147 (1)	South West (1)
aac(6')-Ib-cr-Δ*rmtF*-*rmtF*-Δ*rmtF*-IS*91*-hypothetical protein-*catB*-glutathione S-transferase family protein	CP032188.1 (22,178-27,055)	1	*K. pneumoniae* (1)	147 (1)	North West (1)

7.3.1.2 *A. baumannii*

7.3.1.2.1 *armA*

All *armA*-positive *A. baumannii* isolates (n=12) had *armA* associated with a truncated Tn*1548*. Ten (83.3%) isolates shared the same genetic environment as the *A. baumannii* strain PKAB07 genome from 2,652,279-2,670,083 bp (accession number: CP006963.1), where *armA* was associated with *mphE* and *msrE* but the class 1 integron was missing due to being replaced by genes encoding proteins involved in cellular functions (Figure 7.7A). The presence of the latter suggests that *armA* was located on the chromosome.

The remaining two (16.7%) isolates shared the same genetic environment as *A. baumannii* strain B11911 plasmid pB11911 from 195,319-198,034 bp (accession number: CP021344.1, Figure 7.7B), where *armA* was only associated with a hypothetical protein and IS*Ec29*. Due to the short length of contigs the association of *armA* with other genes found in Tn*1548*, such as *mphE* and *msrE*, could not be confirmed nor the genomic location of the *armA* genes.

The ten isolates that shared the same genetic environment as *A. baumannii* strain PKAB07 belonged to International Clone II (ST2), where nine isolates were isolated from two laboratories in London and the remaining isolate was from the East. The remaining two isolates that shared the same genetic environment as plasmid pB11911 belonged to ST113 and ST622 and were isolated from two laboratories in London.

7.3.1.2.2 *rmtE3*

Analysis of the genetic environment of the *rmtE3* gene found that it had a similar genetic environment to the *rmtE2* gene found in *E. coli* strain S68 plasmid pS68 (accession number: KU130396.1) from 78,042-81,683 bp (Figure 7.8). However, IS*CR20*, which was upstream of the two tRNA-guanine transglycosidases in pS68, was missing due to fragmentation of the contig, as it was only 4,200 bp in length, and could not be identified elsewhere in the genome. Also, there was a Tn*3* transposase instead of IS*26* downstream of the IS*Vs1*-like element.

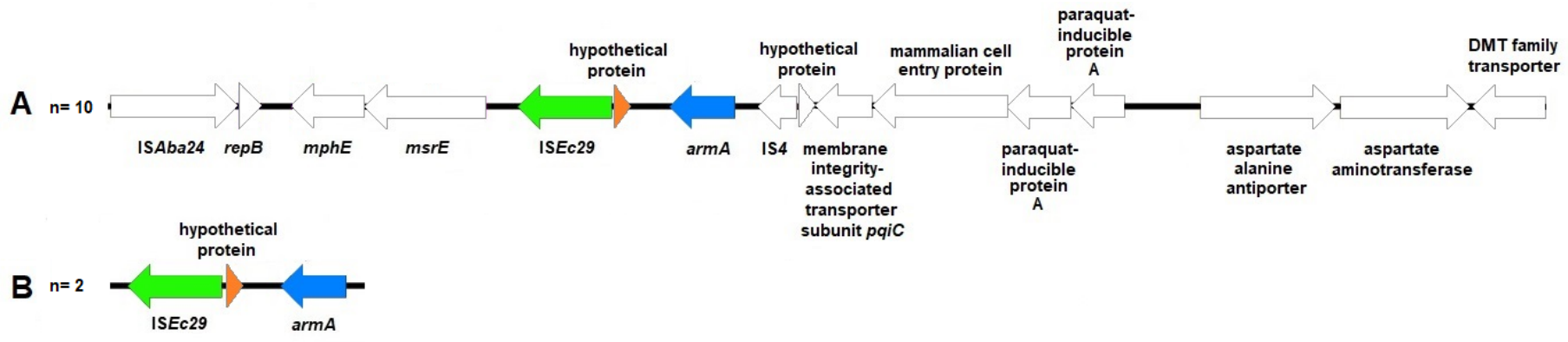


Figure 7.7: Genetic environments of *armA* on single contigs in *A. baumannii*. A: *A. baumannii* strain PKAB07 genome from 2,652,279-2,670,083 bp (accession number: CP006963.1) and B: *A. baumannii* strain B11911 plasmid pB11911 from 195,319-198,034 bp (accession number: CP021344.1). Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side.

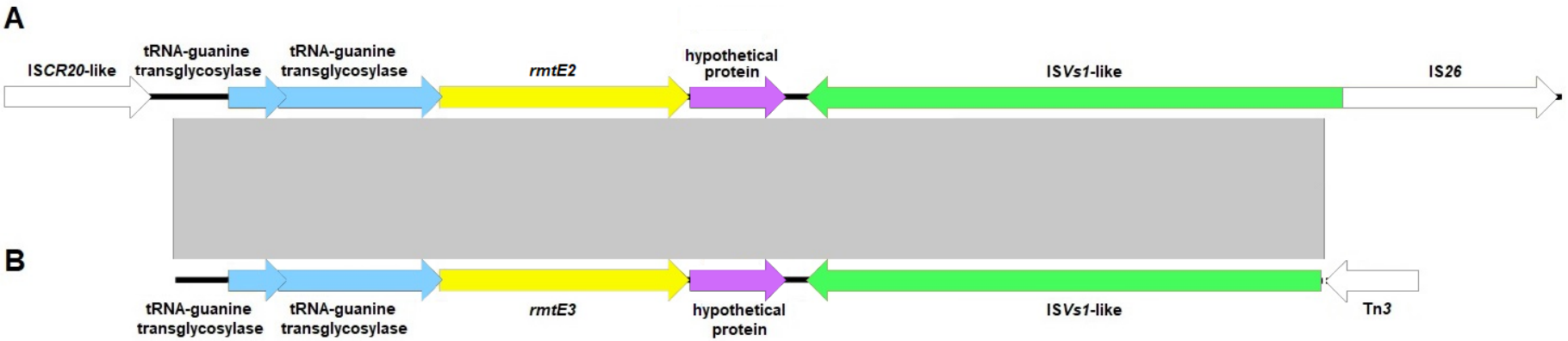


Figure 7.8: Alignment of the genetic environments of *rmtE2* and *rmtE3*. A: *rmtE2*-positive *E. coli* strain S68 plasmid pS68 from 76,414-82,388 bp (accession number: KU130396.1); B: *rmtE3*-positive *A. baumannii* ST79. The grey shading indicates 99.9% sequence identity.

The genetic location of *rmtE3* is unknown as repeated attempts at electroporation in *E. coli* TOP10 failed and the contig contained no chromosomal or plasmid-specific genes.

7.3.1.3 *P. aeruginosa*

7.3.1.3.1 *rmtB1* and *rmtB4*

Analysis of the single *rmtB1* contig identified *rmtB1* had a novel genetic environment as it was associated with *floR2*, *sul1* and *tet*(G) as well as IS6*100* and IS*91* (Figure 7.9A). This genetic environment was deposited in Genbank under the accession number MT019964. The *rmtB1* gene was found on a 334,872 bp contig and appeared to be present on a plasmid as 1-320,547 bp shared 99.9% identity to *Pseudomonas koreensis* strain P19E3 plasmid p1 (accession number: CP027478.1) from 76,175-378,721 bp and 320,548-325,206 bp shared 100% identity to *P. aeruginosa* strain AR441 plasmid unnamed3 (accession number: CP029094.1) from 253,265-257,923 bp. The region 325,291-334,872 bp encoded the genetic environment surrounding *rmtB1* (accession number: MT019964).

Analysis of single contigs harbouring *rmtB4* identified that the genetic environment in all *rmtB4*-positive *P. aeruginosa* isolates (n=11) was identical to *P. aeruginosa* strain ST773 (accession number: CP041945.1) from 2,438,442-2,446,741 bp (Figure 7.9B), where *rmtB4* was associated with two IS*91* insertion sequences. However, both IS*91* insertion sequences were truncated in one ST654 isolate and single isolates belonging to STs 233, 316, 357 and 773 had truncated IS*91* insertion sequences downstream of *groEL* due to fragmentation of the contigs. Five isolates appeared to have *rmtB4* on chromosomes. Four (80.0%) isolates shared identity to *P. aeruginosa* strain 1334/14 (accession number: CP035739.1), where the *rmtB4*-encoding region in one ST733 isolate was flanked by regions identical to 1,273,289-1,278,093 bp and 1,294,052-1,294,147 bp and downstream of the *rmtB4*-encoding region, single ST316, ST357 and ST773 isolates were identical to 1,285,062-1,288,975 bp, 1,284,586-1,288,962 bp and 1,294,109-1,296,467 bp, respectively. One ST233 isolate shared identity to *P. aeruginosa* strain PASGNDM699 (accession number: CP020704.1) downstream of the *rmtB4* encoding region from 3,422,807-3,424,930 bp.

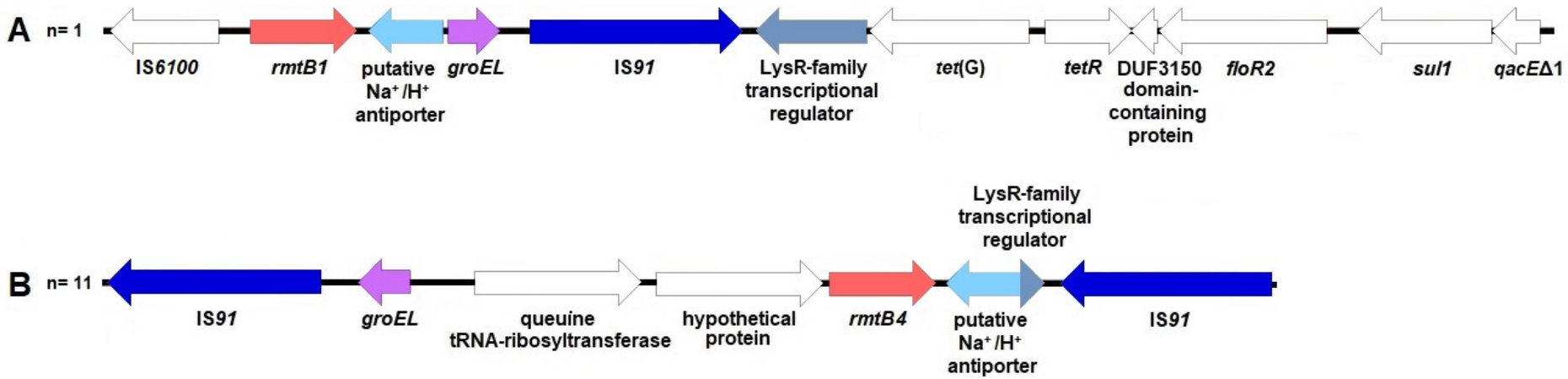


Figure 7.9: Genetic environments of *rmtB1* and *rmtB4* in *P. aeruginosa*. A: Genetic environment surrounding *rmtB1* from this study (accession number: MT019964) and B: *P. aeruginosa* strain ST773 from 2,438,442-2,446,741 bp (accession number: CP041945.1). Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side.

However, the genetic location of *rmtB4* was unknown for six isolates due to no chromosomal or plasmid DNA being found on the contigs. This included *P. aeruginosa* ST316 (n=2) and ST733 (n=1) isolates from London, two ST654 isolates from London and the South West of England and one ST357 isolate from the East of England.

7.3.1.3.2 *rmtC*

In the single *rmtC*-positive *P. aeruginosa* ST357 isolate, *rmtC* was found associated with the insertion sequence IS*91* and endonuclease III (Figure 7.10). This genetic environment appeared to be novel as there were no published DNA sequences in GenBank that matched the length of the contig (3,373 bp). Therefore, this genetic environment was deposited in Genbank under the accession number MN991292. It was not possible to determine whether *rmtC* was located on a plasmid or the chromosome due to its presence on such a short contig.

7.3.1.3.3 *rmtD1 and rmtD3*

The single *rmtD1*-positive *P. aeruginosa* isolate, belonging to ST277, had *rmtD1* on a 5,826 bp contig, which shared 100% identity to *P. aeruginosa* strain PA0905 (accession number: DQ914960.2), from 6,698-11,099 bp (Figure 7.11A). This encoded IS*CR14*, *rmtD1*, a putative tRNA ribosyltransferase and *groEL*. Due to the short length of the contig the genetic location of *rmtD1* could not be determined.

Analysis of the single contigs harbouring *rmtD3* in *P. aeruginosa* isolates belonging to ST357 (50.0%, 2/4), ST308 (25.0%, 1/4) and ST654 (25.0%, 1/4) identified that *rmtD3* was located in an identical genetic environment to *P. aeruginosa* strain MyNCGM481 (accession number: LC229801.1) from 1-8,347 bp. *rmtD3* was found associated with two IS*91* insertion sequences but no other antibiotic resistance genes were located on the contigs (Figure 7.11B). Single ST654 and ST308 isolates appeared to have truncated IS*91* insertion sequences both upstream and downstream of *rmtD3*. The two *P. aeruginosa* ST357 isolates, which were both isolated from the same laboratory in the North West of England over a three-year period, had *rmtD3* present on the chromosome. *rmtD3* was found on contigs that were ~14,000 bp long,

where DNA upstream and downstream of the genetic environment of *rmtD3* shared ~99.0% identity to the *P. aeruginosa* strain PA83 chromosome (accession number: CP017293.1) from ~2,801,000-2,808,100 bp and ~6,223,500-6,227,700 bp, respectively. However, the genetic location of *rmtD3* for the *P. aeruginosa* ST308 isolate from London and the *P. aeruginosa* ST654 isolate from Yorkshire and the Humber could not be determined due to DNA encoding the genetic environment of *P. aeruginosa* strain MyNCGM481 only being found on the contigs.

7.3.1.3.4 *rmtF2*

Two (66.7%) of the three *rmtF2*-positive *P. aeruginosa* isolates, which belonged to ST316 and ST664 and were isolated from the North East and the South East of England, respectively, shared an identical genetic environment with *P. aeruginosa* strain IOMTU487 (accession number: LC224309.1) from 1-11,556 bp (Figure 7.12A). *rmtF2* was associated with Tn*1721* as well as the antibiotic resistance genes bla_{OXA-10}, *aac(6')-Ib-cr* and bla_{PAC-1}. Both isolates had *rmtF2* present on the chromosome as the ST664 isolate had *rmtF2* on a 38,561 bp contig, where 1-26,890 bp of the contig shared 99.2% identity to 5,979,815-6,006,711 bp of the *P. aeruginosa* strain S04 90 chromosome (accession number: CP011369.1) and the ST316 isolate had *rmtF2* on a 109,206 bp contig, where 11,940-97,569 bp of the contig matched to 331,493-417,122 bp of the *P. aeruginosa* strain Ocean-1155 chromosome (accession number: CP022526.1).

The other *rmtF2*-positive *P. aeruginosa* isolate, which belonged to ST654 and was isolated from London, had a novel genetic environment (Figure 7.12B), where *rmtF2* was associated with IS*91* and Tn*3* as well as the antibiotic resistance genes *aac(6')-Ib-cr*, the ESBL gene bla_{GES-9} and *catB7*. This genetic environment was deposited in Genbank under the accession number MN991291. *rmtF2* was present on the chromosome as it was found on a 62,396 bp contig, where 12,265-60,409 bp matched to 3,391,991-3,440,027 bp of the chromosome from *P. aeruginosa* strain H26027 (accession number: CP033684.1) and 60,409-62,396 bp of the contig matched to 3,442,900-3,444,887 bp of the chromosome from *P. aeruginosa* strain H26027 (accession number: CP033684.1).

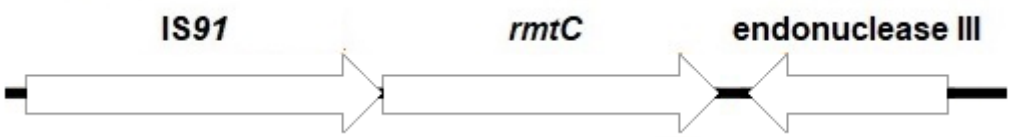


Figure 7.10: Genetic environment of *rmtC* in the *P. aeruginosa* ST357 isolate from this study (accession number: MN991292).

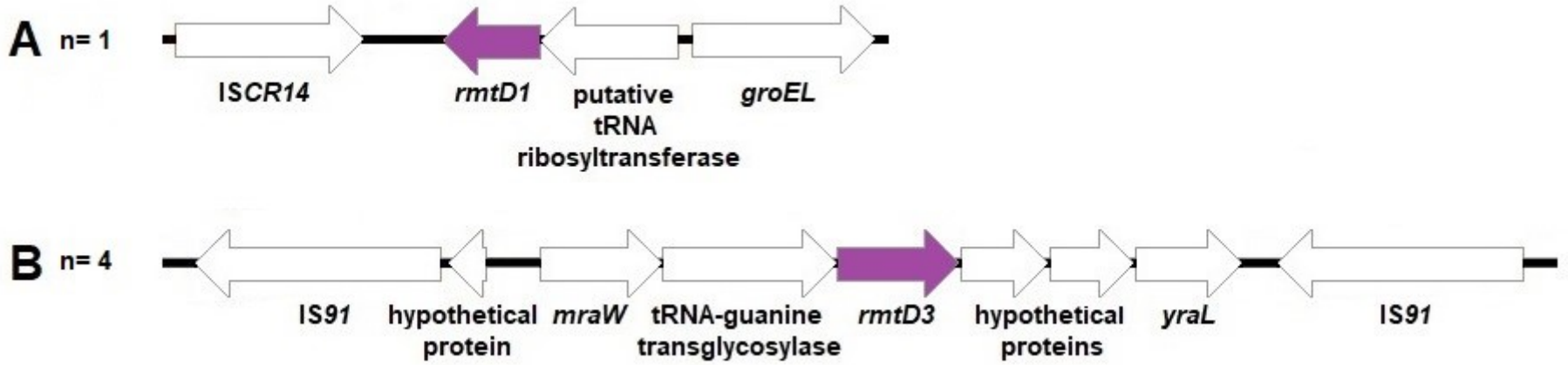


Figure 7.11: Genetic environments of *rmtD1* and *rmtD3* identified on a single contig in *P. aeruginosa*. A: *P. aeruginosa* strain PA0905 from 6,698-11,099 bp (accession number: DQ914960.2) and B: *P. aeruginosa* strain MyNCGM481 from 1-8,347 bp (accession number: LC229801.1). Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side.

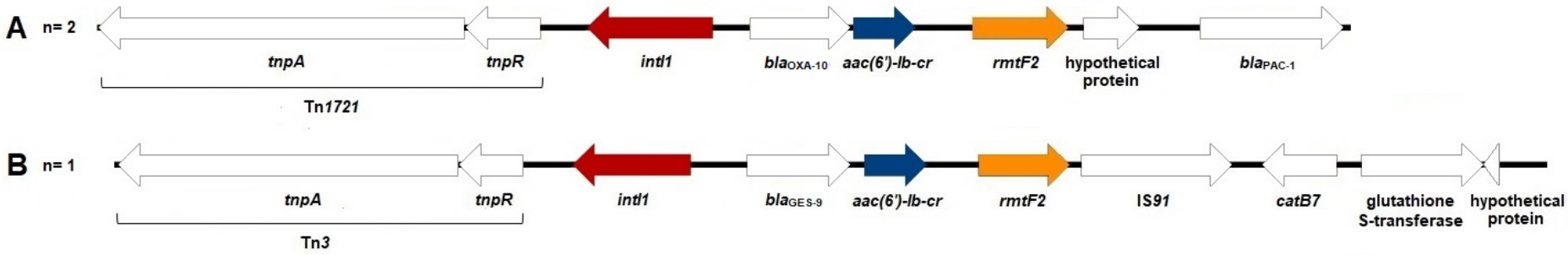


Figure 7.12: Genetic environments of *rmtF2* identified on a single contig in *P. aeruginosa*. A: *P. aeruginosa* strain IOMTU487 from 1-11,556 bp (accession number: LC224309.1) and B: novel *rmtF2* genetic environment in *P. aeruginosa* ST654 from this study (accession number: MN991291). Genes that are not shared between genetic environments are coloured white. The number of isolates can be found on the left-hand side.

7.3.2 Analysis of plasmids harbouring 16S RMTase genes

7.3.2.1 Isolation of plasmids from 16S RMTase-producing bacteria

Plasmid transformants harbouring 16S RMTase genes were generated for 40.0% (22/55) of isolates from the AMRHAI Reference Unit and 49.4% (39/79) isolates from the prospective surveillance study (Tables 7.8-7.11). This included one *rmtB* + *rmtF*-positive *E. coli* ST4108 isolate where two transformants were obtained: one positive for *rmtB* and the other for *rmtB* + *rmtF*. Herein, these plasmids (*rmtB* and *rmtB* + *rmtF*) will be analysed separately. Transformants could not be produced for the remaining plasmids (54.5%, 73/134).

PCR analysis and WGS of the transformants identified that 33.9% (21/62) plasmids contained *rmtB* (Table 7.9), 30.6% (19/62) harboured *rmtF* (Table 7.11), 19.4% (12/62) harboured *rmtC* (Table 7.10), 14.5% (9/62) harboured *armA* (Table 7.8) and 1.6% (1/62) harboured *rmtB* + *rmtF* (Table 7.11).

A total of 87.5% (21/24) *rmtB*, 85.7% (12/14) *rmtC*, 82.6% (19/23) *rmtF*, 50.0% (1/2) *rmtB* + *rmtF* and 13.6% (9/66) *armA*-positive donor isolates had plasmids harbouring 16S RMTase genes successfully transferred in an *E. coli* host.

7.3.2.2 Identification of plasmid replicon types

Plasmid replicon typing identified 11 replicon types (Tables 7.8-7.11) where IncFII (48.4%, 30/62) was the most common, followed by IncFIB (16.1%, 10/62), IncFIA (12.9%, 8/62), IncA/C (9.7%, 6/62), IncR (6.5%, 4/62), IncN (3.2%, 2/62) and single plasmids had the replicon types IncHI1A, IncHI1B, IncI, IncL/M and IncX3. Ten plasmids were untypeable.

Single plasmid replicons were identified in 30 (48.4%) plasmids, whereas 14 (22.6%) had multiple plasmid replicons with the combination IncFIB + IncFII (35.7%, 5/14) the most common, followed by IncFIA + IncFIB + IncFII (21.4%, 3/14) and IncFII + IncR (14.3%, 2/14); single plasmids had the combinations IncA/C + IncR, IncI + IncFIA, IncFIA + IncFIB and IncHI1A + IncHI1B. IncN plasmids (n=2) were only associated with *armA* genes, whereas no

other replicon types were found to be specific to certain 16S RMTase genes, host species or STs.

Table 7.8: Species, STs, geographical distribution, predicted plasmid size (kb), replicon types, TA system genes and antibiotic resistance genes, associated with *armA*-containing plasmids.

Number of isolates	Donor species	Donor ST	Geographical distribution (no. of laboratories)	Predicted plasmid size (kb)	Replicon type(s)	TA system genes	Plasmid-associated resistance genes
2	*E. cloacae* complex	265 (2)	Scotland (2)	26.7 and 30.9	Unknown	-	*armA*, *aadA2*, *bla*$_{TEM-1}$, *mphE*, *msrE*, *sul1* and *dfrA12*
1	*E. coli*	405	London (1)	126.4	HI1A and HI1B	-	*armA*, *bla*$_{NDM-1}$, *bla*$_{DHA-1}$, *mphE*, *msrE* and *sul1*
1	*K. oxytoca*	Unknown	London (1)	102.1	N	-	*armA*, *aac(6')-Ib-cr*, *bla*$_{OXA-1}$, *mphE*, *msrE*, *dfrA14* and *catB4*
1	*K. pneumoniae*	14	East (1)	101.4	FII and FIB	-	*armA*, *aac(6')-Ib-cr*, *strA*, *strB*, *mphE*, *msrE*, *sul1* and *sul2*
1	*K. pneumoniae*	15	London (1)	69.3	R	-	*armA*, *aac(3)-IId*, *aac(6')-Ib-cr*, *aadA1*, *aadA2*, *bla*$_{CTX-M-15}$, *bla*$_{OXA-9}$, *bla*$_{SHV-11}$, *mphE*, *msrE*, *sul1* and *dfrA12*
1	*K. pneumoniae*	37	London (1)	64.6	R	-	*armA*, *ant(2'')-Ia*, *aph(3')-Ia*, *bla*$_{DHA-1}$, *mphE*, *msrE* and *sul1*
1	*K. pneumoniae*	307	London (1)	50.1	N	-	*armA*, *aac(6')-Ib-cr*, *aadA16*, *sul1*, *dfrA27* and *arr-3*
1	*K. pneumoniae*	307	London (1)	87.3	L/M	*pemK*/*pemI*	*armA*, *aac(3)-IId*, *bla*$_{NDM-1}$, *bla*$_{DHA-1}$, *bla*$_{TEM-1}$, *mphE*, *msrE* and *sul1*

Table 7.9: Species, STs, geographical distribution, predicted plasmid size (kb), replicon types, TA system genes and antibiotic resistance genes, associated with *rmtB*- and *rmtB* + *rmtF*-containing plasmids.

Number of isolates	Donor species	Donor ST	Geographical distribution (no. of laboratories)	Predicted plasmid size (kb)	Replicon type(s)	TA system genes	Plasmid-associated resistance genes
3	*K. pneumoniae* (2)	138 (2)	Scotland (2)	83.6 and 83.8	FII	*hok*/*sok*	*rmtB*, *bla*$_{NDM-5}$, *bla*$_{TEM-1}$, *ermB* and *mphA*
	E. coli (1)	405	Scotland (1)	80.0			
2	*E. cloacae* complex (1)	78	East Midlands (1)	86.2	FII	*hok*/*sok*	*rmtB*, *aadA2*, *bla*$_{NDM-1}$, *bla*$_{TEM-1}$, *sul1* and *dfrA12*
	E. coli (1)	90	North West (1)	89.3		-	
2	*E. coli*	46 (1) and 410 (1)	London (2)	69.7 and 78.9	FII	*hok*/*sok*	*rmtB*, *aadA2*, *bla*$_{NDM-5}$, *bla*$_{TEM-1}$, *sul1* and *dfrA12*
1	*E. coli*	167	West Midlands (1)	100.4	FII	*hok*/*sok*	*rmtB*, *aadA2*, *bla*$_{NDM-5}$, *bla*$_{TEM-1}$, *ermB*, *mphA*, *sul1* and *dfrA12*
1	*E. coli*	167	London (1)	138.0	FII, FIA and FIB	*hok/sok* and *srnB/srnC*	*rmtB*, *aac(3)-IId*, *aadA5*, *bla*$_{TEM-1}$, *qepA1, mphA*, *sul1* and *dfrA17*
1	*E. coli*	405	East Midlands (1)	83.2	FII	*hok*/*sok*	*rmtB*, *bla*$_{NDM-1}$, *bla*$_{TEM-1}$, *ermB* and *mphA*
1	*E. coli*	405	London (1)	83.9	I and FIA	*ccdB/ccdA* and *pndA/pndB*	*rmtB*, *aadA2*, *bla*$_{NDM-5}$, *bla*$_{CMY-42}$, *bla*$_{TEM-1}$, *mphA*, *sul2* and *dfrA12*
1	*E. coli*	405	North West (1)	121.5	FIA	*ccdB/ccdA* and *srnB/srnC*	*rmtB*, *aac(6')-Ib-cr*, *aadA5*, *strA*, *strB*, *bla*$_{NDM-5}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *mphA*, *sul1*, *sul2*, *drA17*, *catB3* and *tet*(A)
1	*E. coli*	410	East (1)	70.7	FII	*hok*/*sok*	*rmtB*, *aac(3)-IIa*, *aadA2*, *bla*$_{NDM-4}$, *bla*$_{TEM-1}$, *sul1* and *dfrA12*
1	*E. coli*	448	London (1)	24.3	R	*ccdB/ccdA*	*rmtB* and *bla*$_{TEM-1}$
1	*E. coli*	448	London (1)	98.3	FII	*hok*/*sok*	*rmtB*, *aadA2*, *bla*$_{NDM-5}$, *bla*$_{TEM-1}$, *ermB*, *mphA*, *sul1* and *dfrA12*
1	*E. coli*	1702	West Midlands (1)	153.9	FII, FIA and FIB	*ccdB/ccdA* and *srnB/srnC*	*rmtB*, *aadA2*, *bla*$_{NDM-1}$, *bla*$_{TEM-1}$, *ermB*, *mphA*, *sul1*, *dfrA12* and *tet*(B)

Table 7.9 continued

Number of isolates	Donor species	Donor ST	Geographical distribution (no. of laboratories)	Predicted plasmid size (kb)	Replicon type(s)	TA system genes	Plasmid-associated resistance genes
1	*E. coli*	2450	London (1)	69.6	FIA and FIB	*ccdB/ccdA*	*rmtB*, *aac(6')-Ib-cr*, *aadA5*, *bla*$_{NDM-1}$, *bla*$_{TEM-1}$, *bla*$_{OXA-1}$, *sul1*, *catB4* and *tet*(B)
1	*E. coli*	4108	London (1)	95.5	FII	*hok*/*sok*	*rmtB*, *bla*$_{TEM-1}$, *bla*$_{SFO-1}$ and *mphA*
1	*K. pneumoniae*	147	East (1)	176.0	A/C and R	-	*rmtB*, *aadA1*, *aadA2*, *ant(2'')-Ia*, *aph(3')-Ia*, *strA*, *strB*, *bla*$_{OXA-10}$, *bla*$_{TEM-1}$, *bla*$_{VEB-1}$, *sul1*, *sul2*, *dfrA12*, *cmlA1*, *arr-2*, *tet*(A) and *tet*(G),
1	*K. pneumoniae*	1248	London (1)	82.6	FII	*hok*/*sok*	*rmtB*, *bla*$_{TEM-1}$, *ermB* and *mphA*
1	*K. pneumoniae*	1587	Yorkshire and the Humber (1)	146.8	FII, FIA and FIB	*hok/sok* and *srnB/srnC*	*rmtB*, *aadA2*, *bla*$_{NDM-1}$, *bla*$_{OXA-232}$, *bla*$_{TEM-1}$, *qepA4*, *ermB*, *mphA*, *sul1*, *dfrA12* and *tet*(B)
1	*E. coli*	4108	London (1)	136.6	FII and R	*hok*/*sok*	*rmtB* + *rmtF*, *aac(6')-Ib-cr*, *bla*$_{TEM-1}$, *bla*$_{SFO-1}$, *mphA* and *arr-2*

Table 7.10: Species, STs, geographical distribution, predicted plasmid size (kb), replicon types, TA system genes and antibiotic resistance genes, associated with *rmtC*-containing plasmids.

Number of isolates	Donor species	Donor ST	Geographical distribution (no. of laboratories)	Predicted plasmid size (kb)	Replicon type(s)	TA system genes	Plasmid-associated resistance genes
7	*K. pneumoniae* (4)	147 (1)	South West (1)	110.8	FIB	*ccdB*/*ccdA*	*rmtC*, bla_{NDM-1} and *sul1*
		29 (1), 89 (1) and 307 (1)	London (3)	106.4	FII		
	E. cloacae complex (2)	78	East Midlands (1)	99.4			
		275	London (1)	106.5			
	C. freundii (1)	22	East (1)	105.8			
3	*E. coli* (2)	131	Yorkshire and the Humber (1)	138	A/C	-	*rmtC*, *aac(6')-Ib-cr*, bla_{NDM-1}, bla_{CMY-6}, and *sul1*
		167	North West (1)	136.7			
	C. freundii (1)	18	London (1)	138			
1	*C. freundii*	18	London (1)	68.5	A/C	-	*rmtC* and bla_{NDM-1}
1	*K. pneumoniae*	11	London (1)	62.9	A/C	-	*rmtC*, *aac(6')-Ib-cr*, bla_{NDM-1}, bla_{DHA-1}, and *sul1*

Table 7.11: Species, STs, geographical distribution, predicted plasmid size (kb), replicon types, TA system genes and antibiotic resistance genes, associated with *rmtF*-containing plasmids.

Number of isolates	Donor species	Donor ST	Geographical distribution (no. of laboratories)	Predicted plasmid size (kb)	Replicon type(s)	TA system genes	Plasmid-associated resistance genes
8	*K. pneumoniae*	336 (2)	London (2)	20.3	Unknown	-	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
		11	North West (1)	24.6	FII and R	-	
		16	North West (1)	53.7	X3	-	
		147	East Midlands (1)	21.4	Unknown	-	
		231	East Midlands (1)	19.7	Unknown	-	
		231	London (1)	23.9	Unknown	-	
		231	London (1)	116.8	FIB and FII	*hok*/*sok*	
2	*K. pneumoniae*	147 (2)	London (1)	92.3	Unknown	*ccdB*/*ccdA*	*rmtF*, *aac(6')-Ib-cr*, *qnrB1*, *dfrA14* and *arr-2*
			Yorkshire and the Humber (1)	132.9	R		
1	*K. pneumoniae*	11	London (1)	59.6	FII	*pemK*/*pemI*	*rmtF*, *aac(6')-Ib-cr*, *aadA2*, *aph(3')-Ia*, bla_{NDM-1}, *qnrB1*, *mphA*, *sul1*, *dfrA12* and *arr-2*,
1	*K. pneumoniae*	231	London (1)	22.3	Unknown	-	*rmtF*, *aac(6')-Ib-cr* and bla_{SHV-12}
1	*K. pneumoniae*	231	North West (1)	28.7	Unknown	-	*rmtF*, *aac(6')-Ib-cr*, $bla_{CTX-M-15}$, bla_{TEM-1} and *arr-2*
1	*K. pneumoniae*	231	London (1)	35.4	Unknown	-	*rmtF*, *aac(6')-Ib-cr*, $bla_{CTX-M-15}$, bla_{TEM-1}, *qnrS1* and *arr-2*
1	*K. pneumoniae*	231	London (1)	118.4	FIB and FII	*hok*/*sok*	*rmtF*, *aac(6')-Ib-cr*, bla_{TEM-1}, *qnrS1*, *catA1* and *arr-2*
1	*K. pneumoniae*	231	London (1)	117.5	FIB and FII	*hok*/*sok*	*rmtF*, *aac(6')-Ib-cr*, bla_{SHV-12}, *catA1* and *arr-2*
1	*K. pneumoniae*	231	West Midlands (1)	144.1	FIB and FII	*hok*/*sok*	*rmtF*, *aac(6')-Ib-cr*, bla_{NDM-1}, bla_{TEM-1}, *qnrB1* and *arr-2*
1	*K. pneumoniae*	395	East (1)	82.7	FIA	-	*rmtF*, *aac(6')-Ib-cr*, *strA*, *strB*, $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1}, *qnrB1*, *sul1*, *sul2*, *dfrA1*, *catB3*, *arr-2* and *tet*(A)

Table 7.11 continued

Number of isolates	Donor species	Donor ST	Geographical distribution (no. of laboratories)	Predicted plasmid size (kb)	Replicon type(s)	TA system genes	Plasmid-associated resistance genes
1	*K. pneumoniae*	437	London (1)	75.2	FIA	*ccdB/ccdA*	*rmtF*, *aac(6')-Ib-cr*, *aadA1*, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-9}}$, *bla*$_{\text{TEM-1}}$, *dfrA14* and *arr-2*

7.3.2.3 Identification of TA system genes

Genes encoding TA systems were identified in 58.1% (36/62) plasmids, which included 90.5% (19/21), 58.3% (7/12), 42.1% (8/19) and 11.1% (1/9) plasmids harbouring *rmtB*, *rmtC*, *rmtF* and *armA*, respectively, as well as the single *rmtB* + *rmtF* plasmid (Tables 7.8-7.11). The most common TA system genes were *hok*/*sok* (52.8%, 19/36), followed by *ccdB*/*ccdA* (38.9%, 4/36), *srnB*/*srnC* (11.1%, 4/36), *pemK*/*pemI* (5.6%, 2/36) and *pndA*/*pndB* (2.8%, 1/36). Plasmids encoding *rmtC* only harboured *ccdB*/*ccdA* genes (Table 7.10). Five plasmids encoding *rmtB* had two sets of TA system genes, where *ccdB*/*ccdA* + *srnB*/*srnC* (40%, 2/5) and *hok*/*sok* + *srnB*/*srnC* (40%, 2/5) were the most common (Table 7.9). TA system genes were mainly found in IncFII plasmids (90.0%, 27/30), where *hok*/*sok* (70.4%, 19/27) was the most common. TA system genes were not identified in plasmids with IncA/C, IncHI1A, IncHI1B, IncN and IncX3 replicons.

7.3.2.4 Identification of antibiotic resistance genes on 16S RMTase plasmids

In total, 51.6% (32/62) plasmids encoding 16S RMTase genes harboured bla_{NDM} genes (Tables 7.8-7.11); these were obtained from 84.2% (32/38) bla_{NDM}-producing donor bacterial isolates. This included one isolate that harboured both bla_{NDM} + $bla_{OXA-232}$, which was obtained from 25.0% (1/4) bla_{NDM} + $bla_{OXA-232}$-producing donor bacterial isolates. bla_{NDM} was identified on all plasmids harbouring *rmtC* (100%, 12/12), 76.2% (16/21) plasmids harbouring *rmtB*, 22.2% (2/9) plasmids harbouring *armA* and 10.5% (2/19) harbouring *rmtF*, but was not identified on the *rmtB* + *rmtF* plasmid. bla_{NDM-1} was identified in all *armA*, *rmtC* and *rmtF* plasmids but *rmtB*-containing plasmids were more frequently associated with bla_{NDM-5} (75.0%, 12/16), than bla_{NDM-1} (18.8%, 3/16) and bla_{NDM-4} (6.3%, 1/16). bla_{NDM} was found on plasmids predicted as ranging in size from 59.6-153.9 kb and was mainly found in plasmids belonging to IncF (78.1%, 25/32), followed by IncA/C (15.6%, 5/32), IncL/M (3.1%, 1/32) and IncHI1A + IncHI1B (3.1%, 1/32) replicon types. All IncA/C plasmids harboured *rmtC*. Plasmids harbouring bla_{NDM} were mainly identified in isolates originating from London (46.9%, 15/32), the East Midlands (9.4%, 3/32), the North West of England (9.4%, 3/32), Scotland (9.4%,

3/32), the West Midlands (9.4%, 3/32), the East of England (6.3%, 2/32), Yorkshire and the Humber (6.3%, 2/32) and the South West of England (3.1%, 1/32). No other carbapenemase gene was identified in the plasmids.

The gene *aac(6')-Ib-cr* was identified in 48.4% (30/62) plasmids (Tables 7.8-7.11) meaning plasmids harbouring *aac(6')-Ib-cr* were obtained from 68.1% (32/47) *aac(6')-Ib-cr*-producing donor bacterial isolates. *aac(6')-Ib-cr* was identified in all *rmtF*-positive plasmids (100%, 19/19), followed by *armA* (44.4%, 4/9), *rmtC* (33.3%, 4/12), *rmtB* (9.5%, 2/21) and was also found in the *rmtB* + *rmtF* plasmid. The gene was found on plasmids predicted to range from 19.7-144.1 kb in size belonging to a range of replicon types (A/C, FIA, FIB, FII, N, R and X). Plasmids harbouring *aac(6')-Ib-cr* were mainly identified in isolates originating from London (60.0%, 18/30), followed by the North West of England (16.7%, 5/30), the East of England (6.7%, 2/30), the East Midlands (6.7%, 2/30), Yorkshire and the Humber (6.7%, 2/30) and the West Midlands (3.3%, 1/30).

bla$_{\text{CTX-M-15}}$ was identified in 9.7% (6/62) plasmids (Tables 7.8-7.11), meaning plasmids harbouring *bla*$_{\text{CTX-M-15}}$ were obtained from 13.3% (6/45) *bla*$_{\text{CTX-M-15}}$-producing donor bacterial isolates. *bla*$_{\text{CTX-M-15}}$ was mainly identified in plasmids harbouring *rmtF* (21.1%, 4/19), followed by *armA* (11.1%, 1/9) and *rmtB* (4.8%, 1/21); it was not identified in *rmtC* plasmids or the *rmtB* + *rmtF* plasmid. *bla*$_{\text{CTX-M-15}}$ was mainly found in IncFIA plasmids (50%, 3/6), followed by IncR (16.7%, 1/6) but replicon types were unknown for two plasmids harbouring *rmtF*. *bla*$_{\text{CTX-M-15}}$ was found on plasmids predicted to range from 28.7-121.5 kb in size, which were isolated from London (50.0%, 3/6), the North West (33.3%, 2/6) and the East of England (16.7%, 1/6).

The PMQR gene *qnrB1* was identified in 8.1% (5/62) plasmids, meaning plasmids harbouring *qnrB1* were obtained from 27.8% (5/18) *qnrB1*-producing donor bacterial isolates. *qnrB1* was only identified in *rmtF* plasmids (26.3%, 5/19) (Table 7.11). These plasmids belonged to a range of replicon types (FIA, FIB, FII and R), were predicted to range in size from 59.6-132.9 kb and were isolated from London (40.0%, 2/5), the East of England (20.0%, 1/5), the West Midlands (20.0%, 1/5) and Yorkshire and the Humber (20.0%, 1/5).

Other genes that were found to be closely associated with 16S RMTase genes within the plasmids included *sul1*, *bla*$_{TEM-1}$, *arr-2, mphE* and *msrE* (Tables 7.8-7.11). *sul1* was associated with 58.1% (36/62) plasmids, where it was mainly found in 91.7% (11/12) *rmtC* plasmids, followed by *armA* (88.9%, 8/9), *rmtB* (71.4%, 15/21) and *rmtF* (10.5%, 2/19) plasmids. *bla*$_{TEM-1}$ was identified in 50.0% (31/62) plasmids, where it was associated with *rmtB* plasmids (100%, 21/21), followed by *rmtF* (36.8%, 7/19) and *armA* (33.3%, 3/9) plasmids. *bla*$_{TEM-1}$ was not identified in *rmtC* plasmids. The gene *arr-2* was identified in 29.0% (18/62) plasmids and was found in most *rmtF*-positive plasmids (89.5%, 17/19) as well as the *rmtB* + *rmtF* plasmid. Additionally, the *mphE* and *msrE* genes were found in 88.9% (8/9) *armA*-plasmids.

7.3.2.5 Identification of plasmids

7.3.2.5.1 *armA*

Only one (11.1%) *armA* plasmid was found to share significant sequence identity with a single plasmid published on GenBank (Table 7.12). One *K. pneumoniae* ST307 isolate from London harboured the plasmid unnamed2 (accession number: CP032193.1) with 99.9% (87,332/87,450 bp) sequence identity. This plasmid was found to harbour *bla*$_{NDM-1}$. Six (66.7%) plasmids were comprised of contigs that shared sequence identity with multiple plasmids (see Table 12 in Appendix 1). The other two (22.2%) *armA* plasmids shared <90.0% identity to published plasmid sequences on GenBank (see Table 13 in Appendix 1).

7.3.2.5.2 *rmtB*

Nine (42.9%) *rmtB* plasmids were found to share significant sequence identity to single published plasmids on GenBank (Table 7.13). Five different plasmids were identified including pM105_FII (accession number: AP018136.1), pLZ135-NDM (accession number: MF353156.1), pM217_FII (accession number: AP018147.1), pMC-NDM (accession number: HG003695.1) and unnamed1 (accession number: CP023923.1). The most common plasmid identified was pM105_FII (accession number: AP018136.1), which was found in five isolates (55.6%, 5/9) (Table 7.13). All other plasmids were identified in single isolates.

Table 7.12: Characteristics of *armA*-positive plasmids published in GenBank identified in this study including replicon type, TA system genes, gaps in the plasmid DNA sequence and missing antibiotic resistance genes.

Reference plasmid (accession number)	Number of isolates	Species	Geographical distribution	Percentage match to reference plasmid	Replicon type(s)	TA system genes	Antibiotic resistance genes	Gaps in plasmid (bp)	Missing antibiotic resistance genes
unnamed2 (CP032193.1)	1	*K. pneumoniae* ST307	London	99.9% (87,332/87,450)	L/M	*pemK*/*pemI*	*armA*, *aac(3)-IId*, bla_{NDM-1}, bla_{TEM-1}, *mphE*, *msrE* and *sul1*	14,848-14,966	-

Table 7.13: Characteristics of *rmtB*-positive plasmids published in GenBank identified in this study including replicon type, TA system genes, gaps in the plasmid DNA sequence and missing or inserted antibiotic resistance genes.

Reference plasmid (accession number)	Number of isolates	Species	Geographical distribution	Percentage match to reference plasmid	Replicon type(s)	TA system genes	Antibiotic resistance genes	Gaps in plasmid (bp)	Inserted antibiotic resistance genes
pM105_FII (AP018136.1)	5	*K. pneumoniae* ST138	Scotland	100% (84,133/84,133)	FII	*hok*/*sok*	*rmtB*, bla_{TEM-1}, *ermB* and *mphA*	-	bla_{NDM-5}
		K. pneumoniae ST138	Scotland	100% (84,133/84,133)				-	
		E. coli ST405	Scotland	100% (84,133/84,133)				-	
		E. coli ST405	East Midlands	100% (84,133/84,133)				-	bla_{NDM-5}, *aadA2*, *sul1* and *dfrA12*
		K. pneumoniae ST1248	London	100% (84,133/84,133)				-	-
pMC-NDM (HG003695.1)	1	*E. coli* ST78	East Midlands	100% (87,619/87,619)	FII	*hok*/*sok*	*rmtB*, *aadA2*, bla_{NDM-1}, bla_{TEM-1}, *sul1* and *dfrA12*	-	-
unnamed1 (CP023923.1)	1	*K. pneumoniae* ST1587	Yorkshire and the Humber	99.3% (139,576/140,557)	FIA, FIB and FII	*hok/sok* and *srnB*/*srnC*	*rmtB, aadA2*, bla_{NDM-1}, bla_{TEM-1}, *ermB*, *mphA*, *sul1*, *dfrA12* and *tet*(B)	11,674-12,269 and 50,428-50,814	$bla_{OXA-232}$ and *qepA*
pM217_FII (AP018147.1)	1	*E. coli* ST167	West Midlands	98.4% (100,415/102,071)	FII	*hok/sok*	*rmtB*, *aadA2*, bla_{NDM-5}, bla_{TEM-1}, *ermB*, *mphA*, *sul1* and *dfrA12*	3,532-4,053, 8,408-8,931, 27,803-28,234 and 47,153-47,334	-
pLZ135-NDM (MF353156.1)	1	*E. coli* ST90	North West	98.0% (89,046/90,845)	FII	-	*rmtB*, *aadA2*, bla_{NDM-1}, bla_{TEM-1}, *sul1* and *dfrA12*	34,903-36,702	-

-

Ten (47.6%) plasmids shared sequence identity with multiple plasmids so appeared to be novel as they were comprised of regions belonging to different plasmids (see Table 14 in Appendix 1). Additionally, three (14.3%) plasmids shared <90.0% identity with published plasmid sequences in GenBank (see Table 15 in Appendix 1).

Reference plasmid pM105_FII (accession number: AP018136.1) lacked a *bla*$_{NDM}$ gene, as did the pM105_FII-like plasmid identified in a *K. pneumoniae* ST1248 isolate from London. The remaining four pM105_FII-like plasmids identified were positive for *bla*$_{NDM-5}$ (Figure 7.13). Further analysis identified the presence of a complex class 1 integron present in the *E. coli* ST405 isolate from the East Midlands, which was identical to pNDM-d2e9 (accession number: CP026201.1) from 47,019-58,217 bp and contained *bla*$_{NDM-5}$, *ble*$_{MBL}$, *aadA2*, *dfrA12* and *sul1* (Figure 7.14A). This class 1 integron was bracketed by two IS*26* elements. However, the other *bla*$_{NDM-5}$-positive isolates had a truncated transposon due to the insertion of IS*26* (Figure 7.14B), where they only harboured the genes *bla*$_{NDM-5}$, *ble*$_{MBL}$, *trpF* and a truncated *tat* gene (which encodes a twin-arginine translocation [TAT] pathway signal sequence domain protein involved in the transfer of folded proteins [500]). Furthermore, the *E. coli* ST405 isolate from the East Midlands and the *K. pneumoniae* ST1248 isolate from London shared an additional region with pNDM-d2e9 (74,511-77,362 bp) encoding two single-stranded DNA binding proteins and a group II intron reverse transcriptase/maturase.

Additionally, the *bla*$_{NDM-1}$-positive plasmid unnamed1 (accession number: CP023923.1), found in a *K. pneumoniae* ST1587 isolate from Yorkshire and the Humber, had *qepA* inserted into it as well as a region from *K. pneumoniae* strain FDAARGOS_440 plasmid unnamed3 (accession number: CP023924.1) ranging from 3,120-9,397 bp, which included *bla*$_{OXA-232}$ (Figure 7.15).

All plasmids belonged to IncFII but unnamed1 (accession number: CP023923.1) also belonged to IncFIA and IncFIB.

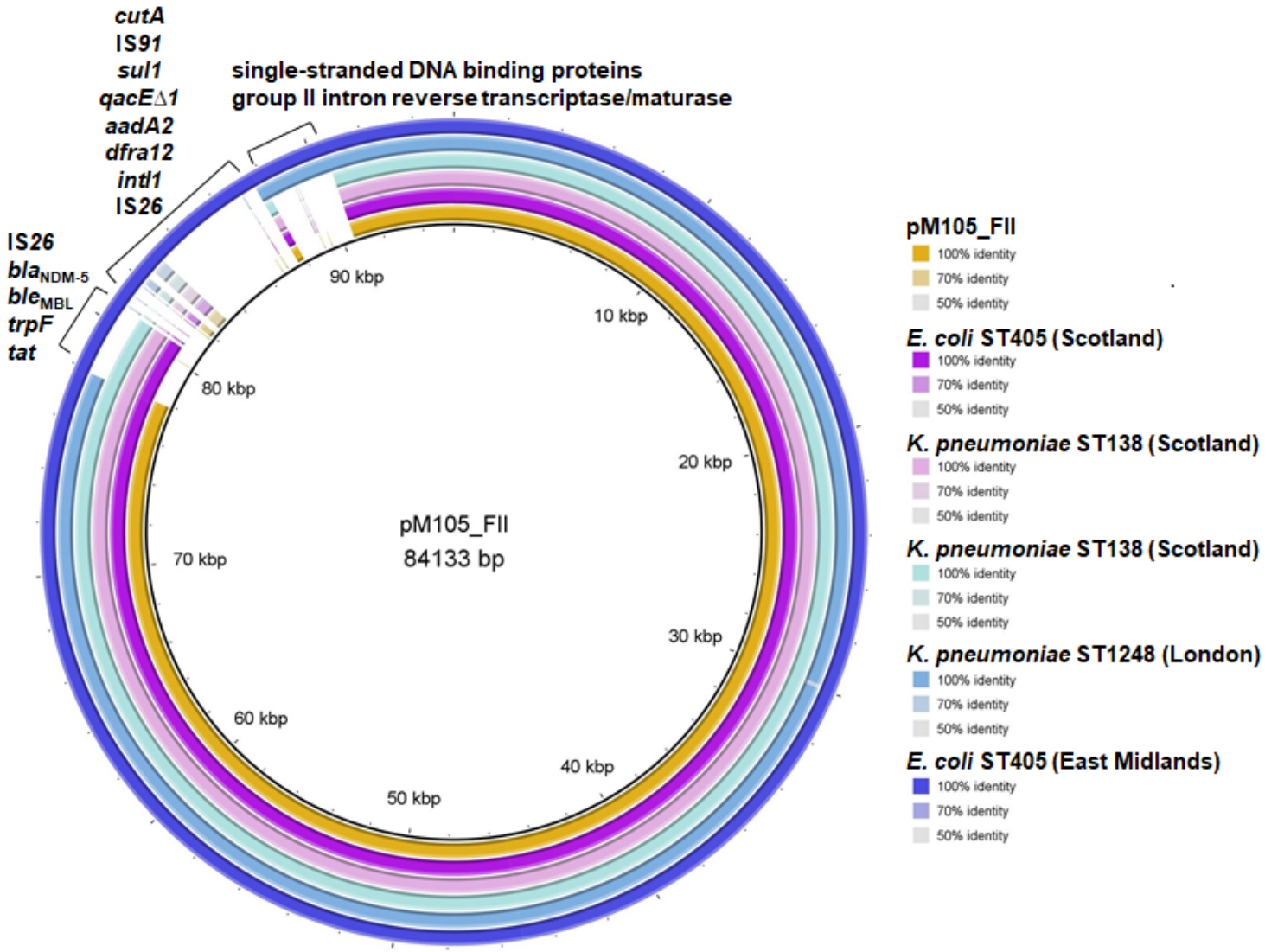


Figure 7.13: Insertions identified in pM105_FII-like plasmids identified in this study. The reference sequence for pM105_FII (accession number: AP018136.1) is signified by the orange ring. Insertions in the plasmid are signified by gaps in the orange ring and inserted genes are labelled.

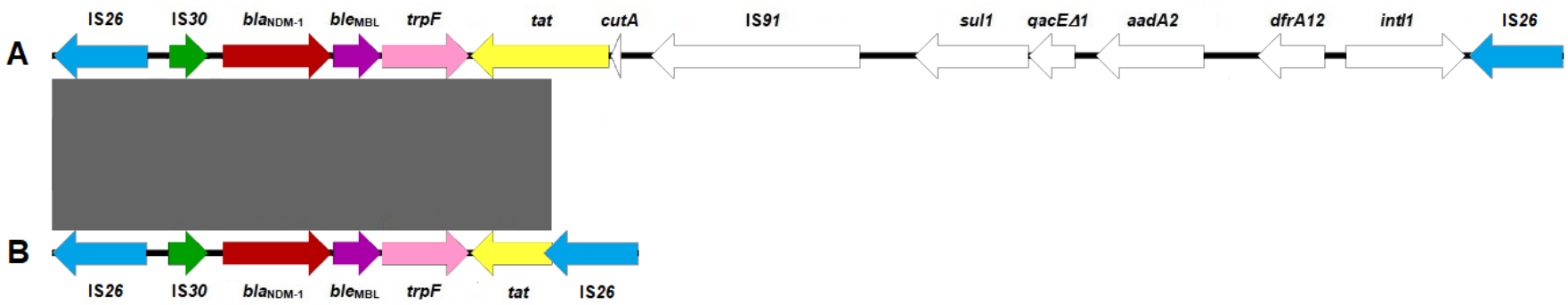


Figure 7.14: Alignment of the *bla*$_{NDM-5}$-containing class 1 integron found in pM105_FII in the *E. coli* ST405 isolate from the East Midlands (A) compared to the truncated class 1 integron identified in pM105_FII isolated from two *K. pneumoniae* ST138 isolates and one *E. coli* ST405 isolate from Scotland (B). The grey shading indicates 100% sequence identity. Genes that are not shared between genetic environments are coloured white.

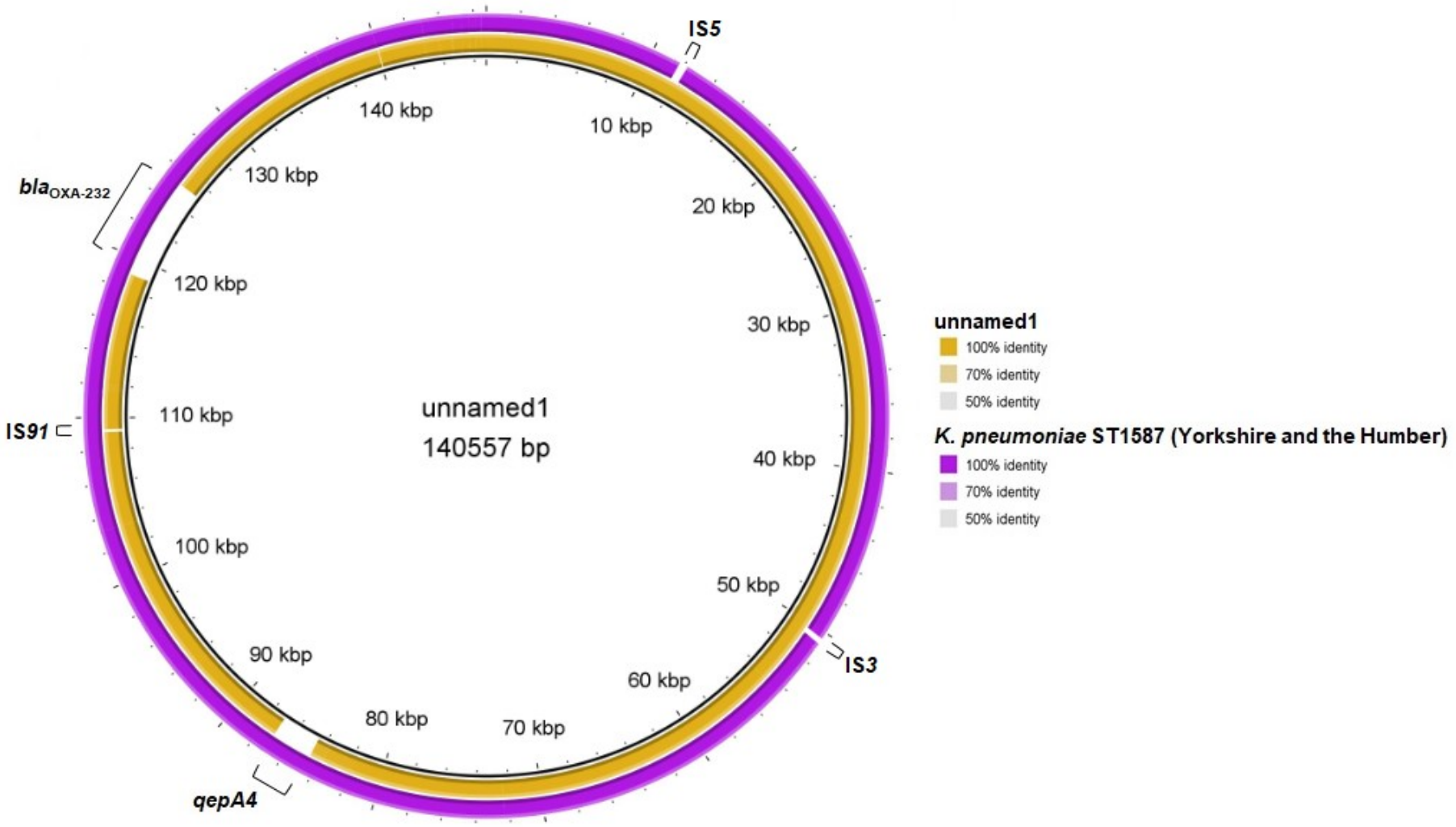


Figure 7.15: Insertions identified in unnamed1 plasmids identified in this study. The reference sequence for unnamed1 (accession number: CP023923.1) is signified by the orange ring. Insertions in the plasmid are signified by gaps in the orange ring and deleted genes are signified by gaps in the purple ring. Both inserted and deleted genes are labelled.

7.3.2.5.3 *rmtC*

Ten (83.3%) *rmtC* plasmids were found to match to single plasmids published on GenBank (Table 7.14). The other two (16.7%) plasmids shared <90.0% sequence identity with published plasmid sequences on GenBank (see Table 16 in Appendix 1).

Four different plasmids were identified including pNDM_22ES (accession number: CM008904.1), pHS36-NDM (accession number: KU726616.1), pEC4-NDM-6 (accession number: KC887916.2) and pECL3-NDM-1 (accession number: KC887917.1).

pNDM_22ES (accession number: CM008904.1) was the most common as it was identified in five (41.7%) isolates, including *K. pneumoniae* ST29, ST89 and ST307 isolates from London, *E. cloacae* complex ST275 from London and *C. freundii* ST22 from the East of England. pHS36-NDM (accession number: KU726616.1) was found in three (25.0%) isolates including *C. freundii* ST22 from London, *E. coli* ST131 from Yorkshire and the Humber and *E. coli* ST167 from the North West of England. All other plasmids were found in single isolates. All plasmids were positive for $bla_{\text{NDM-1}}$.

All three isolates that harboured pHS36-NDM-like plasmids (accession number: KU726616.1), a plasmid that had a truncated *rmtC* gene, had insertions of the complete *rmtC* gene and *aac(6')-Ib-cr* but all were missing *aadA2* and *dfrA12* (Figure 7.16). The *K. pneumoniae* ST147 isolate carrying a pEC4-NDM-6-like plasmid (accession number: KC887916.2) harboured $bla_{\text{NDM-1}}$ rather than $bla_{\text{NDM-6}}$.

Table 7.14: Characteristics of *rmtC*-positive plasmids published in GenBank identified in this study including replicon type, TA system genes, gaps in the plasmid DNA sequence and missing or inserted antibiotic resistance genes.

Plasmid (accession number)	Number of isolates	Species	Geographical distribution	Percentage match to plasmid	Replicon type(s)	TA system genes	Antibiotic resistance genes	Gaps in plasmid (bp)	Missing antibiotic resistance genes	Inserted antibiotic resistance genes
pNDM_22ES (CM008904.1)	5	*K. pneumoniae* ST89	London	100% (106,482/106,482)	FII	*ccdB*/*ccdA*	*rmtC*, bla_{NDM-1} and *sul1*	-	-	-
		K. pneumoniae ST307								
		E. cloacae complex ST275	London	99.7% (106,128/106,482)				65,157-65,511		
		K. pneumoniae ST29	London	99.6% (106,020/106,482)				65,049-65,511		
		C. freundii ST22	East	99.4% (105,819/106,482)				62,538-63,201		
pHS36-NDM (KU726616.1)	3	*C. freundii* ST22	London	98.7% (136,196/137,952)	A/C	-	bla_{NDM-1}, bla_{CMY-6} and *sul1*	35,200-36,956	*aadA2* and *dfrA12*	*rmtC* and *aac(6')-Ib-cr*
		E. coli ST131	Yorkshire and the Humber							
		E. coli ST167	North West							
pEC4-NDM-6 (KC887916.2)	1	*K. pneumoniae* ST147	South West	100% (110,768/110,786)	FIB	*ccdB*/*ccdA*	*rmtC*, bla_{NDM-1} and *sul1*	-	-	-
pECL3-NDM-1 (KC887917.2)	1	*E. cloacae* complex ST78	East Midlands	100% (99,435/99,435)	FII	*ccdB*/*ccdA*	*rmtC*, bla_{NDM-1} and *sul1*	-	-	-

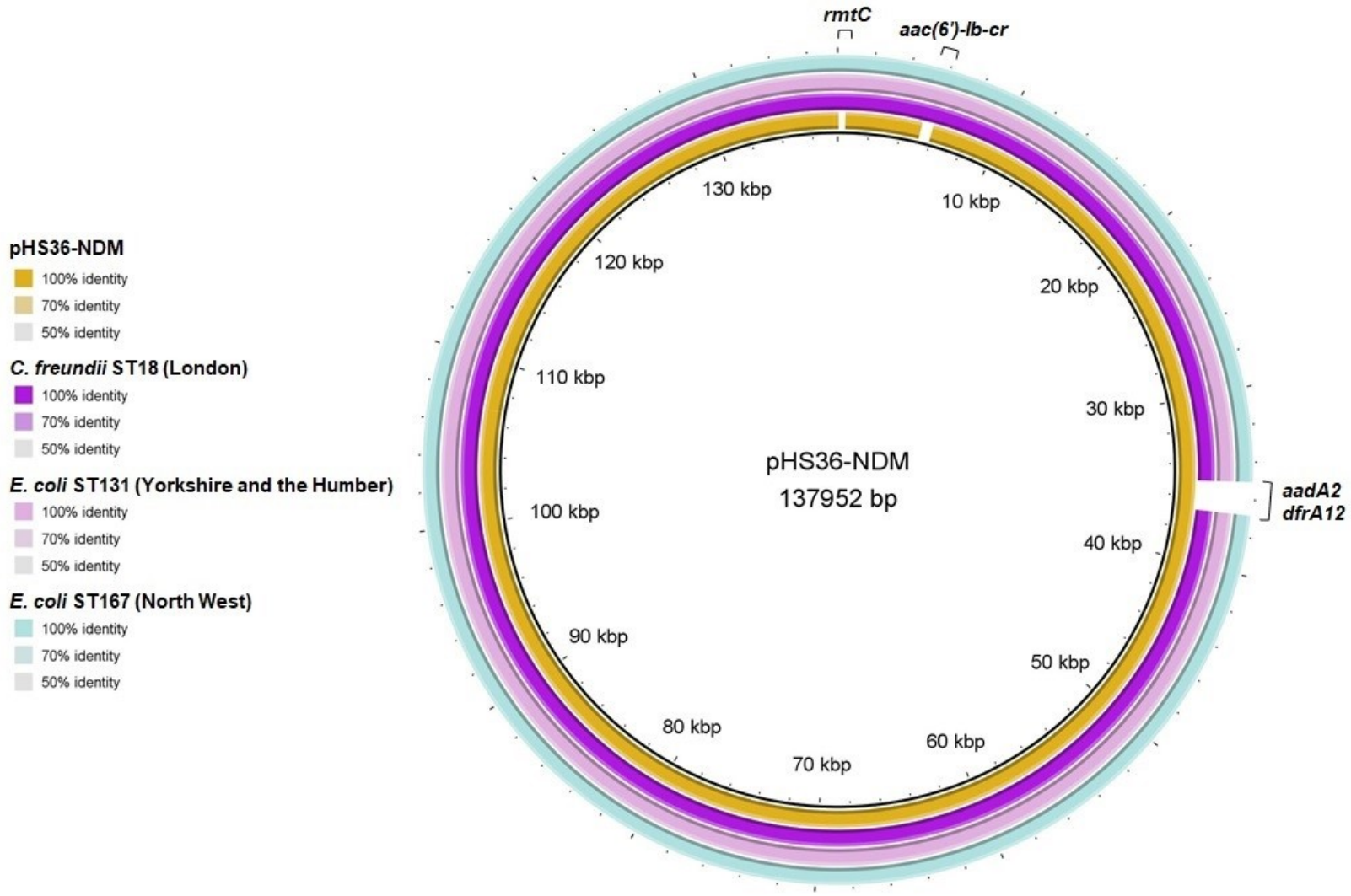


Figure 7.16: Insertions identified in pHS36-NDM plasmids identified in this study. The reference sequence for pHS36-NDM (accession number: KU726616.1) is signified by the orange ring. Insertions in the plasmid are signified by gaps in the orange ring and deleted genes are signified by gaps in the purple, pink and blue rings. Both inserted and deleted genes are labelled.

7.3.2.5.4 *rmtF*

Only one (5.3%) *rmtF* plasmid was found to share significant sequence identity to a single published plasmid on GenBank (Table 7.15). Seventeen (89.5%) plasmids were comprised of contigs that shared sequence identity with multiple plasmids (see Table 17 in Appendix 1). One (5.3%) plasmid shared <90.0% identity to published plasmid sequences on GenBank (see Table 18 in Appendix 1).

One *K. pneumoniae* ST231 isolate from London harboured a pIncFIBpQil-like plasmid (accession number: CP036321.1) with 95.4% (121,454/127,300 bp) sequence identity. The plasmid was missing $bla_{CTX\text{-}M\text{-}15}$; Tn*3*; IS*NCY*, a histidine kinase, adenylyl cyclases, methyl-binding proteins and phosphatases (HAMP) domain-containing protein (where HAMP domains are α-helical coiled coils found in chemoreceptors that are involved in signal transduction [501]); a cupin fold metalloprotein (which is a metal-binding protein that contains a β-barrel fold at the metal binding site [502]) and the DNA polymerase V subunit UmuC (which is the catalytic subunit of the polymerase involved in DNA repair in prokaryotic bacteria [503]) (Figure 7.17).

7.3.2.5.5 *rmtB + rmtF*

Analysis of the two transformants obtained from the *rmtB* + *rmtF E. coli* ST4108 isolate from London indicated that *rmtB* + *rmtF* were found on two separate plasmids as plasmid sequences found in the *rmtB*-positive transformant were identical to the *rmtB* + *rmtF* transformant. However, the *rmtB* or *rmtF* plasmids did not share significant identity with single plasmid sequences on GenBank as contigs were found to share identity with different plasmids (Table 7.16).

Table 7.15: Characteristics of the *rmtF*-positive plasmid published in GenBank identified in this study including replicon type, TA system genes, gaps in the plasmid DNA sequence and missing antibiotic resistance genes.

Plasmid (accession number)	Number of isolates	Species	Geographical distribution	Percentage match to plasmid	Replicon type(s)	TA system genes	Antibiotic resistance genes	Gaps in plasmid (bp)	Missing antibiotic resistance genes
pIncFIBpQil (CP036321.1)	1	*K. pneumoniae* ST231	London	95.4% (121,454/127,300)	FIB and FII	*hok*/*sok*	*rmtF*, *aac(6')-Ib-cr*, bla_{TEM-1}, *qnrS1*, *catA1* and *arr-2*	30,327-32,502, 48,738-52,246 and 53,069-53,232	$bla_{CTX-M-15}$

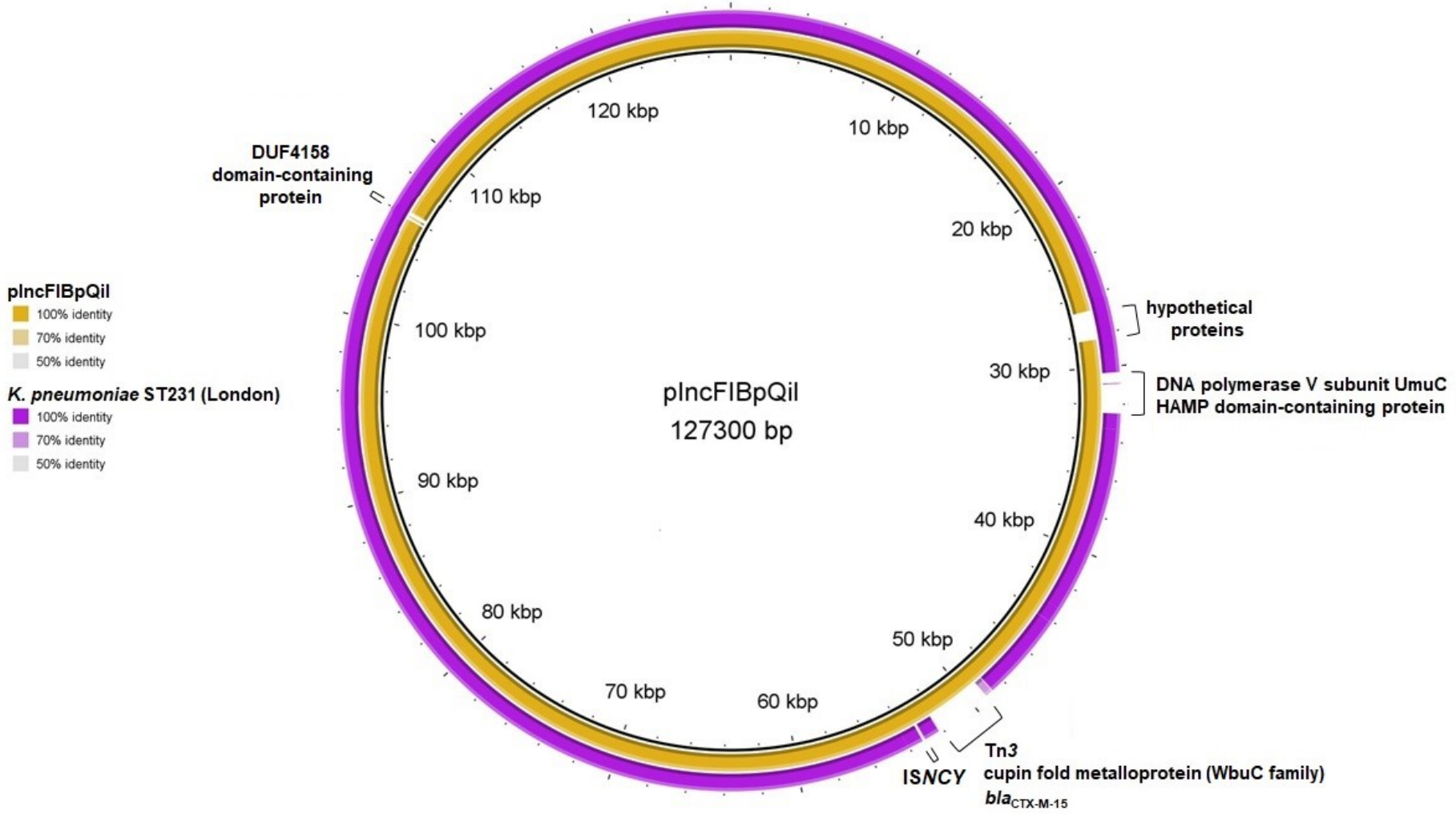


Figure 7.17: Insertions identified in pIncFIBpQil plasmids identified in this study. The reference sequence for pIncFIBpQil (accession number: CP036321.1) is signified by the orange ring. Insertions in the plasmid are signified by gaps in the orange ring and deleted genes are signified by gaps in the purple ring. Both inserted and deleted genes are labelled.

Table 7.16: Plasmid DNA sequences identified in *rmtB* and *rmtF* plasmids in the *rmtB* + *rmtF*-positive *E. coli* ST4108 isolate, where regions of the plasmids shared identity to different plasmids.

Plasmid	Name	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched	Antibiotic resistance genes	TA system genes
rmtB	pMC-NDM	HG003695.1	87,619	58,248	66.5	1-802, 11,962-29,159, 31,082-47,450 and 63,738-87,619	*rmtB*, bla_{TEM-1}, bla_{SFO-1} and *mphA*	*hok/sok*
	pKP1814-1	KX839207.1	299,858	23,253	7.8	166,661-182,245, 205,543-213,031 and 213,491-213,672		-
	unnamed8	CP029121.1	3,174	3,174	100	1-3,174	-	-
	unnamed1	CP033761.1	111,938	10,831	9.7	90,425-90,769 and 175,696-186,183	-	-
rmtF	unitig_1	CP022612.1	334,957	31,158	9.3	133,522-133,701, 148,277-148,439, 157,023-178,300, 197,630-199,090, 200,286-204,133 and 216,351-220,584	-	-
	pCRKP-1215_1	CP024839.1	130,922	8,201	6.3	157,538-158,406 and 158,618-165,951	-	-
	unnamed1	CP029723.1	183,663	7,334	4.0	158,618-165,951	*rmtF*, *aac(6')-Ib-cr* and *arr-2*	-
	p48896_2	CP024431.1	114,815	2,545	2.2	74,957-77,501	-	-
	unnamed3	CP029105.1	2,101	2,101	100	1-2,101	-	-
	pNDM-BJ03	MF415608.1	60,125	695	1.2	23,165-22,821 and 23,643-23,294	-	-

The *rmtB* plasmid was composed of contigs that shared identity with regions belonging to four different plasmids (Table 7.16), where 68.8% (58,220/84,567 bp) of its DNA sequence was identical to pMC-NDM (accession number: HG003695.1), which harboured the genes *rmtB*, bla_{TEM-1}, bla_{SFO-1} *and mphA*; 26.6% (22,478/84,567 bp) was identical to pKP1814-1 (accession number: KX839207.1), 3.8% (3,174/84,567 bp) was identical to unnamed8 (accession number: CP029121.1) and 0.8% (695/84,567 bp) was identical to unnamed1 (accession number: CP033761.1).

The *rmtF* plasmid appeared to be composed of contigs that shared identity with regions belonging to six plasmids: unitig_1 (accession number: CP022612.1), pCRKP-1215_1 (accession number: CP024839.1), unnamed1 (accession number: CP029723.1), p48896_2 (accession number: CP024431.1), unnamed3 (accession number: CP029105.1) and pNDM-BJ03 (accession number: MF415608.1). A total of 61.4% (32,182/52,422 bp) of the *rmtF* plasmid composed of unitig_1, followed by 14.4% (7,565/52,422 bp) of pCRKP-1215_1, 14.0% (7,334/52,422 bp) of unnamed1 (which harboured the *rmtF*, *aac(6')-Ib-cr* and *arr-2* genes), 4.9% (2,545/52,422 bp) of p48896_2, 4.0% (2,101/52,422 bp) of unnamed3 and 1.3% (695//52,422 bp) of pNDM-BJ03.

7.4 Discussion

This study was conducted to identify which transposable elements and plasmids are associated with 16S RMTase genes and contributing to their spread throughout the UK as well as which antibiotic resistance genes may drive the spread of 16S RMTase genes through co-selection. This study has shown that 16S RMTase genes are associated with transposable elements commonly reported to be associated with 16S RMTase genes (such as Tn*1548*, Tn*2* and IS*Ecp1*) as well as plasmids. Moreover, 16S RMTase genes appear to be highly associated with NDM carbapenemase genes on plasmids. Therefore, all of these factors will enable the spread of 16S RMTase genes within the UK.

armA genes were found to be part of the composite transposon Tn*1548*, which has been reported as associated with *armA* in the literature and is disseminating worldwide [161,165,206,248,319,504-506]. However, Tn*1548* transposons could not be fully constructed in 84.8% (217/256) isolates due to fragmented contigs, where either the macrolide resistance genes *mphE* and *msrE*, the whole class 1 integron or only the gene cassettes within the class 1 integron were missing. Yet, contigs harbouring the missing genes were present in 82.9% (180/217) isolates indicating that the full Tn*1548* structure may have been present. The association of *armA* with Tn*1548,* which can carry a range of genes encoding resistance to antibiotics such as β-lactams, carbapenems, chloramphenicol, macrolides, rifampicin, sulphonamides, trimethoprim, streptomycin or spectinomycin [165,248], means that the use of these antibiotics could drive selection of Tn*1548* [248] and may be the reason behind why *armA* is the most common 16S RMTase gene to be identified in this study as well as globally.

Around 37.8% (31/82) *rmtB1* genes in the Enterobacterales were associated with complete Tn*2* transposons, where Tn*2* is the most frequently reported transposable element associated with *rmtB* [266]. However, in 48.8% (40/82) isolates Tn*2* was located on separate contigs, meaning that Tn*2* may have been associated with *rmtB* in these isolates but could not be confirmed. Tn*2* was not associated with *rmtB1* in *P. aeruginosa*, although it has been previously reported in this species [252], as IS*6100* was associated with *rmtB1*. To our knowledge this is the first report of an association between *rmtB1* and IS*6100*. Two *E. coli* isolates were found to have *rmtB* genetically linked to *qepA*, which encodes a quinolone efflux pump. *qepA* is commonly associated with IS*CR3* (as found in this study) [215,314,507-509] and is frequently identified with *rmtB* in the same conformation identified in this study (Tn*2*-*rmtB*-*groEL*-IS*CR3*-*qepA*) [215,314,507,508]. *rmtB* and *qepA* have mainly been identified in *E. coli* isolated from companion animals and farm animals in China [170,270,510] but are believed to be spreading worldwide due to their dissemination on IncF plasmids in Enterobacterales, which was supported by this study [169,510,511].

The *rmtB4*-positive *P. aeruginosa* isolates shared the same genetic environment as *P. aeruginosa* strain ST773 (accession number: CP041945.1), which was isolated from a patient in the USA who received medical treatment in India [512]. This genetic environment is identical to that found in the *rmtB4*-positive *P. aeruginosa* strain IOMTU 304, which was identified in Nepal (accession number: LC052325.1) [396], but the latter is missing the LysR-family transcriptional regulator and IS*91* downstream of *rmtB4*. The isolate from Nepal belonged to ST235 [396] and the isolate from the USA belonged to ST773 [512], whereas the *rmtB4* isolates from this study belonged to STs 233, 316, 357, 654 and 773. This genetic environment surrounding *rmtB4* therefore appears to be widespread among *P. aeruginosa* STs. Additionally, most patients (54.5%, 6/11) with *rmtB4*-positive *P. aeruginosa* isolates had an unknown travel history so it is unknown if they had previously travelled to India or Nepal. However, four (36.4%) patients had previously travelled to India and one (9.1%) patient travelled to Nigeria, indicating that *rmtB4*-positive *P. aeruginosa* are circulating outside of India and Nepal.

rmtC has been reported to be associated with IS*Ecp1* [137,163,193,200,311,339,513], which was supported by this study as IS*Ecp1* was associated with 67.4% (58/86) *rmtC* genes. As previously reported by Hopkins *et al*. [200], who found *rmtC* associated with partially deleted IS*Ecp1* elements in *S. enterica* serovar Virchow isolates from PHE's culture collection, *rmtC* was associated with partially deleted IS*Ecp1* elements in 56.9% (33/58) isolates in this study. Therefore, there are *rmtC* genes associated with partially deleted IS*Ecp1* elements circulating around the UK, meaning these *rmtC* genes may not be able to be spread via transposition due to partial loss of the MGE. Additionally, as the 3' end was deleted, which encodes the −35 and −10 promoter sequences [514], the ability of IS*Ecp1* to promote the expression of *rmtC* is lost in these isolates. Therefore, the expression of these *rmtC* genes must be controlled by alternative promoters. However, 27.9% (24/86) *rmtC* genes were associated with IS*91*-like elements, which are IS elements that are similar to IS*Ecp1* as they also transpose by rolling-circle replication [330]. Again, due to variation in length of the contigs encoding *rmtC*, the

association between *rmtC* and IS*91* could not be confirmed in three *rmtC* isolates. The association between IS*91*-like elements and *rmtC* has also been reported in Australia [513], Canada [194,513], India [513], New Zealand [513], Romania [515], Taiwan [516] and the USA [273], where it has been found on bla_{NDM}-containing plasmids isolated from *E. cloacae* complex, *Klebsiella* spp. and *S. marcescens* isolates. In this study, the association was found in multiple species belonging to diverse STs throughout all regions of England apart from the East Midlands, indicating that this association is widespread.

The single *rmtD1*-positive *P. aeruginosa* was found to have *rmtD1* associated with IS*CR14*, which has previously been reported to be associated with *rmtD1* in *K. pneumoniae* and *P. aeruginosa* isolates from Brazil [338]. The isolate in this study belonged to ST277, a 'high-risk' clone known to be endemic in Brazil [286,309], which is where the patient (who the bacterial isolate was isolated from) had previously received medical treatment [309]. As *rmtD1* was found associated with IS*CR14* in a 'high-risk' clone, this means that *rmtD1* is more likely to disseminate to other bacteria due to transposition by IS*CR14* and through the spread of *P. aeruginosa* ST277. The four *rmtD3*-positive *P. aeruginosa* isolates had the same genetic environment as *P. aeruginosa* strain MyNCGM481 from Myanmar [140], where *rmtD3* was found between two IS*91* insertion sequences. *P. aeruginosa* strain MyNCGM481 from Myanmar belonged to ST316 [140] and *P. aeruginosa* 1334/14 from Poland belonged to ST234 [275] but the *rmtD3*-positive isolates in this study belonged to ST308 as well as the 'high-risk' clones ST357 and ST654 [387]. This means that *rmtD3* is likely to spread to other bacteria due to transposition by IS*91* and the dissemination of these 'high-risk' clones. Out of the patients infected with *rmtD3*-positive *P. aeruginosa* isolates, two (50.0%) had an unknown travel history so it is unknown if they had acquired their infection from Myanmar or Poland but one patient (25.0%) had previously travelled to India and one (25.0%) had not travelled outside of the UK. Therefore, *rmtD3*-positive *P. aeruginosa* are currently circulating outside of Myanmar and Poland.

The *rmtE3* gene identified in *A. baumannii* ST79 shared a similar genetic environment to *rmtE2* but it appeared to be missing the IS*CR20* element that was found upstream of *rmtE2* [142] as it was not identified on the *rmtE3* contig nor in the genome of *A. baumannii* ST79. This genetic environment surrounding *rmtE3* was found in another *A. baumannii* isolate (accession number: NZ_ULHD01000074.1), which was also missing the IS*CR20* element. This means that another transposable element may be involved in the transposition of *rmtE3*. Also, this suggests that *A. baumannii* acts as a reservoir of *rmtE3* as it has not yet been identified in another species. In this study, the genetic location of *rmtE3* could not be determined as several attempts of electroporation failed to generate transformants. The genetic location of the *rmtE3* gene in the *A. baumannii* isolate from Mexico was also unknown as well as its ST so it is unclear whether the spread of *rmtE3* is due to clonal expansion.

Most *rmtF1* (88.5%, 100/113) genes in the Enterobacterales were found to be associated with IS*91* elements. Additionally, 45.1% (51/113) isolates were associated with *catB* genes and 27.4% (31/113) isolates were associated with *aac(6')-Ib-cr*, which have previously been reported to be associated with *rmtF* in Enterobacterales from Egypt [205], India [206], La Réunion Island [143], Singapore [208], South Africa [212], South Korea [209] and the USA [517]. However, variation in the length of *rmtF*-encoding contigs meant that the association of *aac(6')-Ib-cr* and *catB* with *rmtF1* could not be confirmed for the remaining isolates. Additionally, *rmtF2* in *P. aeruginosa* was found to be genetically linked to *aac(6')-Ib-cr*, therefore, as *rmtF1* and *rmtF2* genes have been found to be genetically linked to *aac(6')-Ib-cr*, this means that the use of fluoroquinolones such as ciprofloxacin could drive selection of *rmtF* genes. *rmtF2* was also found associated with $bla_{\text{OXA-10}}$ and $bla_{\text{PAC-1}}$, in the two isolates with the same genetic environment as *P. aeruginosa* strain IOMTU487 (accession number: LC224309.1) [396] and the other *rmtF2*-positive isolate had *rmtF2* associated with the ESBL gene $bla_{\text{GES-9}}$ and *catB7*. This means that the use of β-lactams as well as chloramphenicol could also drive selection of *rmtF2* genes. As all of the patients with *rmtF2*-positive *P.*

aeruginosa isolates had an unknown travel history, it is unknown if *rmtF2* is circulating in the UK or was obtained from other countries.

Analysis of plasmids harbouring 16S RMTase genes showed that they were found on a variety of broad-host range (A/C, L/M, N and R) and narrow-host range (F, HIA, HIB, I and X) plasmids. Most of these plasmids belonged to the replicon type IncF (58.1%, 36/62), which is one of the most common replicon types associated with 16S RMTase genes in Enterobacterales [127,266,340,355,518]. This may be due to IncF plasmids providing host cells with increased fitness through carriage of genes encoding antibiotic resistance or virulence, as well as the use of addiction systems to ensure their maintenance and stability within the cells [344]. However, *armA* appeared to be most frequently identified on broad-host range plasmids belonging to Inc groups R, N and L/M. This may explain why *armA* is the most common 16S RMTase gene as its dissemination on broad-host range plasmids allows it to spread to multiple species of bacteria, compared with narrow-host range plasmids such as IncF, which will only allow spread between Enterobacterales [344].

A high proportion of bla_{NDM} (84.2%, 32/38) genes appeared to be co-located with 16S RMTase genes on plasmids. bla_{NDM-1} was present on all *rmtC* plasmids, which is an association that has appeared frequently in the literature as *rmtC* and bla_{NDM-1} have been found on plasmids in Canada [513,519], India [194,206,513], Kenya [193], Mauritius [520], New Zealand [198], Singapore [521], the UK [206] and the UAE [522]. What is concerning is that 41.7% (5/12) *rmtC* plasmids in this study (as well as many *rmtC* and bla_{NDM-1} plasmids reported elsewhere [193,194,513,520]) belong to IncA/C. IncA/C plasmids have a very broad-host range as they have been identified in Enterobacterales, *Pseudomonas* spp., *Aeromonas* spp., *Photobacterium* spp., *Vibrio* spp. and *Yersinia* spp. [523-525], therefore providing *rmtC* and bla_{NDM-1} the perfect vehicle to spread pan-aminoglycoside and carbapenem resistance to multiple species.

bla_{NDM} was also found on a high number of *rmtB* plasmids (76.2%, 16/21), particularly bla_{NDM-5} (56.3%, 9/16). Most of these plasmids belonged to IncFII (77.8%, 7/9), which appears to be

common for plasmids co-harbouring *rmtB* and *bla*$_{NDM-5}$ as they have been identified in South Korea [209], Spain [224], the UAE [526] and the USA [527]. In contrast to *rmtB*, *bla*$_{NDM-1}$ was the only *bla*$_{NDM}$ variant associated with *armA* and *rmtF*, where it was found on IncL/M and IncHI1A + IncHI1B plasmids with *armA*, and IncF plasmids with *rmtF*. IncHI1 plasmids have frequently been reported to be involved in the dissemination of *bla*$_{NDM-1}$ [525], and have previously been found to encode *armA* as well as *bla*$_{NDM-1}$ in India [306] and Spain [307]. *bla*$_{NDM-1}$ is also known to be disseminated on IncF plasmids [525], particularly with 16S RMTase genes [182,352,443,528], and has been associated with *rmtF* on an IncFII plasmid in Canada [529]. Overall, the association of *bla*$_{NDM}$ with 16S RMTase genes on plasmids supports the findings from the AMRHAI Reference Unit (see Chapter 4), BSAC Resistance Surveillance Project (see Chapter 5) and the prospective surveillance study (see Chapter 6), which suggested that 16S RMTase genes were genetically linked to carbapenemase genes such as *bla*$_{NDM}$.

Generally, 16S RMTase genes appear to be found on separate plasmids to *bla*$_{OXA-48-like}$ genes [227,233,251,265,318,530], as suggested by this study as only one plasmid harboured *rmtB* and *bla*$_{OXA-232}$ along with *bla*$_{NDM-1}$. However, the association of a 16S RMTase gene with a *bla*$_{OXA-48-like}$ gene on a plasmid has previously been reported in Singapore, where *armA* and *bla*$_{OXA-181}$ were found on the same plasmid [531]. In contrast to other studies where *bla*$_{KPC}$ has been reported on plasmids harbouring 16S RMTase genes [532,533], no *bla*$_{KPC}$ genes were found on plasmids harbouring 16S RMTase genes in this study, suggesting they may have been on separate plasmids, as also seen in Brazil [534], China [535], India [536] and Poland [537]. Therefore, these findings suggest that 16S RMTase genes in the UK are not frequently genetically linked to carbapenemase genes other than *bla*$_{NDM}$.

As well as carbapenemase genes, *aac(6')-Ib-cr*, *bla*$_{CTX-M-15}$ and *qnrB1* were the most common resistance genes associated with 16S RMTase genes in bacterial isolates from the AMRHAI Reference Unit (see Chapter 4), BSAC Resistance Surveillance Project (see Chapter 5) and the prospective surveillance study (see Chapter 6). Overall, a high proportion of *aac(6')-Ib-cr*

genes were transferred with 16S RMTase genes on plasmids (68.1%, 32/47), indicating they are commonly associated with 16S RMTase genes on plasmids, which is supported by a study by Wei *et al*. [431] in China who found *aac(6')-Ib-cr* in 100% (7/7) plasmids harbouring 16S RMTase genes. As predicted, *aac(6')-Ib-cr* was found to be genetically linked to *rmtF*, as it was found on all *rmtF* plasmids in this study and has been reported on plasmids isolated in Egypt [205], La Réunion Island [143] and South Africa [212]. However, *qnrB1* and *bla*$_{\text{CTX-M-15}}$ were found to be transferred with 16S RMTase genes on plasmids at much lower levels as they were only transferred in 27.8% (5/18) and 13.3% (6/45) plasmids, respectively. Low levels of *bla*$_{\text{CTX-M-15}}$ and *qnrB* genes on plasmids harbouring 16S RMTase genes have also been reported in a study by Poirel *et al*. [227] in Angola, where 18.8% (3/16) *bla*$_{\text{CTX-M-15}}$ and 12.5% (2/16) *qnrB* genes were found on plasmids harbouring 16S RMTase genes. Therefore, this suggests that 16S RMTase genes are more likely to spread through co-selection with *bla*$_{\text{NDM}}$ carbapenemase genes than ESBL or PMQR genes (other than *aac(6')-Ib-cr*).

Most plasmids isolated from this study (53.2%, 33/62) appeared to be novel as they were comprised of regions encoded by different published plasmids, especially *rmtF* plasmids as 89.5% (17/19) consisted of multiple plasmid DNA sequences. However, out of the 46.8% (29/62) plasmids that could be identified, pM105_FII (accession number: AP018136.1), pNDM_22ES (accession number: CM008904.1) and pHS36-NDM (accession number: KU726616.1) were the most common. The *rmtB*-positive pM105_FII was originally isolated from a clinical *E. coli* isolate from Myanmar in 2015 in a study by Sugawara *et al*. [538] but unlike other *bla*$_{\text{NDM-5}}$-harbouring plasmids identified in the study, pM105_FII lacked *bla*$_{\text{NDM-5}}$. This loss was believed to be due to transposition as the region containing *bla*$_{\text{NDM-5}}$ was thought to be bracketed by two IS*26* elements [538]. However, in this study 80.0% (4/5) pM105_FII plasmids had *bla*$_{\text{NDM-5}}$ meaning that *bla*$_{\text{NDM-5}}$ and surrounding genes were ether reintroduced into pM105_FII or these plasmids were the precursor to pM105_FII before the loss of *bla*$_{\text{NDM-5}}$. As two of the patients had no record of travel outside of the UK, this suggests that pM105_FII is currently circulating outside of Myanmar. However, two patients had an unknown travel

history and one patient had previously travelled but the location was unknown, therefore bacteria harbouring pM105_FII may have been obtained from Myanmar.

The plasmid pNDM_22ES was the most common *rmtC* plasmid (41.7%, 5/12) identified. It was initially reported in a clinical *E. cloacae* complex isolate from an outbreak of bla_{NDM-1}-positive *S. marcescens* and Enterobacterales in a neonatal ward in Romania in 2013 [515]. However, two of the patients in this study had not previously travelled to Romania as one had not travelled outside the UK and the other travelled to the Philippines, indicating that this plasmid is currently circulating outside of Romania. The other three patients had an unknown travel history, so it is not known if they obtained bacteria harbouring this plasmid from Romania. The *rmtC*-positive pHS36-NDM was first identified in a *S. enterica* subsp. enterica serovar Stanley isolate isolated from an 11-month-old child with diarrhoea from China in 2012 [201,539]. However, in this study the pHS36-NDM plasmids were missing the antibiotic resistance genes *aadA2* and *dfrA12* but had acquired *aac(6')-Ib-cr*. Huang *et al*. [201] identified that pHS36-NDM shared 99.9% identity to plasmids pNDM-KN (accession number: JN157804.1), which was isolated in Kenya; pNDM10505 (accession number: JF503991.1), which was isolated in Canada, and pNDM10469 (accession number: JN861072.1) isolated in Japan. They suggested that this plasmid may be frequently transmitted between virulent Enterobacterales [194], which may be why this plasmid is present in the UK as the association of pHS36-NDM with virulent Enterobacterales, such as the 'high-risk' clone *E. coli* ST131 (which was identified in this study), may have led to its global dissemination.

Many (58.1%, 36/62) of the plasmids harboured TA system genes, which encode a toxin and an antitoxin used for plasmid maintenance. The loss of a resistance plasmid encoding a TA system leads to cell death due to the inability of the bacterial cell to express the antitoxin to neautralise the toxin, therefore maintaining antibiotic resistance [23,24]. TA systems allow the maintainenance of resistance plasmids even when there is no selective pressure from antibiotic use and allow strains harbouring these plasmids to outcompete strains that do not harbour the plasmids. This is particularly concerning for plasmids encoding 16S RMTase

genes as 72.2% (26/36) and 36.1% (13/36) plasmids encoding TA systems harboured carbapenemase and PMQR genes, respectively. Therefore, plasmids encoding resistance to aminoglycosides, β-lactams and fluoroquinolones, which are key antibiotics used to treat Gram-negative bacterial infections, are being maintained in bacterial populations [58,59]. These plasmids can be spread to other bacteria through horizontal transfer promoting multidrug resistance and make the treatment of Gram-negative bacterial infections more difficult. However, as TA systems are potential drug targets any future drugs designed to target MDR plasmids harbouring TA sytems could help prevent the spread of 16S RMTase genes [540].

The main limitation of this study is that short-read sequencing was used to sequence the bacterial isolates and plasmids. Although short-read sequencing is widely used by research groups and public health laboratories due to its low cost and low per-base error rates (<1.0% for Illumina sequencing) [52], the use of short reads makes *de novo* genome assembly difficult. Bacterial genomes, as well as plasmids, harbour sections of tandem repeats as well as MGEs, which can lead to fragmented contigs, mis-assemblies or gaps as reads can be shorter than the regions of tandem repeats and multiple copies of identical MGEs can be found throughout a genome (or plasmid) [52,53]. Unfortunately, most published DNA sequences (on GenBank for example) have been generated by short-read sequencing [52,54], meaning that there may be gaps or misalignments in these published DNA sequences. Therefore, the genetic environments of 16S RMTase genes or plasmids identified in this study using published DNA sequences from GenBank as a reference may be incorrect and will need further investigation to verify them.

To resolve this issue long-read sequencing technologies such as single-molecule, real-time (SMRT) sequencing (Pacific BioSciences) or the MinION (Oxford Nanopore Technologies) could be used. However, the high cost of long-read sequencing (e.g. $1000 per isolate for the MinION) restricts its use to small numbers of samples [541] so it was not feasible for this study. When used, long-read sequencing can provide single continuous reads of up to >15 kb [542]

(compared with 150 bp used by the Illumina HiSeq 2500 System in this study). This means that regions containing MGEs or tandem repeats can be properly mapped and assembled [53,542]. Long-read sequencing can also be used for *de novo* assembly of plasmids, whereas short-read sequencing relies on the use of reference-based read mapping, which may be problematic if no reference is available [541,542]. The latter was seen with the novel plasmids identified in this study so long-read sequencing should be used in the future to correctly assemble these plasmids so they can be characterised. Another limitation was the inability to extract and characterise additional plasmids harbouring 16S RMTase genes due to financial and time constraints so there may be plasmids that were not identified in this study circulating in the UK.

In conclusion, this study was able to prove that 16S RMTase genes are associated with commonly identified transposable elements, including Tn*1548*, Tn*2*, IS*Ecp1* and IS*91*. Furthermore, 62 plasmids harbouring 16S RMTase genes *armA*, *rmtB*, *rmtC* and *rmtF* were isolated, where 53.2% (33/62) appeared to be novel. This study was also able to confirm that 16S RMTase genes are genetically linked to bla_{NDM} on plasmids. This indicates that transposition and co-selection with bla_{NDM} carbapenemase genes are driving the emergence of 16S RMTase genes in the UK.

Chapter 8: Discussion

16S RMTases are an emerging mechanism of aminoglycoside resistance in Gram-negative bacteria and confer high-level resistance (MICs >256 mg/L) to all clinically-relevant aminoglycosides with only a single gene [127,304]. Prior to this study, 16S RMTase-producing bacteria were known to be present in the UK, however, no surveillance studies had been performed so their prevalence in the UK was unknown. Therefore, the aims of this study were to determine the prevalence of 16S RMTase-producing bacteria as well as investigate the mechanisms that are contributing to the spread of 16S RMTase genes i.e. clonal expansion and co-location on plasmids alongside other antibiotic resistance genes.

8.1 Main findings

Initially, to determine the occurrence of 16S RMTase genes in the UK, Gram-negative bacterial isolates from 2003-2015 from the AMRHAI Reference Unit at PHE were studied (see Chapter 4). 16S RMTase genes were identified in 83.2% (1,312/1,576) of pan-aminoglycoside-resistant (amikacin MIC >64 mg/L; gentamicin MIC >32 mg/L and tobramycin MIC >32 mg/L) bacteria and were frequently associated with carbapenemase genes (94.3%, 1,237/1,312), although this strain collection was biased towards carbapenemase producers. The most common carbapenemase genes identified amongst 16S RMTase-producing bacteria were bla_{NDM} alleles (48.7%, 602/1,237).

To avoid bias towards carbapenemase-producers, WGS data from Gram-negative bacterial isolates from 2001-2013 from the Bacteraemia Programme of the BSAC Resistance Surveillance Project (see Chapter 5) were studied to determine the prevalence of 16S RMTase genes in a collection not pre-selected on the basis of any resistance pattern. Analysis of WGS data identified a prevalence of 0.3% (4/1,566) amongst isolates from bloodstream infections. It also supported the observation that 16S RMTase and carbapenemase genes may be genetically linked as carbapenemase genes were found at a low prevalence amongst the 16S RMTase-negative isolates (1.5%, 23/1,562). The prevalence of carbapenemase producers was low in this strain collection as carbapenemase producers are not frequently isolated from

bloodstream infections in the UK. In 2017, only 3.6% (109/3,000) of CPE referred to the AMRHAI Reference Unit originated from bloodstream infections [94].

To calculate the current prevalence of 16S RMTase genes in the UK and identify risk factors associated with the acquisition of 16S RMTase-producing bacteria, a six-month prospective surveillance study involving UK hospitals was set up from 1st May to 31st October 2016 (see Chapter 6). A period prevalence rate of 0.1% (79/71,063) was identified and age (≥65 years), being male, being an inpatient or a non-UK resident and receiving medical treatment abroad were identified as potential risk factors. These risk factors are similar to those associated with the acquisition of carbapenemase-producing bacteria, which include advanced age, travel abroad and residency or receiving medical treatment in countries that are reservoirs of carbapenemase producers (such as India and the Middle East) [453,543,544]. However, 63.9% (106/166) questionnaires were incomplete and the patient cohort was rather small so these results need to be interpreted with caution. Nevertheless, this is the first study to identify potential risk factors for the acquisition of 16S RMTase-producing bacteria and could be used as a foundation for future studies. 16S RMTase genes were closely associated with carbapenemase genes (87.3%, 69/79), where bla_{NDM} (66.7%, 46/69) was the most common, which supported findings from analysis of the AMRHAI Reference Unit strain collection.

Analysis of data from all three studies identified that *armA* is the most common 16S RMTase gene circulating in the UK and is predominantly found in *A. baumannii* and *K. pneumoniae*. This is likely due to carriage on broad-host range plasmids (such as IncR, IncN and IncL/M) and association with Tn*1548* driving the spread of *armA*. *rmtA*, *rmtG*, *rmtH* and *npmA* were not detected, therefore, these genes do not appear to be currently circulating in the UK. Additionally, the 16S RMTase gene variants *rmtB4*, *rmtD3* and *rmtF2*, which were initially identified in *P. aeruginosa* in Nepal [144], Myanmar [140] and Nepal [144], respectively, were identified in *P. aeruginosa* in this study. Furthermore, the variant *rmtE3* was identified in *A. baumannii*, and has also been identified in *A. baumannii* in Mexico. However, the patient with the *rmtE3*-positive *A. baumannii* isolate had not travelled outside of the UK. Neither had one

patient with an *rmtD3*-positive *P. aeruginosa* isolate, suggesting that, if these two travel histories are correct, there may be unidentified reservoirs of these genes in the UK.

MIC data demonstrated that 16S RMTase-producing bacteria were MDR, where most isolates were resistant to carbapenems, 3rd generation cephalosporins and fluoroquinolones. These, as well as aminoglycosides, are the main antibiotics used to treat Gram-negative infections so alternative treatment options will need to be used to treat 16S RMTase-producing bacteria. Fortunately, this study observed lower levels of resistance for tigecycline and colistin in 16S RMTase-producing *A. baumannii*, Enterobacterales and *P. aeruginosa* compared with 3rd generation cephalosporins, carbapenems and ciprofloxacin. Therefore, colistin and tigecycline can still be used as treatment options for 16S RMTase-producing Gram-negative bacteria as well as newer agents that are currently in phase III clinical trials, such as cefiderocol [377]; 16S RMTase producers are, however, resistant to the new aminoglycoside plazomicin.

A total of 83.7% (885/1,057) 16S RMTase-producing bacteria were identified to be 'high-risk' bacterial clones by MLST or through PCR (in the case of *A. baumannii* belonging to International Clone II). These 'high-risk' bacterial clones included *A. baumannii* International Clone II; *E. coli* STs 167, 405 and 410; *K. pneumoniae* STs 14, 147 and 231 and *P. aeruginosa* STs 357, 654 and 773. PFGE and VNTR provided further discrimination within these STs (apart from *K. pneumoniae* ST231 and *P. aeruginosa* ST773), indicating that different strains are circulating in the UK. This is concerning as 'high-risk' clones are generally MDR and/or virulent strains with the ability to spread within healthcare settings [446,447], therefore their acquisition of 16S RMTase genes underscores the limited treatment options for these bacteria. The association of 16S RMTase genes with 'high-risk' bacterial clones suggests that clonal expansion is contributing to the emergence of 16S RMTase genes within the UK.

To determine which MGEs and antibiotic resistance genes were associated with 16S RMTase genes, the genetic environment of 16S RMTase genes was investigated *in silico*. As seen in the literature, Tn*1548* [161,165,206,248,319,504-506], Tn*2* [266], IS*Ecp1* [137,163,193,200,311,339,513], IS*CR14* [338] and IS*91* [194,206,273,513,515,516] were

found to be associated with *armA*, *rmtB1*, *rmtC, rmtD1* and *rmtC* and *rmtF*, respectively. A total of 47.1% (269/571) 16S RMTase genes were associated with Tn*1548*, Tn*2*, IS*Ecp1*, IS*CR14* or IS*91* on single contigs. However, fragmentation of contigs prevented the identification of complete Tn*1548* and Tn*2* transposons that were associated with *armA* (84.8%, 217/256) and *rmtB* (48.8%, 40/82), respectively. Therefore, the total number of 16S RMTase genes associated with these MGEs is likely to be higher than reported. Nevertheless, the association of 16S RMTase genes with MGEs is likely to be contributing to their spread. In Tn*1548*, *armA* was associated with a range of antibiotic resistance genes conferring resistance to antibiotics including β-lactams, chloramphenicol, macrolides, rifampicin, sulphonamides, trimethoprim, streptomycin and spectinomycin meaning the use of these antibiotics could drive the spread of *armA*. Other associations found were *rmtB* with $bla_{\text{TEM-1}}$ (due to its association with Tn*2*), *rmtC* with *sul1* and *rmtF* with *catB*. Therefore, the use of β-lactams, sulphonamides and chloramphenicol will likely drive the spread of *rmtB*, *rmtC* and *rmtF*, respectively.

As carbapenemase genes (particularly bla_{NDM}), *aac(6')-Ib-cr*, $bla_{\text{CTX-M-15}}$ and *qnrB1* were commonly identified in 16S RMTase-producing bacteria, plasmids were extracted and characterised in order to determine if these genes were genetically linked and driving the spread of 16S RMTase genes through co-selection (see Chapter 7). Analysis of the plasmids identified a high proportion of bla_{NDM} genes (84.2%, 32/38) were co-located with 16S RMTase genes on plasmids as well as *aac(6')-Ib-cr* (68.1%, 32/47), but *qnrB1* (27.8%, 5/18) and $bla_{\text{CTX-M-15}}$ (13.3%, 6/45) were co-transferred at lower frequencies. Only a single $bla_{\text{OXA-232}}$ gene was co-transferred along with $bla_{\text{NDM-1}}$ but no other carbapenemase genes were found on 16S RMTase plasmids. This suggests that the spread of 16S RMTase genes is mainly driven through co-location with bla_{NDM} on plasmids rather than other antibiotic resistance genes.

Further analysis identified that most plasmids (53.2%, 33/62) appeared to be novel as they were composed of DNA sequences from multiple published plasmids, where the highest number of novel plasmids was found for *rmtF* (89.5%, 17/19). On the other hand, a variety of

previously identified plasmids (n=20) were observed to be circulating within the UK, with the *rmtB*-positive pM105_FII (accession number: (AP018136.1) and *rmtC*-positive pNDM_22ES (accession number: CM008904.1) the most common (25.0%, 5/20). However, none of the patients were known to have travelled to the countries where these plasmids have been previously reported. Isolates harbouring these plasmids may therefore have spread due to patients interacting with others who have visited the aforementioned countries; alternatively, they may be circulating in the UK due to an unknown reservoir, where horizontal transfer has led to the spread of these plasmids in different species and STs.

8.2 Future Work

Based on the results from the prospective surveillance study, an additional prospective surveillance study involving all regions in the UK should be undertaken, ideally with a larger patient cohort where all patient data are available, in order to identify potential risk factors more reliably and determine a prevalence rate of 16S RMTase genes that is more representative of the whole of the UK. Alternatively, an in-depth study could be conducted in London, which had the highest prevalence of 16S RMTase-producing bacteria, where patient records of those infected with 16S RMTase-producing bacteria as well as a control group of patients with 16S RMTase-negative bacterial infections could be studied in detail. This could help determine how 16S RMTase-producing bacteria are acquired, the type of infections they are associated with and if they contribute to increased morbidity or mortality.

Additionally, 16S RMTase-negative isolates that displayed high-level pan-aminoglycoside resistance (amikacin MIC >256 mg/L; gentamicin MIC >256 mg/L and tobramycin MIC >256 mg/L) should be investigated further to rule out production of novel 16S RMTases. This could be done through the use of MALDI-TOF MS to detect methylation of 16S rRNA in the bacterial ribosomes, which would signify the presence of 16S RMTase activity [545]. This method could have been performed under the supervision of Professor Bruno González-Zorn at the Complutense University of Madrid but this was not possible due to time and finanical constraints. Although the 16S RMTase-negative isolates harboured multiple AMEs that could

account for the pan-aminoglycoside resistance, the presence of a novel AME that could cause high-level pan-aminoglycoside resistance could be investigated as described by Doi *et al*. [546]. In this study a novel AAC gene, *aac(6')-Iad*, was identified by analysing transformants harbouring plasmids containing inserts of genomic DNA from an aminoglycoside-resistant *Acinetobacter* genospecies strain. Analysis of the amino acids following sequencing of the inserts led to the identification of an amino acid sequence sharing identity to AAC(6') enzymes in one transformant and high-performance liquid chromatography (HPLC) was able to confirm the ability of the novel enzyme to perform the acetylation of aminoglycosides.

Furthermore, plasmids extracted from this study should undergo long-read sequencing, which could not be used in this study due to financial and time constraints, in order to allow the assembly and characterisation of the plasmids that appear to be novel. Long-read sequencing should also be used to confirm the genetic environments of 16S RMTase genes, as the generation of fragmented contigs via short-read sequencing made their identification difficult. Long-read sequencing should also be used to identify if the 16S RMTase genes are on plasmids or chromosomes.

To date, the fitness cost of 16S RMTase genes has only been investigated for *armA* [547], *rmtB* [548], *rmtC* [549] and *npmA* [547], which was tested through the use of growth curves and growth competition assays, where *E. coli* strains harbouring 16S RMTase genes were compared with the wild-type strains. *armA*, *rmtB* and *npmA* had a negative effect on bacterial growth (although the effect on growth rate was small for *npmA* [547]), which is believed to be due to 16S RMTases interfering with the ability of endogenous methyltransferases (such as RsmH and RsmI in *E. coli*) to methylate the 16S rRNA [547]. No fitness cost was imposed by *rmtC* [549]. However, Lioy *et al*. [547] believed that the lack of a fitness cost for *rmtC* compared with *armA* and *npmA* in their study was due to differences in experimental conditions. Gutierrez *et al*. [549], who found no fitness cost for *rmtC*, measured the growth rate every hour for a 12-hour period as opposed to every three minutes during the start of the exponential phase so the whole growth cycle was not analysed [547]. Moreover, Gutierrez *et al*. [549]

analysed only 100 bacterial colonies to find resistant strains in the growth competition assays as opposed to 500-1000 colonies in the study by Lioy *et al*. [547]. Conversely, the difference in results may be due to *rmtC* being chromosomally inserted [549], whereas the other studies used plasmids harbouring 16S RMTase genes so the results may have been biased due to variable plasmid copy number [547,548]. Future studies should be conducted to identify the fitness cost of 16S RMTase genes that were chromosomally inserted in order to prevent multiple copies of the plasmids influencing the results. Analysis of the fitness costs of *rmtA* and *rmtD*-*rmtH* may identify why some genes, such as *rmtE* and *rmtH*, have only been identified sporadically.

In this study, many plasmids (58.1%, 36/62) encoded TA systems. These are recognised to contribute to plasmid maintenance and strain maintenance as the loss of a plasmid encoding a TA system leads to cell death due to the inability of the bacterial cell to express the antitoxin [23,24]. Currently, the stability of plasmids from this study with or without genes that encode TA systems is unknown. A study by Yang *et al*. [168] investigated the genetic stability of *armA*- and *rmtB*-harbouring plasmids through continuous passage of conjugants harbouring the plasmids. They identified the plasmids, which mainly encoded *rmtB* (90.6%, 29/32), had high genetic stability as 93.8% (30/32) plasmids were not lost from the host strain after 30 passages. However, the type of media used was unknown and the presence of TA system genes was not investigated, although most plasmids were IncF plasmids (60.0%, 18/30), which are known to harbour addiction systems such as TA systems [344]. This is supported by this study where 90.0% (27/30) plasmids encoding a TA system belonged to IncF, therefore, this may be why the plasmids identified by Yang *et al*. [168] had high genetic stability. Continuous passage of plasmids harbouring 16S RMTase genes using non-selective and selective media (e.g. containing 256 mg/L amikacin) could be used to confirm the stability of plasmids from this study. Furthermore, the fitness cost of plasmids encoding 16S RMTase genes should be investigated as it could explain why some 16S RMTase genes were more successfully transferred on plasmids than others (such as *rmtB* [87.5%, 21/24] compared with

armA [13.6%, 9/66]). Yang *et al*. [168] observed that the plasmids encoding 16S RMTase genes *armA* and *rmtB* had a low fitness cost as no negative effects on the growth rate were found in 87.5% (28/32) conjugants compared with the wild-type strain during growth competition assays. However, as only plasmids encoding *armA* and *rmtB* were tested, it is unknown if plasmids encoding *rmtC* or *rmtF* impose a fitness cost.

Future studies should also consider the occurrence of 16S RMTase genes in other reservoirs such as animals, the food chain and wastewater to see if they are contributing to the emergence in the UK. The occurrence of 16S RMTase genes in bacteria from companion and farm animals has not been reported in the UK but reports from other countries have identified *armA* and *rmtB* in farm animals (including cattle [360,361], poultry [162,166-168,173,354-359] and swine [169,266,315,332]) as well as companion animals (such as cats and dogs [172,351,358,550]). Two studies in the UK have examined the occurrence of 16S RMTases in *Salmonella* spp. [164,200], where one *rmtC*-positive *S. enterica* serovar Virchow isolate was isolated from frozen produce. Additional studies should be undertaken to screen other foodborne pathogens, supermarket meat as well as abattoirs and abattoir workers for the occurrence of 16S RMTase-producers to understand if they are being spread in the food chain. No studies have been conducted in the UK to identify 16S RMTase genes in the environment, although reports from other countries have identified *npmA* in toilet wastewater from planes arriving in an international airport in Copenhagen, Denmark [363]; *armA* and *rmtB* in wastewater from hospitals as well as treatment plant influent and effluent from sewage sites in Basel, Switzerland [225] and *rmtD* from the Tietê River in Brazil [282]. Screening of wastewater and other water samples should therefore be conducted to identify if 16S RMTase-producers are present in environmental reservoirs.

Finally, further research should be undertaken to develop inhibitors of 16S RMTases or novel aminoglycosides that are not affected by 16S RMTases to retain the use of aminoglycosides in treating Gram-negative bacterial infections. Vinal *et al*. [372] discovered three regions within the methyltransferase core fold of NpmA (the β2/β3 linker, the β5/β6 linker and the β6/β7

linker) that were important for binding to the bacterial 30S ribosomal unit or the substrate SAM. Further studies should be conducted to identify the role of these regions in N7-G1405 16S RMTases in ribosome binding and substrate specificity so they can be targeted by future N7-G1405 16S RMTase inhibitors.

8.3 Overall conclusion

Does the emergence of 16S RMTase genes in the UK mean the end for aminoglycosides? Currently, 16S RMTases do not appear to pose a threat to the overall use of aminoglycosides in the UK. The prevalence rate of 16S RMTase-producers in the UK appears to be very low (0.1%), meaning that other aminoglycoside resistance mechanisms such as efflux pumps (e.g. MexXY-OprM in *P. aeruginosa*) or a combination of AMEs (which, in combination, were shown to cause high-level pan-aminoglycoside resistance in this study) play a greater role in aminoglycoside resistance in the UK. At present, it is not necessary for UK diagnostic laboratories to screen for 16S RMTases routinely due to their low prevalence. However, as 16S RMTase genes have been identified in 'high-risk' clones and associated with MGEs, as well as plasmids harbouring other antibiotic resistance genes (predominantly bla_{NDM}), the prevalence of 16S RMTase genes will likely increase over time. Therefore, clinicians and laboratory staff should be made aware of 16S RMTases as a cause of pan-aminoglycoside resistance, particularly in carbapenemase producers. At present, patients harbouring isolates that carry carbapenemase genes are isolated when admitted to hospital. Clinicians will need to be aware that patients colonised or infected with bacteria harbouring 16S RMTase genes may in future need to be isolated due to the strong association of 16S RMTase genes with carbapenemase genes and carriage on MDR plasmids; such dually-resistant strains would be challenging in the event of an outbreak.

The association of 16S RMTase genes with carbapenemase genes as well as other antibiotic resistance genes (such as ESBL and PMQR genes) results in fewer treatment options for MDR Gram-negative bacterial infections. To avoid recourse to last-line potentially toxic antibiotics, such as colistin, newer agents with alternative targets are required. Meantime,

continued surveillance supported by appropriate infection prevention and control measures are needed to limit spread of such high-level aminoglycoside-resistant organisms.

References

1. Fleming A. On antibacterial action of culture of penicillium, with special reference to their use in isolation of *B. influenzae*. *British Journal of Experimental Pathology*. 1929; 10: 226-236.
2. Rossolini GM, Arena F, Pecile P, Pollini S. Update on the antibiotic resistance crisis. *Current Opinion in Pharmacology*. 2014; 18: 56-60.
3. vanHoek AHAM, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJM. Acquired antibiotic resistance genes: an overview. *Frontiers in Microbiology*. 2011; 2: 203. Available from: doi: 10.3389/fmicb.2011.00203.
4. Aminov RI. A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in Microbiology*. 2010; 1: 134. Available from: doi: 10.3389/fmicb.2010.00134.
5. O'Neill J. *Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance.* Available from: https://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf [Accessed 18th January 2019].
6. European Centre for Disease Prevention and Control/European Medicines Agency (ECDC/EMEA) Joint Working Group. *The bacterial challenge: time to react. A call to narrow the gap between multidrug-resistant bacteria in the EU and the development of new antibacterial agents.* Available from: https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf [Accessed 18th January 2019].
7. Centres for Disease Control and Prevention (CDC). *Antibiotic resistance threats in the United States, 2013*. Available from: http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf [Accessed 18th January 2019].
8. McNulty CA, Boyle P, Nichols T, Clappison P, Davey P. The public's attitudes to and compliance with antibiotics. *Journal of Antimicrobial Chemotherapy*. 2007; 60(1): 63-68.
9. WHO. *The safety of medicines in public health programmes: pharmacovigilance an essential tool*. Available from: https://www.who.int/medicines/areas/quality_safety/safety_efficacy/PharmacovigilanceB.pdf [Accessed 3rd March 2019].
10. Kelesidis T, Kelesidis I, Rafailidis PI, Falagas ME. Counterfeit or substandard antimicrobial drugs: a review of the scientific evidence. *Journal of Antimicrobial Chemotherapy*. 2007; 60(2): 214-236.
11. Zaman SB, Hussain MA, Nye R, Mehta V, Mamun KT, Hossain N. A review on antibiotic resistance: alarm bells are ringing. *Cureus*. 2017; 9(6): e1403. Available from: doi: 10.7759/cureus.1403.
12. Holmes AH, Moore LS, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, *et al*. Understanding the mechanisms and drivers of antimicrobial resistance. *The Lancet*. 2016; 387(10014): 176-187.
13. Rath S, Patra BK. Dispersal of antibiotic resistant bacteria into aquatic environment - an overview. *Journal of Water Pollution and Control*. 2018; 1(1:2): 1-3.
14. Magnet S, Blanchard JS. Molecular insights into aminoglycoside action and resistance. *Chemical Reviews*. 2005; 105(2): 477-498.
15. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*. 2012; 18: 268-281.

16. Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology*. 2002; 292(2): 107-113.
17. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents*. 2010; 35(4): 322-332.
18. Hall CW, Mah TF. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Reviews*. 2017; 41(3): 276-301.
19. Mandakhalikar KD, Rahmat JN, Chiong E, Neoh KG, Shen L, Tambyah PA. Extraction and quantification of biofilm bacteria: method optimized for urinary catheters. *Scientific Reports.* 2018; 8(1): 8069. Available from: doi: 10.1038/s41598-018-26342-3.
20. Sultan I, Rahman S, Jan AT, Siddiqui MT, Mondal AH, Haq QMR. Antibiotics, resistome and resistance mechanisms: a bacterial perspective. *Frontiers in Microbiology*. 2018; 9: 2066.
21. Harms A, Maisonneuve E, Gerdes K. Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science*. 2016; 354(6318): aaf4268. Available from: doi: 10.1126/science.aaf4268.
22. Hall AM, Gollan B, Helaine S. Toxin-antitoxin systems: reversible toxicity. *Current Opinions in Microbiology*. 2017; 36: 102-110.
23. Yang QE, Walsh TR. Toxin-antitoxin systems and their role in disseminating and maintaining antimicrobial resistance. *FEMS Microbiology Reviews*. 2017; 41(3): 343-353.
24. Kamruzzaman M, Shoma S, Thomas CM, Partridge SR, Iredell JR. Plasmid interference for curing antibiotic resistance plasmids *in vivo*. *PLoS One*. 2017; 12(2): e0172913. Available from: doi: 10.1371/journal.pone.0172913.
25. Gerdes K, Rasmussen PB, Molin S. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1986; 83(10): 3116-3120.
26. Zhivich A. Fighting bacterial resistance: approaches, challenges, and opportunities in the search for new antibiotics. Part 1. Antibiotics used in clinical practice: mechanisms of action and the development of bacterial resistance. *Microbiology Independent Research Journal.* 2017; 4(1): 31-51.
27. CDC. *Examples of how antibiotic resistance spreads*. Available from: https://www.cdc.gov/drugresistance/resources/digital_materials.html [Accessed 3rd March 2019].
28. Syal K, Mo M, Yu H, Iriya R, Jing W, Guodong S, *et al*. Current and emerging techniques for antibiotic susceptibility tests. *Theranostics*. 2017; 7(7): 1795-1805.
29. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*. 2008; 3(2): 163-175.
30. Bonev B, Hooper J, Parisot J. Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. *Journal of Antimicrobial Chemotherapy*. 2008; 61(6): 1295-1301.
31. Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases*. 2009; 49(11): 1749-1755.
32. EUCAST. *New definitions of S, I and R*. Available from: http://www.eucast.org/newsiandr/ [Accessed 13th May 2019].
33. Pulido MR, García-Quintanilla M, Martín-Peña R, Cisneros JM, McConnell MJ. Progress on the development of rapid methods for antimicrobial susceptibility testing. *Journal of Antimicrobial Chemotherapy*. 2013; 68(12): 2710-2717.
34. Roschanski N, Fischer J, Guerra B, Roesler U. Development of a multiplex real-time PCR for the rapid detection of the predominant beta-lactamase genes CTX-M, SHV,

TEM and CIT-type AmpCs in Enterobacteriaceae. *PLoS One*. 2014; 9(7): e100956. Available from: doi: 10.1371/journal.pone.0100956.
35. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. *Diagnostic Microbiology and Infectious Disease*. 2011; 70(1): 119-123.
36. Oviaño M, Gómara M, Barba MJ, Revillo MJ, Barbeyto LP, Bou G. Towards the early detection of β-lactamase-producing Enterobacteriaceae by MALDI-TOF MS analysis. *Journal of Antimicrobial Chemotherapy*. 2017; 72(8): 2259-2262.
37. Maxson T, Taylor-Howell CL, Minogue TD. Semi-quantitative MALDI-TOF for antimicrobial susceptibility testing in *Staphylococcus aureus*. *PLoS One*. 2017; 12(8): e0183899. Available from: doi: 10.1371/journal.pone.0183899.
38. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrobial Agents and Chemotherapy*. 2014; 58(1): 212-220.
39. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, *et al*. Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy*. 2012; 67(11): 2640-2644.
40. Inouye M, Dashnow H, Raven LA, Schultz MB, Pope BJ, Tomita T, *et al*. SRST2: rapid genomic surveillance for public health and hospital microbiology labs. *Genome Medicine*. 2014; 6: 90. Available from: doi: 10.1186/s13073-014-0090-6.
41. Gilad J, Giladi M, Poch F, Aharoni Y, Schwartz D. “All-in-one-plate” E-test and disk diffusion susceptibility co-testing for multiresistant *Acinetobacter baumannii*. *European Journal of Clinical Microbiology and Infectious Diseases*. 2006; 25(12): 799-802.
42. van Belkum A, Tassios PT, Dijkshoorn L, Haeggman S, Cookson B, Fry NK, *et al*. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clinical Microbiology and Infection*. 2007; 13(S3): 1-46. Available from: doi: 10.1111/j.1469-0691.2007.01786.x.
43. Li W, Raoult D, Fournier PE. Bacterial strain typing in the genomic era. *FEMS Microbiology Reviews*. 2009; 33(5): 892-916.
44. Woodford N, Turton JF, Livermore DM. Multi-resistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiology Reviews*. 2011; 35(5): 736-755.
45. Goering RV. Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. *Infection, Genetics and Evolution*. 2010; 10(7): 866-875.
46. Herschleb J, Ananiev G, Schwartz DC. Pulsed-field gel electrophoresis. *Nature Protocols*. 2007; 2(3): 677-684.
47. Lindstedt BA. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. *Electrophoresis*. 2005; 26(13): 2567-2582.
48. Sabat AJ, Budimir A, Nashev D, Sá-Leão R, van Dijl Jm, Laurent F. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Euro Surveillance*. 2013; 18(4): 20380.
49. Maiden MC, Jansen van Rensburg MJ, Bray JE, Earle SG, Ford SA, Jolley KA, *et al*. MLST revisited: the gene-by-gene approach to bacterial genomics. *Nature Reviews Microbiology*. 2013; 11(10): 728-736.
50. Seidl K, Leimer N, Palheiros Marques M, Furrer A, Holzmann-Bürgel A, Senn G, *et al*. Clonality and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* at the University Hospital Zurich, Switzerland between 2012 and 2014. *Annals of Clinical*

Microbiology and Antimicrobials. 2015; 14: 14. Available from: doi: 10.1186/s12941-015-0075-3.
51. Salipante SJ, SenGupta DJ, Cummings LA, Land TA, Hoogestraat DR, Cookson BT. Application of whole-genome sequencing for bacterial strain typing in molecular epidemiology. *Journal of Clinical Microbiology*. 2015; 53(4): 1072-1079.
52. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Computational Biology*. 2017; 13(6): e1005595. Available from: doi: 10.1371/journal.pcbi.1005595.
53. Arredondo-Alonso S, Willems RJ, van Schaik W, Schürch AC. On the (im)possibility of reconstructing plasmids from whole-genome short-read sequencing data. *Microbial Genomics*. 2017; 3(10): e000128. Available from: doi: 10.1099/mgen.0.000128.
54. Koren S, Phillippy AM. One chromosome, one contig: complete microbial genomes from long-read sequencing and assembly. *Current Opinion in Microbiology*. 2015; 23: 110-120.
55. Pollard MO, Gurdasani D, Mentzer AJ, Porter T, Sandhu MS. Long reads: their purpose and place. *Human Molecular Genetics*. 2018; 27(R2): R234-R241. Available from: doi: 10.1093/hmg/ddy177.
56. Brown D. Antibiotic resistance breakers: can repurposed drugs fill the antibiotic discovery void? *Nature Reviews Drug Discovery*. 2015; 14: 821-832.
57. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, *et al*. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet*. 2018; 18(3): 318-327.
58. Livermore DM. Current epidemiology and growing resistance of Gram-negative pathogens. *Korean Journal of Internal Medicine*. 2012; 27(2): 128-142.
59. Tamma PD, Cosgrove SE, Maragakis LL. Combination therapy for treatment of infections with Gram-negative bacteria. *Clinical Microbiology Reviews*. 2012; 25(3): 450-470.
60. Durante-Mangoni E, Grammatikos A, Utili R, Falagas ME. Do we still need aminoglycosides? *International Journal of Antimicrobial Agents*. 2009; 33(3): 201-205.
61. Shakil S, Khan R, Zarrilli R, Khan AU. Aminoglycosides versus bacteria-a description of the action, resistance mechanism, and nosocomial battleground. *Journal of Biomedicine Science*. 2008; 15(1): 5-14.
62. WHO. *WHO list of critically important antimicrobials for human medicine*. Available from: https://www.who.int/foodsafety/publications/cia2017.pdf [Accessed 4th March 2019].
63. Vakulenko SB, Mobashery S. Versatility of aminoglycosides and prospects for their future. *Clinical Microbiology Reviews*. 2003; 16(3): 430-450.
64. Jackson J, Chen C, Buising K. Aminoglycosides: how should we use them in the 21st century? *Current Opinion in Infectious Diseases*. 2013; 26(6): 516-525.
65. Serio AW, Keepers T, Andrews L, Krause KM. Aminoglycoside revival: review of a historically important class of antimicrobials undergoing rejuvenation. *EcoSal Plus*. 2018; 8(1). Available from: doi: 10.1128/ecosalplus.ESP-0002-2018.
66. Krause KM, Serio AW, Kane TR, Connolly LE. Aminoglycosides: an overview. *Cold Spring Harbor Perspectives in Medicine*. 2016; 6(6): a027029. Available from: doi: 10.1101/cshperspect.a027029.
67. Jones D, Metzger HJ, Schatz A, Waksman SA. Control of Gram-negative bacteria in experimental animals by streptomycin. *Science*. 1944; 100(2588): 103-105.
68. Medical Research Council. Treatment of pulmonary tuberculosis with streptomycin and para-aminosalicylic acid. *British Medical Journal*. 1950; 2: 1073-1085.

69. Takahashi Y, Igarashi M. Destination of aminoglycoside antibiotics in the 'post-antibiotic era'. *The Journal of Antibiotics.* [Preprint] 2017. Available from: doi: 10.1038/ja.2017.117.
70. Kawaguchi H, Naito T, Nakagawa S, Fujisawa KI. BB-K8, a new semisynthetic aminoglycoside antibiotic. *The Journal of Antibiotics.* 1972; 25(12): 695-708.
71. Waksman SA, Lechevalier HA. Neomycin, a new antibiotic active against streptomycin-resistant bacteria, including tuberculosis organisms. *Science*. 1949; 109(2830): 305-307.
72. Pittenger RC, Wolfe RN, Hoehn MM, Marks PN, Daily WA, McGuire JM. Hygromycin. I. Preliminary studies on the production and biologic activity of a new antibiotic. *Antibiotics & Chemotherapy*. 1953; 3(12): 1268-1278.
73. Umezawa H. Kanamycin: its discovery. *Annals of the New York Academy of Sciences*. 1958; 76(2): 20-26.
74. Frohardt R, Haskell TH, John E, Penner K. Antibiotic and methods for obtaining same: United states patent office; 1959. US2916485A.
75. Mason DJ, Dietz A, Smith RM. Actinospectacin, a new antibiotic. I. Discovery and biological properties. *Antibiotics & Chemotherapy*. 1961; 11: 118-122.
76. Weinstein MJ, Luedemann GM, Oden EM, Wagman GH, Rosselet JP, Marquez JA, *et al*. Gentamicin, a new antibiotic complex from *Micromonospora*. *Journal of Medicinal Chemistry*. 1963; 6(4): 463-464.
77. Higgins CE, Kastner RE. Nebramycin, a new broad-spectrum antibiotic complex. II. Description of *Streptomyces tenebrarius*. *Antimicrobial Agents and Chemotherapy*. 1967; 7: 324-331.
78. Wick WE, Welles JS. Nebramycin, a new broad-spectrum antibiotic complex. IV. *In vitro* and *in vivo* laboratory evaluation. *Antimicrobial Agents and Chemotherapy*. 1967; 7: 341-348.
79. Shomura T, Ezaki N, Tsuruoka T, Niwa T, Akita E. Studies on antibiotic SF-733, a new antibiotic. I. Taxonomy, isolation and characterization. *Journal of Antibiotics*. 1970; 23(3): 155-161.
80. Weinstein MJ, Marquez JA, Testa RT, Wagman GH, Oden EM, Waitz JA. Antibiotic 6640, a new *Micromonospora*-produced aminoglycoside antibiotic. *Journal of Antibiotics*. 1970; 23(11): 551-554.
81. Oda T, Mori T, Itŏ H, Kunieda T, Munakata K. Studies on new antibiotic lividomycins. I. Taxonomic studies on the lividomycin-producing strain *Streptomyces lividus* nov. sp. *Journal of Antibiotics*. 1971; 24(6): 333-338.
82. Nara T, Yamamoto M, Kawamoto I, Takayama K, Okachi R, Takasawa S, *et al*. Fortimicins A and B, new aminoglycoside antibiotics. I. Producing organism, fermentation and biological properties of fortimicins. *Journal of Antibiotics*. 1977; 30(7): 533-540.
83. Kondo S, Hotta K. Semisynthetic aminoglycoside antibiotics: development and enzymatic modifications. *Journal of Infection and Chemotherapy*. 1999; 5(1): 1-9.
84. Rahal JJ, Simberkoff MS, Kagan K, Moldover NH. Bactericidal efficacy of Sch 20569 and amikacin against gentamicin-sensitive and -resistant organisms. *Antimicrobial Agents and Chemotherapy*. 1976; 9(4): 595-599.
85. Oizumi K, Ariji F, Kumano N, Oka S, Konno K. Action mechanism of 3',4'-dideoxykanamycin B (DKB) on *Klebsiella pneumoniae*. *The Scientific Reports of the Research Institutes, Tohoku University*. 1974; 21(3-4): 47-53.
86. Wright J, Daniels PJL. Aminoacyl derivatives of aminoglycoside antibiotics; 1975. BE824657.

87. Aggen JB, Armstrong ES, Goldblum AA, Dozzo P, Linsell MS, Gliedt MJ, *et al*. Synthesis and spectrum of the neoglycoside ACHN-490. *Antimicrobial Agents and Chemotherapy*. 2010; 54(11): 4636-4642.
88. Spelman DW, McDonald M, Spicer WJ. Aminoglycoside antibiotic agents: a review. *The Medical Journal of Australia.* 1989; 151(6): 346-349.
89. Jana S, Deb JK. Molecular understanding of aminoglycoside action and resistance. *Applied Microbiology and Biotechnology*. 2006; 70(2): 140-150.
90. Ramirez MS, Tolmasky ME. Amikacin: uses, resistance, and prospects for inhibition. *Molecules*. 2017; 22: e2267. Available from: doi: 10.3390/molecules22122267.
91. Freeman CD, Nicolau DP, Belliveau PP, Nightingale CH. Once-daily dosing of aminoglycosides: review and recommendations for clinical practice. *Journal of Antimicrobial Chemotherapy*. 1997; 39(6): 677-686.
92. Mingeot-Leclercq MP, Tulkens PM. Aminoglycosides: nephrotoxicity. *Antimicrobial Agents and Chemotherapy*. 1999; 43(5): 1003-1012.
93. Lanvers-Kaminsky C, Zehnhoff-Dinnesen AA, Parfitt R, Ciarimboli G. Drug-induced ototoxicity: mechanisms, pharmacogenetics, and protective strategies. *Clinical Pharmacology and Therapeutics*. 2017; 101(4): 491-500.
94. PHE. *English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) report 2018*. Available from: https://www.gov.uk/government/publications/english-surveillance-programme-antimicrobial-utilisation-and-resistance-espaur-report [Accessed 4th March 2019].
95. Davis BD. Mechanism of bactericidal action of aminoglycosides. *Microbiological Reviews*. 1987; 51: 341-350.
96. Arenz S, Wilson DN. Bacterial protein synthesis as a target for antibiotic inhibition. *Cold Spring Harbor Perspectives in Medicine*. 2016; 6(9): a025361. Available from: doi: 10.1101/cshperspect.a025361.
97. Wilson DN. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nature Reviews Microbiology*. 2013; 12(1): 35-48.
98. Robinson A, van Oijen AM. Bacterial replication, transcription and translation: mechanistic insights from single-molecule biochemical studies. *Nature Reviews Microbiology*. 2013; 11(5): 303-315.
99. Hancock RE, Farmer SW, Li ZS, Poole K. Interaction of aminoglycosides with the outer membranes and purified lipopolysaccharide and OmpF porin of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. 1991; 35(7): 1309-1314.
100. Bryan LE, Kwan S. Roles of ribosomal binding, membrane potential, and electron transport in bacterial uptake of streptomycin and gentamicin. *Antimicrobial Agents and Chemotherapy*. 1983; 23: 835-845.
101. Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. Aminoglycosides: activity and resistance. *Antimicrobial Agents and Chemotherapy*. 1999; 43(4): 727-737.
102. Kondo J, Westhof E. Aminoglycoside antibiotics: structural decoding of inhibitors targeting the ribosomal decoding A site. In: Gualerzi CO, Brandi L, Fabbretti A, Pon CL. *Antibiotics: targets, mechanisms and resistance.* Weinheim, Germany: Wiley-VCH; 2013. 453-470.
103. Garneau-Tsodikova S, Labby KJ. Mechanisms of resistance to aminoglycoside antibiotics: overview and perspectives. *Medchemcomm*. 2016; 7(1): 11-27.
104. ECDC. *Surveillance of antimicrobial resistance in Europe 2017*. Available from: https://ecdc.europa.eu/sites/portal/files/documents/EARS-Net-report-2017-update-jan-2019.pdf [Accessed 4th March 2019].
105. Fernández L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE. Adaptive resistance to the “last hope” antibiotics polymyxin B and colistin in *Pseudomonas*

aeruginosa is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrobial Agents and Chemotherapy*. 2010; 54(8): 3372-3382.
106. Fei Y, Ma V, Maerkl N, You P. The down regulation of *E. coli* OmpF in response to sub-inhibitory concentrations of kanamycin is not mediated by MarA. *Journal of Experimental Microbiology and Immunology*. 2012; 16: 101-107.
107. Sato M, Machida K, Arikado E, Saito H, Kakegawa T, Kobayashi H. Expression of outer membrane proteins in *Escherichia coli* growing at acid pH. *Applied and Environmental Microbiology*. 2000; 66(3): 943-947.
108. Kaeriyama M, Machida K, Kitakaze A, Wang H, Lao Q, Fukamachi T, *et al*. OmpC and OmpF are required for growth under hyperosmotic stress above pH 8 in *Escherichia coli*. *Letters in Applied Microbiology*. 2006; 42(3): 195-201.
109. Rosenberg EY, Ma D, Nikaido H. AcrD of *Escherichia coli* is an aminoglycoside efflux pump. *Journal of Bacteriology*. 2000; 182(6): 1754-1756.
110. Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrobial Agents and Chemotherapy*. 2001; 45(12): 3375-3380.
111. Islam S, Jalal S, Wretlind B. Expression of the MexXY efflux pump in amikacin-resistant isolates of *Pseudomonas aeruginosa*. *Clinical Microbiology and Infection*. 2004; 10: 877-883.
112. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. *Drug Resistance Updates*. 2010; 13: 151-171.
113. Novick RP, Clowes RC, Cohen SN, Curtiss R, Datta N, Falkow S. Uniform nomenclature for bacterial plasmids: a proposal. *Bacteriological Reviews*. 1976; 40(1): 168-189.
114. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews*. 1993; 57(1): 138-163.
115. Vanhoof R, Hannecart-Pokorni E, Content J. Nomenclature of genes encoding aminoglycoside-modifying enzymes. *Antimicrobial Agents and Chemotherapy*. 1998; 42(2): 483.
116. Robicsek A, Strahlilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, *et al*. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nature Medicine*. 2006; 12: 83-88.
117. Frasson I, Cavallaro A, Bergo C, Richter SN, Palù G. Prevalence of *aac(6')-Ib-cr* plasmid-mediated and chromosome-encoded fluoroquinolone resistance in Enterobacteriaceae in Italy. *Gut Pathogens*. 2011; 3(1): 12. Available from: doi: 10.1186/1757-4749-3-12.
118. Ferretti JJ, Gilmore KS, Courvalin P. Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. *Journal of Bacteriology*. 1986; 167(2): 631-638.
119. Aghazadeh M, Rezaee MA, Nahaei MR, Mahdian R, Pajand O, Saffari F, *et al*. Dissemination of aminoglycoside-modifying enzymes and 16S rRNA methylases among *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates. *Microbial Drug Resistance*. 2013; 19(4): 282-288.
120. Wright GD. Aminoglycoside-modifying enzymes. *Current Opinion in Microbiology*. 1999; 2(5): 499-503.
121. Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clinical Microbiology Reviews*. 1995; 8(4): 496-514.
122. Honoré N, Cole ST. Streptomycin resistance in mycobacteria. *Antimicrobial Agents and Chemotherapy*. 1994; 38(2): 238-242.

123. Böttger EC. Resistance to drugs targeting protein synthesis in mycobacteria. *Trends in Microbiology*. 1994; 2(10): 416-421.
124. Springer B, Kidan YG, Prammananan T, Ellrott K, Böttger EC, Sander P. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. *Antimicrobial Agents and Chemotherapy*. 2001; 45(10): 2877-2884.
125. Lee H, Yong D, Yum JH, Roh KH, Lee K, Yamane K, *et al*. Dissemination of 16S rRNA methylase-mediated highly amikacin-resistant isolates of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in Korea. *Diagnostic Microbiology and Infectious Disease*. 2006; 56(3): 305-312.
126. Liou GF, Yoshizawa S, Courvalin P, Galimand M. Aminoglycoside resistance by ArmA-mediated ribosomal 16S methylation in human bacterial pathogens. *Journal of Molecular Biology*. 2006; 359(2): 358-364.
127. Wachino J, Arakawa Y. Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: an update. *Drug Resistance Updates*. 2012; 15(3): 133-148.
128. Cundliffe E. How antibiotic-producing organisms avoid suicide. *Annual Review of Microbiology*. 1989; 43: 207-233.
129. Gniadkowski M, Schneider I, Palucha A, Jungwirth R, Mikiewicz B, Bauernfeind A. Cefotaxime-resistant Enterobacteriaceae isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing β-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. *Antimicrobial Agents and Chemotherapy*. 1998; 42(4): 827-832.
130. Galimand M, Courvalin P, Lambert T. Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation. *Antimicrobial Agents and Chemotherapy*. 2003; 47(8): 2565-2571.
131. Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, *et al*. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. *The Lancet*. 2003; 362: 1888-1893.
132. Wachino J, Shibayama K, Kimura K. Yamane K, Suzuki S, Arakawa Y. RmtC introduces G1405 methylation in 16S rRNA and confers high-level aminoglycoside resistance on Gram-positive microorganisms. *FEMS Microbiology Letters*. 2010; 311(1): 56-60.
133. Marsh JW, Pacey MP, Ezeonwuka C, Ohm SL, Snyder D, Cooper VS, *et al*. *Clostridioides difficile*: a potential source of NpmA in the clinical environment. *Journal of Antimicrobial Chemotherapy*. 2019; 74(2): 521-523.
134. Martinez FJ, Leffler DA, Kelly CP. *Clostridium difficile* outbreaks: prevention and treatment strategies. *Risk Management and Healthcare Policy*. 2012; 5: 55-64.
135. Doi Y, Yokoyama K, Yamane K, Wachino J, Shibata N, Yagi T, *et al*. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high level resistance to aminoglycosides. *Antimicrobial Agents and Chemotherapy*. 2004; 48(2): 491-496.
136. Costello SE, Deshpande LM, Davis AP, Mendes RE, Castanheria M. Aminoglycoside-modifying enzymes and 16S ribosomal RNA methyltransferases-encoding genes among a global collection of Gram-negative isolates. *Journal of Global Antimicrobial Resistance*. 2019; 16: 278-285.
137. Wachino J, Yamane K, Shibayama K, Kurokawa H, Shibata N, Suzuki S, *et al*. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. *Antimicrobial Agents and Chemotherapy*. 2006; 50(1): 178-184.
138. Doi Y, de Oliveira Garcia D, Adams J, Paterson DL. Coproduction of novel 16S rRNA methylase RmtD and metallo-β-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. *Antimicrobial Agents and Chemotherapy*. 2007; 51(3): 852-856.

139. Tijet N, Andres P, Chung C, Lucero C, WHONET-Argentina Group, Low DE, *et al*. *rmtD2*, a new allele of a 16S rRNA methylase gene, has been present in Enterobacteriaceae isolates from Argentina for more than a decade. *Antimicrobial Agents and Chemotherapy*. 2011; 55: 904-909.
140. Tada T, Shimada K, Mya S, Zan KN, Kuwahara K, Kirikae T, *et al.* A new variant of 16S rRNA methylase, RmtD3, in a clinical isolate of *Pseudomonas aeruginosa* in Myanmar. *Antimicrobial Agents and Chemotherapy*. 2018; 62(1): e01806-17. Available from: doi: 10.1128/AAC.01806-17.
141. Davis MA, Baker KN, Orfe LH, Shah DH, Besser TE, Call DR. Discovery of a gene conferring multiple-aminoglycoside tesistance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. 2010; 54(6): 2666-2669.
142. Xia J, Sun J, Li L, Fang LX, Deng H, Yang RS, *et al*. First report of the IncI1/ST898 conjugative plasmid carrying *rmtE2* 16S rRNA methyltransferase gene in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. 2015; 59(12): 7921-7922.
143. Galimand M, Courvalin P, Lambert T. RmtF, a new member of the aminoglycoside resistance 16S rRNA N7 G1405 methyltransferase family. *Antimicrobial Agents and Chemotherapy*. 2012; 56: 3960-3962.
144. Tada T, Shimada K, Satou K, Hirano T, Pokhrel BM, Sherchand JB, *et al*. Metallo-β-lactamases (DIM-1, NDM-1, VIM-2) and a 16S rRNA methyltransferase (RmtB4, RmtF2) producing *Pseudomonas aeruginosa* in Nepal. *Antimicrobial Agents and Chemotherapy*. 2017; 61: e00694-17. Available from: doi: 10.1128/AAC.00694-17.
145. Bueno MFC, Francisco GR, O'Hara JA, de Oliveira Garcia D, Doi Y. Coproduction of 16S rRNA methyltransferase RmtD or RmtG with KPC-2 and CTX-M group extended-spectrum β-lactamases in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*. 2013; 57(5): 2397-2400.
146. O'Hara JA, McGann P, Snesrud EC, Clifford RJ, Waterman PE, Lesho EP, *et al*. Novel 16S rRNA methyltransferase RmtH produced by *Klebsiella pneumoniae* associated with war-related trauma. *Antimicrobial Agents and Chemotherapy*. 2013; 57(5): 2413-2416.
147. Wachino J, Shibayama K, Kurokawa H, Kimura K, Yamane K, Suzuki S, *et al*. Novel plasmid-mediated 16S rRNA m1A1408 methyltransferase, NpmA, found in a clinically isolated *Escherichia coli* strain resistant to structurally diverse aminoglycosides. *Antimicrobial Agents and Chemotherapy*. 2007; 51(12): 4401-4409.
148. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*. 2018; 35(6): 1547-1549.
149. Doi Y, Wachino JI, Arakawa Y. Aminoglycoside resistance: the emergence of acquired 16S ribosomal RNA methyltransferases. *Infectious Disease Clinics of North America*. 2016; 30(2): 523-537.
150. Tada T, Miyoshi-Akiyama T, Shimada K, Nga TT, Thu le TA, Son NT, *et al*. Dissemination of clonal complex 2 *Acinetobacter baumannii* stains co-producing carbapenemases and 16S rRNA methylase *armA* in Vietnam. *BMC Infectious Diseases*. 2015; 15: 433. Available from: doi: 10.1186/s12879-015-1171-x.
151. Hasani A, Sheikhalizadeh V, Ahangarzadeh Rezaee M, Rahmati-Yamchi M, Hasani A, Ghotaslou R, *et al*. Frequency of aminoglycoside-modifying enzymes and *armA* among different sequence groups of *Acinetobacter baumannii* in Iran. *Microbial Drug Resistance*. 2016; 22(5): 347-353.
152. Kang HY, Kim KY, Kim J, Lee JC, Lee YC, Cho DT, *et al*. Distribution of conjugative-plasmid-mediated 16S rRNA methylase genes among amikacin-resistant Enterobacteriaceae isolates collected in 1995 to 1998 and 2001 to 2006 at a university hospital in South Korea and identification of conjugative plasmids mediating

dissemination of 16S rRNA methylase. *Journal of Clinical Microbiology*. 2008; 46(2): 700-706.
153. Bogaerts P, Galimand M, Bauraing C, Deplano A, Vanhoof R, De Mendonca R, *et al*. Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. *Journal of Antimicrobial Chemotherapy*. 2007; 59(3): 459-464.
154. Fritsche TR, Castanheira M, Miller GH, Jones RN, Armstrong ES. Detection of methyltransferases conferring high-level resistance to aminoglycosides in Enterobacteriaceae from Europe, North America, and Latin America. *Antimicrobial Agents and Chemotherapy*. 2008; 52(5): 1843-1845.
155. Wu JJ, Chen HM, Ko WC, Wu HM, Tsai SH, Yan JJ. Prevalence of extended-spectrum β-lactamases in *Proteus mirabilis* in a Taiwanese university hospital, 1999 to 2005: identification of a novel CTX-M enzyme (CTX-M-66). *Diagnostic Microbiology and Infectious Disease*. 2008; 60(2): 169-175.
156. Lee HW, Kang HY, Shin KS, Kim J. Multidrug-resistant *Providencia* isolates carrying bla_{PER-1}, bla_{VIM-2}, and *armA*. *Journal of Microbiology*. 2007; 45(3): 272-274.
157. Zhou Y, Yu H, Guo Q, Xu X, Ye X, Wu S, *et al*. Distribution of 16S rRNA methylases among different species of Gram-negative bacilli with high-level resistance to aminoglycosides. *European Journal of Clinical Microbiology & Infectious Diseases*. 2010; 29(11): 1349-1353.
158. Bouzidi N, Aoun L, Dekhil M, Granier SA, Poirel L, Brisabois A, *et al*. Co-occurrence of aminoglycoside resistance gene *armA* in non-Typhi *Salmonella* isolates producing CTX-M-15 in Algeria. *Journal of Antimicrobial Chemotherapy*. 2011; 66(1): 2180-2181.
159. Granier SA, Hidalgo L, San Millan A, Escudero JA, Gutierrez B, Brisabois A, *et al*. ArmA methyltransferase in a monophasic *Salmonella enterica* isolate from food. *Antimicrobial Agents and Chemotherapy*. 2011; 55(11): 5262-5266.
160. Moissenet D, Weill FX, Arlet G, Harrois D, Girardet JP, Vu-Thien H. *Salmonella enterica* serotype Gambia with CTX-M-3 and *armA* resistance markers: nosocomial infections with a fatal outcome. *Journal of Clinical Microbiology*. 2011; 49(4): 1676-1678.
161. Naas T, Bentchouala C, Cuzon G, Yaou S, Lezzar A, Smati F, *et al*. Outbreak of *Salmonella enterica* serotype Infantis producing ArmA 16S RNA methylase and CTX-M-15 extended-spectrum β-lactamase in a neonatology ward in Constantine, Algeria. *International Journal of Antimicrobial Agents*. 2011; 38: 135-139.
162. Du XD, Li DX, Hu GZ, Wang Y, Shang YH, Wu CM, *et al*. Tn*1548*-associated *armA* is co-located with *qnrB2*, *aac(6')-Ib-cr* and $bla_{CTX-M-3}$ on an IncFII plasmid in a *Salmonella enterica* subsp. *enterica* serovar Paratyphi B strain isolated from chickens in China. *Journal of Antimicrobial Chemotherapy*. 2012; 67: 246-248.
163. Folster JP, Rickert R, Barzilay EJ, Whichard JM. Identification of the aminoglycoside resistance determinants *armA* and *rmtC* among non-typhi *Salmonella* Isolates from humans in the United States. *Antimicrobial Agents and Chemotherapy*. 2009; 53(10): 4563-4564.
164. Hidalgo L, Hopkins KL, Wareham DW, Gutierrez B, González-Zorn B. Association of extended-spectrum β-Lactamase VEB-5 and 16S rRNA methyltransferase ArmA in *Salmonella enterica* from the United Kingdom. *Antimicrobial Agents and Chemotherapy*. 2012; 56(9): 4985-4987.
165. Galimand M, Sabtcheva S, Courvalin P, Lambert T. Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn*1548*. *Antimicrobial Agents and Chemotherapy*. 2005; 49: 2949-2953.
166. Du XD, Wu CM, Liu HB, Li XS, Beier RC, Xiao F, *et al*. Plasmid-mediated ArmA and RmtB 16S rRNA methylases in *Escherichia coli* isolated from chickens. *Journal of Antimicrobial Chemotherapy*. 2009; 64(6): 1328-1330.

167. Du XD, Liu HB, Wu CM, Li XS, Cao XY, Cui BA, *et al*. The genetic environment of *armA* on pHNE, an IncN plasmid, in one *Escherichia coli* isolate from a chicken. *Journal of Antimicrobial Chemotherapy*. 2010; 65(12): 2694-2695.
168. Yang Y, Zhang A, Lei C, Wang H, Guan Z, Xu C, *et al*. Characteristics of plasmids coharboring 16S rRNA methylases, CTX-M, and virulence factors in *Escherichia coli* and *Klebsiella pneumoniae* isolates from chickens in China. *Foodborne Pathogens and Disease*. 2015; 12(11): 873-880.
169. Deng Y, Zeng Z, Chen S, He L, Liu Y, Wu C, *et al*. Dissemination of IncFII plasmids carrying *rmtB* and *qepA* in *Escherichia coli* from pigs, farm workers and the environment. *Clinical Microbiology and Infection*. 2011; 17(11): 1740-1745.
170. Liu JH, Deng YT, Zeng ZL, Gao JH, Chen L, Arakawa Y, *et al*. Coprevalence of plasmid-mediated quinolone resistance determinants QepA, Qnr, and AAC(6')-Ib-cr among 16S rRNA methylase RmtB-producing *Escherichia coli* isolates from pigs. *Antimicrobial Agents and Chemotherapy*. 2008; 52(8): 2992-2993.
171. Choi MJ, Lim SK, Nam HM, Kim AR, Jung SC, Kim MN. Apramycin and gentamicin resistances in indicator and clinical *Escherichia coli* isolates from farm animals in Korea. *Foodborne Pathogens and Disease*. 2011; 8(1): 119-123.
172. Xia J, Fang LX, Cheng K, Xu GH, Wang XR, Liao XP, *et al*. Clonal Spread of 16S rRNA methyltransferase-producing *Klebsiella pneumoniae* ST37 with high prevalence of ESBLs from companion animals in China. *Frontiers in Microbiology*. 2017; 8: 529. Available from: doi: 10.3389/fmicb.2017.00529.
173. Wang Y, Zhang A, Yang Y, Lei C, Jiang W, Liu B, *et al*. Emergence of *Salmonella enterica* serovar Indiana and California isolates with concurrent resistance to cefotaxime, amikacin and ciprofloxacin from chickens in China. *International Journal of Food Microbiology*. 2017; 262: 23-30.
174. Nagasawa M, Kaku M, Kamachi K, Shibayama K, Arakawa Y, Yamaguchi K, *et al*. Loop-mediated isothermal amplification assay for 16S rRNA methylase genes in Gram-negative bacteria. *Journal of Infection and Chemotherapy*. 2014; 20(10): 635-638.
175. Yamane K, Wachino J, Doi Y, Kurokawa H, Arakawa Y. Global spread of multiple aminoglycoside resistance genes. *Emerging Infectious Diseases*. 2015; 11(6): 951-953.
176. Yamane K, Wachino J, Suzuki S, Shibata N, Kato H, Shibayama K, *et al*. 16S rRNA methylase producing, Gram-negative pathogens, Japan. *Emerging Infectious Diseases*. 2007; 13: 642-646.
177. Shin SY, Kwon KC, Park JW, Song JH, Ko YH, Sung JY, *et al*. Characteristics of *aac(6')-Ib-cr* gene in extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from Chungnam area. *Korean Journal of Laboratory Medicine*. 2009; 29(6): 541-550.
178. Jin JS, Kwon KT, Moon DC, Lee JC. Emergence of 16S rRNA methylase *rmtA* in colistin-only-sensitive *Pseudomonas aeruginosa* in South Korea. *International Journal of Antimicrobial Agents*. 2009; 33(5): 490-491.
179. Upadhyay S, Joshi SR, Khyriem AB, Bhattacharya P. Acquired 16S methyl transferase associated high level aminoglycoside resistance in *Acinetobacter baumannii* recovered from ICU patients from a tertiary referral hospital of northeast India. *International Journal of Infectious Diseases*. 2016; 45(1): 46. Available from: doi: 10.1016/j.ijid.2016.02.144.
180. Wangkheimayum J, Paul D, Dhar D, Nepram R, Chetri S, Bhowmik D, *et al*. Occurrence of acquired 16S methyltransferase mediated aminoglycoside resistance in clinical isolates of Enterobacteriaceae within a tertiary referral hospital of northeast India. *Antimicrobial Agents and Chemotherapy*. 2017; 61(6): e01037-16. Available from: doi: 10.1128/AAC.01037-16.

181. Dakhl ZF, Alwan SK. Dissemination of aminoglycosides resistance in *Pseudomonas aeruginosa* isolates in Al-Diwaniya hospitals. *International Journal of Advanced Research*. 2015; 3(11): 376-384.
182. Poirel L, Schrenzel J, Cherkaoui A, Bernabeu S, Renzi G, Nordmann P. Molecular analysis of NDM-1-producing enterobacterial isolates from Geneva, Switzerland. *Journal of Antimicrobial Chemotherapy*. 2011; 66(8): 1730-1733.
183. Chen F, Wang L, Wang M, Xie Y, Xia X, Li X, *et al*. Genetic characterization and *in vitro* activity of antimicrobial combinations of multidrug-resistant *Acinetobacter baumannii* from a general hospital in China. *Oncology Letters*. 2018; 15(2): 2305-2315.
184. Islam MA, Talukdar PK, Hoque A, Huq M, Nabi A, Ahmed D, *et al*. Emergence of multidrug-resistant NDM-1-producing Gram-negative bacteria in Bangladesh. *European Journal of Clinical Microbiology & Infectious Diseases.* 2012; 31(10): 2593-2600.
185. Sidjabat HE, Townell N, Nimmo GR, George NM, Robson J, Vohra R, *et al*. Dominance of IMP-4-producing *Enterobacter cloacae* among carbapenemase-producing Enterobacteriaceae in Australia. *Antimicrobial Agents and Chemotherapy*. 2015; 59(7): 4059-4066.
186. Heang V, Hout B, Prouty MG, Supraprom C, Ford GW, Newell SW, *et al*. Detection of *qnrVC* and *rmtB* genes from a multidrug-resistant *Ralstonia pickettii* wound infection isolate in Cambodia. *International Journal of Antimicrobial Agents*. 2014; 44(1): 84-85.
187. Al-Gallas N, Abbassi MS, Gharbi B, Manai M, Ben Fayala MN, Bichihi R, *et al*. Occurrence of plasmid-mediated quinolone resistance determinants and *rmtB* gene in *Salmonella enterica* serovar enteritidis and Typhimurium isolated from food-animal products in Tunisia. *Foodborne Pathogens and Disease*. 2013; 10(9): 813-819.
188. Castanheira M, Deshpande LM, Melano RG, Mendes RE, Jones RN. Characterization of two new variants of 16S rRNA methylase encoding genes, *rmtB2* and *rmtB3*. Poster session presented at: 22nd European Congress of Clinical Microbiology & Infectious Diseases (ECCMID); 2012 April 1st; London, United Kingdom.
189. Al Sheikh YA, Marie MA, John J, Krishnappa LG, Dabwab KH. Prevalence of 16S rRNA methylase genes among β-lactamase-producing Enterobacteriaceae clinical isolates in Saudi Arabia. *Libyan Journal of Medicine*. 2014; 9: 24432. Available from: doi: 10.3402/ljm.v9.24432.
190. Sartor AL, Raza MW, Abbasi SA, Day KM, Perry JD, Paterson DL, *et al*. Molecular epidemiology of NDM-1-producing Enterobacteriaceae and *Acinetobacter baumannii* isolates from Pakistan. *Antimicrobial Agents and Chemotherapy*. 2014; 58(9): 5589-5593.
191. Haldorsen BC, Simonsen GS, Sundsfjord A, Samuelsen O; Norwegian Study Group on Aminoglycoside Resistance. Increased prevalence of aminoglycoside resistance in clinical isolates of *Escherichia coli* and *Klebsiella* spp. in Norway is associated with the acquisition of AAC(3)-II and AAC(6′)-Ib. *Diagnostic Microbiology and Infectious Disease*. 2014; 78(1): 66-69.
192. Kapmaz M, Erdem F, Abulaila A, Yeniaras E, Oncul O, Aktas Z. First detection of NDM-1 with CTX-M-9, TEM, SHV and *rmtC* in *Escherichia coli* ST471 carrying IncI2, A/C and Y plasmids from clinical isolates in Turkey. *Journal of Global Antimicrobial Resistance*. 2016; 7: 152-153.
193. Carattoli A, Villa L, Poirel L, Bonnin RA, Nordmann P. Evolution of IncA/C bla_{CMY-2} carrying plasmids by acquisition of the bla_{NDM-1} carbapenemase gene. *Antimicrobial Agents and Chemotherapy*. 2012; 56(2): 783-786.
194. Tijet N, Richardson D, MacMullin G, Patel SN, Melano RG. Characterization of multiple NDM-1-producing Enterobacteriaceae isolates from the same patient. *Antimicrobial Agents and Chemotherapy*. 2015; 59(6): 3648-3651.

195. Kiaei S, Moradi M, Hosseini Nave H, Hashemizadeh Z, Taati-Moghadam M, Kalantar-Neyestanaki D. Emergence of co-existence of *bla*$_{NDM}$ with *rmtC* and *qnrB* genes in clinical carbapenem-resistant *Klebsiella pneumoniae* isolates in burning center from southeast of Iran. *Folia Microbiologica*. 2018; 64(1): 55-62.
196. Al-Kadmy IMS, Musafer HK, Al-Kaabi MHAA, Al-Jubori SS. Detection of 16S ribosomal RNA methylation in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* clinical isolates from Baghdad hospitals. *Iraqi Journal of Biotechnology*. 2015; 14(1): 53-62.
197. Tada T, Miyoshi-Akiyama T, Dahal RK, Mishra SK, Ohara H, Shimada K, *et al*. Dissemination of multidrug-resistant *Klebsiella pneumoniae* clinical isolates with various combinations of carbapenemases (NDM-1 and OXA-72) and 16S rRNA methylases (ArmA, RmtC and RmtF) in Nepal. *International Journal of Antimicrobial Agents.* 2013; 42(4): 367-378.
198. Williamson DA, Sidjabat HE, Freeman JT, Roberts SA, Silvey A, Woodhouse R, *et al.* Identification and molecular characterisation of New Delhi metallo-β-lactamase-1 (NDM-1)- and NDM-6-producing Enterobacteriaceae from New Zealand hospitals. *International Journal of Antimicrobial Agents.* 2012; 39(6): 529-533.
199. Tada T, Tsuchiya M, Shimada K, Nga TTT, Thu LTA, Phu TT, *et al*. Dissemination of carbapenem-resistant *Klebsiella pneumoniae* clinical isolates with various combinations of carbapenemases (KPC-2, NDM-1, NDM-4, and OXA-48) and 16S rRNA methylases (RmtB and RmtC) in Vietnam. *BMC Infectious Diseases*. 2017; 17(1): 467. Available from: doi: 10.1186/s12879-017-2570-y.
200. Hopkins KL, Escudero JA, Hidalgo L, Gonzalez-Zorn B. 16S rRNA methyltransferase RmtC in *Salmonella enterica* serovar Virchow. *Emerging Infectious Diseases*. 2010; 16(4): 712-715.
201. Huang J, Deng S, Ren J, Tu J, Ye M, Wang M. Characterization of a *bla*$_{NDM-1}$-harboring plasmid from a *Salmonella enterica* clinical isolate in China. *Molecular Medicine Reports.* 2017; 16(2): 1087-1092.
202. Saranathan R, Kumari R, Kalaivani R, Suresh S, Rani A, Purty S, *et al*. Detection of IS*Aba1* in association with a novel allelic variant of the β-lactamase ADC-82 and class D β-lactamase genes mediating carbapenem resistance among the clinical isolates of MDR *A. baumannii*. *Journal of Medical Microbiology*. 2017; 66(2): 103-111.
203. Bado I, Papa-Ezdra R, Delgado-Blas JF, Gaudio M, Gutiérrez C, Cordeiro NF, *et al*. Molecular characterization of carbapenem-resistant *Acinetobacter baumannii* in the intensive care unit of Uruguay's university hospital identifies the first *rmtC* gene in the species. *Microbial Drug Resistance*. 2018; 24(7): 1012-1019.
204. Mohanam L, Menon T. Emergence of *rmtC* and *rmtF* 16S rRNA methyltransferase in clinical isolates of *Pseudomonas aeruginosa*. *Indian Journal of Medical Microbiology.* 2017; 35(2): 282-285.
205. Gamal D, Fernández-Martínez M, Salem D, El-Defrawy I, Montes LÁ, Ocampo-Sosa AA, *et al*. Carbapenem-resistant *Klebsiella pneumoniae* isolates from Egypt containing *bla*$_{NDM-1}$ on IncR plasmids and its association with *rmtF*. *International Journal of Infectious Diseases*. 2016; 43: 17-20.
206. Hidalgo L, Hopkins KL, Gutierrez B, Ovejero CM, Shukla S, Douthwaite S, *et al*. Association of the novel aminoglycoside resistance determinant RmtF with NDM carbapenemase in Enterobacteriaceae isolated in India and the UK. *Journal of Antimicrobial Chemotherapy*. 2013; 68(7): 1543-1550.
207. Filgona F, Banerjee T, Anupurba S. Incidence of the novel *rmtF* and *rmtG* methyltransferases in carbapenem-resistant Enterobacteriaceae from a hospital in India. *Journal of Infection in Developing Countries*. 2015; 9(9): 1036-1039.

208. Khong WX, Marimuthu K, Teo J, Ding Y, Xia E, Lee JJ, *et al*. Tracking inter-institutional spread of NDM and identification of a novel NDM-positive plasmid, pSg1-NDM, using next-generation sequencing approaches. *Journal of Antimicrobial Chemotherapy.* 2016; 71(11): 3081-3089.
209. Yoon EJ, Kang DY, Yang JW, Kim D, Lee H, Lee KJ, *et al*. New Delhi metallo-β-lactamase-producing Enterobacteriaceae in South Korea between 2010 and 2015. *Frontiers in Microbiology*. 2018; 9: 571. Available from: doi: 10.3389/fmicb.2018.00571.
210. Mancini S, Poirel L, Corthesy M, Greub G, Nordmann P. *Klebsiella pneumoniae* co-producing KPC and RmtG, finally targeting Switzerland. *Diagnostic Microbiology and Infectious Disease*. 2017; 90(2): 151-152.
211. Lee CS, Vasoo S, Hu F, Patel R, Doi Y. *Klebsiella pneumoniae* ST147 coproducing NDM-7 carbapenemase and RmtF 16S rRNA methyltransferase in Minnesota. *Journal of Clinical Microbiology*. 2014; 52(11): 4109-4110.
212. Rubin JE, Peirano G, Peer AK, Govind CN, Pitout JD. NDM-1-producing Enterobacteriaceae from South Africa: moving towards endemicity? *Diagnostic Microbiology and Infectious Disease*. 2014; 79(3): 378-380.
213. Hu F, Munoz-Price LS, DePascale D, Rivera JI, Doi Y. *Klebsiella pneumoniae* sequence type 11 isolate producing RmtG 16S rRNA methyltransferase from a patient in Miami, Florida. *Antimicrobial Agents and Chemotherapy*. 2014; 58(8): 4980-4981.
214. Beyrouthy R, Robin F, Hamze M, Bonnet R. IncFIIk plasmid harbouring an amplification of 16S rRNA methyltransferase-encoding gene *rmtH* associated with mobile element IS*CR2*. *Journal of Antimicrobial Chemotherapy*. 2017; 72(2): 402-406.
215. Berçot B, Poirel L, Nordmann P. Plasmid-mediated 16S rRNA methylases among extended-spectrum β-lactamase-producing Enterobacteriaceae isolates. *Antimicrobial Agents and Chemotherapy*. 2008; 52(12): 4526-4527.
216. Strateva T, Markova B, Marteva-Proevska Y, Ivanova D, Mitov I. Widespread dissemination of multidrug-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemase and ArmA 16S ribosomal RNA methylase in a Bulgarian university hospital. *Brazilian Journal of Infectious Diseases*. 2012; 16(3): 307-310.
217. El-Sayed-Ahmed MA, Amin MA, Tawakol WM, Loucif L, Bakour S, Rolain JM. High prevalence of bla_{NDM-1} carbapenemase-encoding gene and 16S rRNA *armA* methyltransferase among *Acinetobacter baumannii* clinical isolates, Egypt. *Antimicrobial Agents and Chemotherapy*. 2015; 59(6): 3602-3605.
218. Poirel L, Savov E, Nazli A, Trifonova A, Todorova I, Gergova I, *et al*. Outbreak caused by NDM-1- and RmtB-producing *Escherichia coli* in Bulgaria. *Antimicrobial Agents and Chemotherapy*. 2014; 58(4): 2472-2474.
219. Poirel L, Labarca J, Bello H, Rioseco ML, Bernabeu S, Nordmann P. Emergence of the 16S rRNA methylase RmtG in an extended-spectrum-β-lactamase-producing and colistin-resistant *Klebsiella pneumoniae* isolate in Chile. *Antimicrobial Agents and Chemotherapy*. 2014; 58(1): 618-619.
220. Ayad A, Drissi M, de Curraize C, Dupont C, Hartmann A, Solanas S, *et al*. Occurrence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in extended-spectrum β-lactamases producing *Escherichia coli* in Algerian hospitals. *Frontiers in Microbiology*. 2016; 7: 1409. Available from: doi: 10.3389/fmicb.2016.01409.
221. Berçot B, Poirel L, Ozdamar M, Hakko E, Türkoglu S, Nordmann P. Low prevalence of 16S methylases among extended-spectrum-β-lactamase-producing Enterobacteriaceae from a Turkish hospital. *Journal of Antimicrobial Chemotherapy*. 2010; 65(4): 797-798.
222. Ho PL, Lo WU, Yeung MK, Lin CH, Chow KH, Ang I, *et al*. Complete sequencing of pNDM-HK encoding NDM-1 carbapenemase from a multidrug-resistant *Escherichia coli*

strain isolated in Hong Kong. *PLoS One*. 2011; 6(3): e17989. Available from: doi: 10.1371/journal.pone.0017989.
223. Samuelsen Ø, Toleman MA, Hasseltvedt V, Fuursted K, Leegaard TM, Walsh TR, *et al*. Molecular characterization of VIM-producing *Klebsiella pneumoniae* from Scandinavia reveals genetic relatedness with international clonal complexes encoding transferable multidrug resistance. *Clinical Microbiology and Infection.* 2011; 17(12): 1811-1816.
224. Pitart C, Solé M, Roca I, Román A, Moreno A, Vila J, *et al*. Molecular characterization of bla_{NDM-5} carried on an IncFII plasmid in an *Escherichia coli* isolate from a nontraveler patient in Spain. *Antimicrobial Agents and Chemotherapy.* 2015; 59(1): 659-662.
225. Zurfluh K, Bagutti C, Brodmann P, Alt M, Schulze J, Fanning S, *et al*. Wastewater is a reservoir for clinically relevant carbapenemase- and 16S rRNA methylase-producing Enterobacteriaceae. *International Journal of Antimicrobial Agents*. 2017; 50(3): 436-440.
226. McGann P, Hang J, Clifford RJ, Yang Y, Kwak YI, Kuschner RA, *et al*. Complete sequence of a novel 178-kilobase plasmid carrying bla_{NDM-1} in a *Providencia stuartii* strain isolated in Afghanistan. *Antimicrobial Agents and Chemotherapy*. 2012; 56(4): 1673-1679.
227. Poirel L, Goutines J, Aires-de-Sousa M, Nordmann P. High rate of association of 16S rRNA methylases and carbapenemases in Enterobacteriaceae recovered from hospitalized children in Angola. *Antimicrobial Agents and Chemotherapy*. 2018; 62(4): e00021-18. Available from: doi: 10.1128/AAC.00021-18.
228. Quiles MG, Rocchetti TT, Fehlberg LC, Kusano EJ, Chebabo A, Pereira RM, *et al*. Unusual association of NDM-1 with KPC-2 and *armA* among Brazilian Enterobacteriaceae isolates. *Brazilian Journal of Medical and Biological Research*. 2015; 48(2): 174-177.
229. Tojo M, Mawatari M, Hayakawa K, Nagamatsu M, Shimada K, Mezaki K, *et al*. Multidrug-resistant *Acinetobacter baumannii* isolated from a traveller returned from Brunei. *Journal of Infection and Chemotherapy*. 2015; 21(3): 212-214.
230. Denisuik AJ, Karlowsky JA, Denisuik T, Nichols WW, Keating TA, Adam HJ, *et al*. *In vitro* activity of ceftazidime-avibactam against 338 molecularly characterized gentamicin-nonsusceptible Gram-negative clinical isolates obtained from patients in Canadian Hospitals. *Antimicrobial Agents and Chemotherapy*. 2015; 59(6): 3623-3626.
231. Cao XL, Cheng L, Zhang ZF, Ning MZ, Zhou WQ, Zhang, *et al*. Survey of clinical extended-spectrum beta-lactamase-producing *Enterobacter cloacae* isolates in a Chinese tertiary hospital, 2012-2014. *Microbial Drug Resistance*. 2017; 23(1): 83-89.
232. Pulss S, Semmler T, Prenger-Berninghoff E, Bauerfeind R, Ewers C. First report of an *Escherichia coli* strain from swine carrying an OXA-181 carbapenemase and the colistin resistance determinant MCR-1. *International Journal of Antimicrobial Agents*. 2017; 50(2): 232-236.
233. Galani I, Anagnostoulis G, Chatzikonstantinou M, Petrikkos G, Souli M. Emergence of *Klebsiella pneumoniae* co-producing OXA-48, CTX-M-15, and ArmA in Greece. *Clinical Microbiology and Infection*. 2016; 22(10): 898-899.
234. Waterman PE, McGann P, Snesrud E, Clifford RJ, Kwak YI, Munoz-Urbizo IP, *et al*. Bacterial peritonitis due to *Acinetobacter baumannii* sequence type 25 with plasmid-borne New Delhi metallo-β-lactamase in Honduras. *Antimicrobial Agents and Chemotherapy*. 2013; 57(9): 4584-4586.
235. Ho PL, Leung LM, Chow KH, Lai EL, Lo WU, Ng TK. Prevalence of aminoglycoside modifying enzyme and 16S ribosomal RNA methylase genes among aminoglycoside-resistant *Escherichia coli* isolates. *Journal of Microbiology, Immunology and Infection*. 2016; 49(1): 123-136.
236. Chatterjee S, Datta S, Roy S, Ramanan L, Saha A, Viswanathan R, *et al*. Carbapenem resistance in *Acinetobacter baumannii* and other *Acinetobacter* spp. causing neonatal

sepsis: focus on NDM-1 and its linkage to IS*Aba125*. *Frontiers in Microbiology*. 2016; 7: 1126. Available from: doi: 10.3389/fmicb.2016.01126.
237. Brigante G, Migliavacca R, Bramati S, Motta E, Nucleo E, Manenti M, *et al*. Emergence and spread of a multidrug-resistant *Acinetobacter baumannii* clone producing both the carbapenemase OXA-23 and the 16S rRNA methylase ArmA. *Journal of Medical Microbiology*. 2012; 61(5): 653-661.
238. Revathi G, Siu LK, Lu PL, Huang LY. First report of NDM-1-producing *Acinetobacter baumannii* in East Africa. *International Journal of Infectious Diseases*. 2013; 17(12): e1255-58. Available from: doi: 10.1016/j.ijid.2013.07.016.
239. Saule M, Samuelsen Ø, Dumpis U, Sundsfjord A, Karlsone A, Balode A, *et al*. Dissemination of a carbapenem-resistant *Acinetobacter baumannii* strain belonging to International Clone II/sequence type 2 and harboring a novel AbaR4-like resistance island in Latvia. *Antimicrobial Agents and Chemotherapy*. 2013; 57(2): 1069-1072.
240. Salloum T, Arabaghian H, Alousi S, Abboud E, Tokajian S. Genome sequencing and comparative analysis of an NDM-1-producing *Klebsiella pneumoniae* ST15 isolated from a refugee patient. *Pathogens and Global Health*. 2017; 111(4): 166-175.
241. Lean SS, Yeo CC, Suhaili Z, Thong KL. Comparative genomics of two ST 195 carbapenem-resistant *Acinetobacter baumannii* with different susceptibility to polymyxin revealed underlying resistance mechanism. *Frontiers in Microbiology*. 2016; 6: 1445. Available from: doi: 10.3389/fmicb.2015.01445.
242. Shrestha S, Tada T, Shrestha B, Kirikae T, Ohara H, Rijal BP, *et al*. Emergence of aminoglycoside resistance due to *armA* methylase in multi-drug resistant *Acinetobacter baumannii* isolates in a university hospital in Nepal. *Journal of Nepal Health Research Council*. 2016; 14(33): 72-76.
243. Karah N, Haldorsen B, Hermansen NO, Tveten Y, Ragnhildstveit E, Skutlaberg DH, *et al*. Emergence of OXA-carbapenemase- and 16S rRNA methylase-producing international clones of *Acinetobacter baumannii* in Norway. *Journal of Medical Microbiology*. 2011; 60(4): 515-521.
244. Dortet L, Poirel L, Al Yaqoubi F, Nordmann P. NDM-1, OXA-48 and OXA-181 carbapenemase-producing Enterobacteriaceae in Sultanate of Oman. *Clinical Microbiology and Infection*. 2012; 18(5): E144-E148. Available from: doi: 10.1111/j.1469-0691.2012.03796.x.
245. Martinez T, Martinez I, Vazquez GJ, Aquino EE, Robledo IE. Genetic environment of the KPC gene in *Acinetobacter baumannii* ST2 clone from Puerto Rico and genomic insights into its drug resistance. *Journal of Medical Microbiology*. 2016; 65(8): 784-792.
246. Dortet L, Bonnin RA, Bernabeu S, Escaut L, Vittecoq D, Girlich D, *et al*. First occurrence of OXA-72-producing *Acinetobacter baumannii* in Serbia. *Antimicrobial Agents and Chemotherapy*. 2016; 60(10): 5724-5730.
247. Blackwell GA, Holt KE, Bentley SD, Hsu LY, Hall RM. Variants of AbGRI3 carrying the *armA* gene in extensively antibiotic-resistant *Acinetobacter baumannii* from Singapore. *Journal of Antimicrobial Chemotherapy*. 2017; 72(4): 1031-1039.
248. González-Zorn B, Catalan A, Escudero JA, Domínguez L, Teshager T, Porrero C, *et al*. Genetic basis for dissemination of *armA*. *Journal of Antimicrobial Chemotherapy*. 2005; 56(3): 583-585.
249. Karah N, Dwibedi CK, Sjöström K, Edquist P, Johansson A, Wai SN, *et al*. Novel aminoglycoside resistance transposons and transposon derived circular forms detected in carbapenem-resistant *Acinetobacter baumannii* clinical isolate. *Antimicrobial Agents and Chemotherapy*. 2016; 60(3): 1801-1818.
250. Sonnevend A, Ghazawi A, Al Munthari N, Pitout M, Hamadeh MB, Hashmey R, *et al*. Characteristics of epidemic and sporadic strains of *Acinetobacter baumannii* isolated in Abu Dhabi hospitals. *Journal of Medical Microbiology*. 2013; 62(4): 582-590.

251. Doi Y, Hazen TH, Boitano M, Tsai YC, Clark TA, Korlach J, *et al*. Whole-genome assembly of *Klebsiella pneumoniae* coproducing NDM-1 and OXA-232 carbapenemases using single-molecule, real-time sequencing. *Antimicrobial Agents and Chemotherapy*. 2014; 58(10): 5947-5953.
252. Tada T, Miyoshi-Akiyama T, Kato Y, Ohmagari N, Takeshita N, Hung NV, *et al*. Emergence of 16S rRNA methylase-producing *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates in hospitals in Vietnam. *BMC Infectious Diseases*. 2013; 13: 251. Available from: doi: 10.1186/1471-2334-13-251.
253. Bakour S, Alsharapy SA, Touati A, Rolain JM. Characterization of *Acinetobacter baumannii* clinical isolates carrying *bla*$_{\text{OXA-23}}$ carbapenemase and 16S rRNA methylase *armA* genes in Yemen. *Microbial Drug Resistance*. 2014; 20(6): 604-609.
254. Yeganeh Sefidan F, Mohammadzadeh-Asl Y, Ghotaslou R. High-level resistance to aminoglycosides due to 16S rRNA methylation in Enterobacteriaceae isolates. *Microbial Drug Resistance*. [Preprint] 2019. Available from: doi: 10.1089/mdr.2018.0171.
255. Kieffer N, Nordmann P, Aires-de-Sousa M, Poirel L. High prevalence of carbapenemase-producing Enterobacteriaceae among hospitalized children in Luanda, Angola. *Antimicrobial Agents and Chemotherapy*. 2016; 60(10): 6189-6192.
256. Bartoloni A, Sennati S, Di Maggio T, Mantella A, Riccobono E, Strohmeyer M, *et al*. Antimicrobial susceptibility and emerging resistance determinants (*bla*$_{\text{CTX-M}}$, *rmtB*, *fosA3*) in clinical isolates from urinary tract infections in the Bolivian Chaco. *International Journal of Infectious Diseases*. 2016; 43: 1-6.
257. Walkty A, Karlowsky JA, Baxter MR, Adam HJ, Boyd D, Bharat A, *et al*. Frequency of 16S ribosomal RNA methyltransferase detection among *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates obtained from patients in Canadian hospitals (CANWARD, 2013–2017). *Diagnostic Microbiology and Infectious Disease*. 2019; 94(2): 199-201.
258. Helaly GF, Abou Shleib H, Fanaki NH, Abdel Kader O, Ali GH. Potential co-prevalence of plasmid-mediated quinolone resistance determinant *qepA* and 16S rRNA methylase *rmtB* among *E. coli* clinical Isolates from Alexandria- Egypt. *The Journal of the Egyptian Public Health Association*. 2010; 85(5-6): 247-272.
259. Galani L, Galani I, Souli M, Karaiskos I, Katsouda E, Patrozou E, *et al*. Nosocomial dissemination of *Providencia stuartii* isolates producing extended-spectrum β-lactamases VEB-1 and SHV-5, metallo-β-lactamase VIM-1, and RNA methylase RmtB. *Journal of Global Antimicrobial Resistance*. 2013; 1(2): 115-116.
260. Shakil S, Akram M, Ali SM, Khan AU. Acquisition of extended-spectrum beta-lactamase producing *Escherichia coli* strains in male and female infants admitted to a neonatal intensive care unit: molecular epidemiology and analysis of risk factors. *Journal of Medical Microbiology*. 2010; 59(8): 948-954.
261. Nematzadeh S, Shahcheraghi F, Iversen A, Giske CG. Successful international clones of *bla*$_{\text{CTX-M-15}}$-producing *Klebsiella pneumoniae* with coexpression of plasmid-mediated quinolone resistance (PMQR) determinants in Tehran hospitals. *Diagnostic Microbiology & Infectious Disease*. 2015; 83(4): 371-374.
262. Taitt CR, Leski TA, Erwin DP, Odundo EA, Kipkemoi NC, Ndonye JN, *et al*. Antimicrobial resistance of *Klebsiella pneumoniae* stool isolates circulating in Kenya. *PLOS one*. 2017; 12(6): e0178880. Available from: doi: 10.1371/journal.pone.0178880.
263. Yan JJ, Wu JJ, Ko WC, Tsai SH, Chuang CL, Wu HM, *et al*. Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from two Taiwanese hospitals. *Journal of Antimicrobial Chemotherapy*. 2004; 54(6): 1007-1012.
264. Sonnevend Á, Ghazawi A, Hashmey R, Haidermota A, Girgis S, Alfaresi M, *et al*. Multihospital occurrence of pan-resistant *Klebsiella pneumoniae* sequence type 147 with an IS*Ecp1*-directed *bla*$_{\text{OXA-181}}$ insertion in the *mgrB* gene in the United Arab

Emirates. *Antimicrobial Agents and Chemotherapy*. 2017; 61(7): e00418-17. Available from: doi: 10.1128/AAC.00418-17.
265. Mancini S, Poirel L, Tritten ML, Lienhard R, Bassi C, Nordmann P. Emergence of an MDR *Klebsiella pneumoniae* ST231 producing OXA-232 and RmtF in Switzerland. *Journal of Antimicrobial Chemotherapy*. 2018; 73(3): 821-823.
266. Yao Q, Zeng Z, Hou J, Deng Y, He L, Tian W, *et al*. Dissemination of the *rmtB* gene carried on IncF and IncN plasmids among Enterobacteriaceae in a pig farm and its environment. *Journal of Antimicrobial Chemotherapy*. 2011; 66(11): 2475-2479.
267. Poirel L, Bonnin RA, Nordmann P. Analysis of the resistome of a multidrug-resistant NDM-1-producing *Escherichia coli* strain by high-throughput genome sequencing. *Antimicrobial Agents and Chemotherapy*. 2011; 55(9): 4224-4229.
268. Ma XJ, Yang HF, Liu YY, Mei Q, Ye Y, Li HR, *et al*. The emergence of the 16S rRNA methyltransferase RmtB in a multidrug-resistant *Serratia marcescens* isolate in China. *Annals of Laboratory Medicine*. 2015; 35: 172-174.
269. Meradji S, Barguigua A, Bentakouk MC, Nayme K, Zerouali K, Mazouz D, *et al*. Epidemiology and virulence of VIM-4 metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolates from burn patients in eastern Algeria. *Burns*. 2016; 42: 906-918.
270. Deng YT, Zeng ZL, Tian W, Yang T, Liu JH. Prevalence and characteristics of *rmtB* and *qepA* in *Escherichia coli* isolated from diseased animals in China. *Frontiers in Microbiology*. 2013; 4: 198. Available from: doi: 10.3389/fmicb.2013.00198.
271. Liu BT, Liao XP, Yue L, Chen XY, Li L, Yang SS, *et al*. Prevalence of β-lactamase and 16S rRNA methylase genes among clinical *Escherichia coli* isolates carrying plasmid-mediated quinolone resistance genes from animals. *Microbial Drug Resistance*. 2013; 19(3): 237-245.
272. Mushtaq S, Irfan S, Sarma JB, Doumith M, Pike R, Pitout J, *et al*. Phylogenetic diversity of *Escherichia coli* strains producing NDM-type carbapenemases. *Journal of Antimicrobial Chemotherapy*. 2011; 66(9): 2002-2005.
273. Li JJ, Munoz-Price LS, Spychala CN, DePascale D, Doi Y. New Delhi metallo-β-lactamase-1–producing *Klebsiella pneumoniae*, Florida, USA. *Emerging Infectious Disease*. 2016; 22(4): 744-748.
274. Guo X, Dillon BB, Ginn AN, Wiklendt AM, Partridge SR, Iredell JR. Simple multiplex real-time PCR for rapid detection of common 16S rRNA methyltransferase genes. *Diagnostic Microbiology and Infectious Disease*. 2014; 80(1): 29-31.
275. Urbanowicz P, Izdebski R, Baraniak A, Żabicka D, Ziółkowski G, Hryniewicz W, *et al*. *Pseudomonas aeruginosa* with NDM-1, DIM-1 and PME-1 β-lactamases, and RmtD3 16S rRNA methylase, encoded by new genomic islands. *Journal of Antimicrobial Chemotherapy*. [Preprint] 2019. Available from: doi: 10.1093/jac/dkz262.
276. Gokemn TG, Nagiyev T, Meral M, Onlen C, Heydari F, Koksal F. NDM-1 and *rmtC*-producing *Klebsiella pneumoniae* isolates in Turkey. *Jundishapur Journal of Microbiology.* 2016; 9(10): e33990. Available from: doi: 10.5812/jjm.33990.
277. Rahman M, Prasad KN, Pathak A, Pati BK, Singh A, Ovejero CM, *et al*. RmtC and RmtF 16S rRNA methyltransferase in NDM-1–producing *Pseudomonas aeruginosa*. *Emerging Infectious Diseases*. 2015; 21(11): 2059-2062.
278. Dortet L, Flonta M, Boudehen YM, Creton E, Bernabeu S, Vogel A, *et al*. Dissemination of carbapenemase-producing Enterobacteriaceae and *Pseudomonas aeruginosa* in Romania. *Antimicrobial Agents and Chemotherapy*. 2015; 59(11): 7100-7103.
279. Bueno MF, Francisco GR, de Oliveira Garcia D, Doi Y. Complete sequences of multidrug resistance plasmids bearing *rmtD1* and *rmtD2* 16S rRNA methyltransferase genes. *Antimicrobial Agents and Chemotherapy*. 2016; 60(3): 1928-1931.

280. Yamane K, Rossi F, Baberino MGMA, Adams-Haduch JM, Doi Y, Paterson DL. 16S ribosomal RNA methylase RmtD produced by *Klebsiella pneumoniae* in Brazil. *Journal of Antimicrobial Chemotherapy.* 2008; 61(3): 746-747.
281. Castanheira M, Fritsche TR, Sader HS, Jones RN. RmtD 16S RNA methylase in epidemiologically unrelated SPM-1-producing *Pseudomonas aeruginosa* isolates from Brazil. *Antimicrobial Agents and Chemotherapy*. 2008; 52(4): 1587-1588.
282. Fontes LC, Neves PR, Oliveira S, Silva KC, Hachich EM, Sato MI, *et al*. Isolation of *Pseudomonas aeruginosa* coproducing metallo-β-lactamase SPM-1 and 16S rRNA methylase RmtD1 in an urban river. *Antimicrobial Agents and Chemotherapy*. 2011; 55(6): 3063-3064.
283. Cavalcanti FLDS, Mirones CR, Paucar ER, Montes LA, Leal-Balbino TC, de Morais, MMC, *et al*. Mutational and acquired carbapenem resistance mechanisms in multidrug resistant *Pseudomonas aeruginosa* clinical isolates from Recife, Brazil. *Memórias do Instituto Oswaldo Cruz*. 2015; 110(8): 1003-1009.
284. Doi Y, Ghilardi AC, Adams J, de Oliveira Garcia D, Paterson DL. High prevalence of metallo-β-lactamase and 16S rRNA methylase coproduction among imipenem-resistant *Pseudomonas aeruginosa* isolates in Brazil. *Antimicrobial Agents and Chemotherapy*. 2007; 51(9): 3388-3390.
285. Lincopan, N, Neves P, Mamizuka EM, Levy CE. Balanoposthitis caused by *Pseudomonas aeruginosa* co-producing metallo-β-lactamase and 16S rRNA methylase in children with hematological malignancies. *International Journal of Infectious Diseases*. 2010; 14(4): e344-e347. Available from: doi: 10.1016/j.ijid.2009.04.016.
286. Silveira MC, Albano RM, Asensi MD, Carvalho-Assef AP. Description of genomic islands associated to the multidrug-resistant *Pseudomonas aeruginosa* clone ST277. *Infection, Genetics and Evolution*. 2016; 42: 60-65.
287. Moura Q, Fernandes MR, Cerdeira L, Santos ACM, de Souza TA, Lenne S, *et al*. Draft genome sequence of a multidrug-resistant *Aeromonas hydrophila* ST508 strain carrying *rmtD* and $bla_{\text{CTX-M-131}}$ isolated from a bloodstream infection. *Journal of Global Antimicrobial Resistance.* 2017; 10: 289-290.
288. Leigue L, Warth JF, Melo LC, Silva KC, Moura RA, Barbato L, *et al*. MDR ST2179-CTX-M-15 *Escherichia coli* co-producing RmtD and AAC(6′)-Ib-cr in a horse with extraintestinal infection, Brazil. *Journal of Antimicrobial Chemotherapy*. 2015; 70(4): 1263-1265.
289. Lee CS, Hu F, Rivera JI, Doi Y. *Escherichia coli* sequence type 354 coproducing CMY-2 cephalosporinase and RmtE 16S rRNA methyltransferase. *Antimicrobial Agents and Chemotherapy*. 2014; 58(7): 4246-4247.
290. Li B, Pacey MP, Doi Y. Chromosomal 16S ribosomal RNA methyltransferase RmtE1 in *Escherichia coli* sequence type 448. *Emerging Infectious Diseases*. 2017; 23(5): 876-878.
291. Rahman M, Shukla SK, Prasad KN, Ovejero CM, Pati BK, Tripathi A, *et al*. Prevalence and molecular characterisation of New Delhi metallo-β-lactamases NDM-1, NDM-5, NDM-6 and NDM-7 in multidrug-resistant Enterobacteriaceae from India. *International Journal of Antimicrobial Agents*. 2014; 44(1): 30-37.
292. Cerdeira L, Fernandes MR, Francisco GR, Bueno MFC, Lenne S, Souzaet TA, *et al*. Draft genome sequence of a hospital-associated clone of *Klebsiella pneumoniae* ST340/CC258 coproducing RmtG and KPC-2 isolated from a paediatric patient. *Genome Announcements*. 2016; 4(6): e01130-16. Available from: doi: 10.1128/genomeA.01130-16.
293. Ramos PIP, Picão RC, de Almeida LGP, Lima NCB, Girardello R, Vivan ACP, *et al*. Comparative analysis of the complete genome of KPC-2-producing *Klebsiella*

pneumoniae Kp13 reveals remarkable genome plasticity and a wide repertoire of virulence and resistance mechanisms. *BMC Genomics*. 2014; 15: 54. Available from: doi: 10.1186/1471-2164-15-54.
294. Bueno MFC, Francisco GR, Cerdeira L, Lenne S, Souza TA, Lincopan N, *et al*. Draft genome sequence of an aminoglycoside-resistant RmtG-producing *Pseudomonas aeruginosa* ST235 isolated from a cystic fibrosis patient. *Journal of Global Antimicrobial Resistance*. 2017; 8: 106-107.
295. Francisco GR, Nora ST, Bueno MF, da Silva Filho LV, de Oliveira Garcia D. Identification of aminoglycoside-resistant *Pseudomonas aeruginosa* producing RmtG 16S rRNA methyltransferase in a cystic fibrosis patient. *Antimicrobial Agents and Chemotherapy*. 2015; 59(5): 2967-2968.
296. Tada T, Hishinuma T, Watanabe S, Uchida H, Tohya M, Kuwahara-Arai K, *et al*. Molecular characterization of multidrug-resistant *Pseudomonas aeruginosa* isolates in hospitals in Myanmar. *Antimicrobial Agents and Chemotherapy*. 2019; 63(5): e02397-18. Available from: doi: 10.1128/AAC.02397-18.
297. Maeyama Y, Taniguchi Y, Hayashi W, Ohsaki Y, Osaka S, Koide S, *et al*. Prevalence of ESBL/AmpC genes and specific clones among the third-generation cephalosporin-resistant Enterobacteriaceae from canine and feline clinical specimens in Japan. *Veterinary Microbiology*. 2018; 216: 183-189.
298. Ghotaslou R, Yeganeh Sefidan F, Akhi MT, Asgharzadeh M, Mohammadzadeh Asl Y. Dissemination of genes encoding aminoglycoside-modifying enzymes and *armA* among Enterobacteriaceae isolates in Northwest Iran. *Microbial Drug Resistance*. 2017; 23(7): 826-832.
299. Sabtcheva S, Saga T, Kantardjiev T, Ivanova M, Ishii Y, Kaku M. Nosocomial spread of *armA*-mediated high-level aminoglycoside resistance in Enterobacteriaceae isolates producing CTX-M-3 β-lactamase in a cancer hospital in Bulgaria. *Journal of Chemotherapy*. 2008; 20(5): 593-599.
300. Piekarska K, Zacharczuk K, Wołkowicz T, Rzeczkowska M, Bareja E, Olak M, *et al*. Distribution of 16S rRNA methylases among different species of aminoglycoside-resistant Enterobacteriaceae in a tertiary care hospital in Poland. *Advances in Clinical and Experimental Medicine*. 2016; 25(3): 539-544.
301. Kim JW, Heo ST, Jin JS, Choi CH, Lee YC, Jeong YG, *et al*. Characterization of *Acinetobacter baumannii* carrying $bla_{\text{OXA-23}}$, $bla_{\text{PER-1}}$ and *armA* in a Korean hospital. *Clinical Microbiology and Infection*. 2008; 14(7): 716-718.
302. Castanheira M, Deshpande LM, Woosley LN, Serio AW, Krause KM, Flamm RK. Activity of plazomicin compared with other aminoglycosides against isolates from European and adjacent countries, including Enterobacteriaceae molecularly characterized for aminoglycoside-modifying enzymes and other resistance mechanisms. *Journal of Antimicrobial Chemotherapy*. 2018; 73(12): 3346-3354.
303. Galani I, Nafplioti K, Adamou P, Karaiskos I, Giamarellou H, Souli M, *et al.* Nationwide epidemiology of carbapenem resistant *Klebsiella pneumoniae* isolates from Greek hospitals, with regards to plazomicin and aminoglycoside resistance. *BMC Infectious Diseases*. 2019; 19(1): 167. Available from: doi: 10.1186/s12879-019-3801-1.
304. Doi Y, Arakawa Y. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. *Clinical Infectious Diseases*. 2007; 45(1): 88-94.
305. Bush K. Carbapenemases: partners in crime. *Journal of Global Antimicrobial Resistance*. 2013; 1(1): 7-16.
306. Dolejska M, Villa L, Poirel L, Nordmann P, Carattoli A. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance-nodulation-cell division/multidrug efflux pump. *Journal of Antimicrobial Chemotherapy*. 2013; 68(1): 34-39.

307. Solé M, Pitart C, Roca I, Fàbrega A, Salvador P, Muñoz L, *et al*. First description of an *Escherichia coli* strain producing NDM-1 carbapenemase in Spain. *Antimicrobial Agents and Chemotherapy*. 2011; 55(9): 4402-4404.
308. Fiett J, Baraniak A, Izdebski R, Sitkiewicz I, Żabicka D, Meler A, *et al*. The first NDM metallo-β-lactamase-producing Enterobacteriaceae isolate in Poland: evolution of IncFII-type plasmids carrying the *bla*$_{NDM-1}$ gene. *Antimicrobial Agents and Chemotherapy*. 2014; 58(2): 1203-1207.
309. Hopkins KL, Meunier D, Findlay J, Mustafa N, Parsons H, Pike R, *et al*. SPM-1 metallo-β-lactamase-producing *Pseudomonas aeruginosa* ST277 in the UK. *Journal of Medical Microbiology*. 2016; 65(7): 696-697.
310. Pitout JD, Laupland KB. Extended-spectrum β-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *The Lancet*. 2008; 8(3): 159-166.
311. Wachino J, Yamane K, Kimura K, Shibata N, Suzuki S, Ike Y, *et al*. Mode of transposition and expression of 16S rRNA methyltransferase gene *rmtC* accompanied by IS*Ecp1*. *Antimicrobial Agents and Chemotherapy.* 2006; 50(9): 3212-3215.
312. Kang HY, Tamang MD, Seol SY, Kim J. Dissemination of plasmid-mediated *qnr*, *aac(6')-Ib-cr*, and *qepA* genes among 16S rRNA methylase producing Enterobacteriaceae in Korea. *Journal of Bacteriology and Virology*. 2009; 39(3): 173-182.
313. Tamang MD, Seol SY, Oh JY, Kang HY, Lee JC, Lee CY, *et al*. Plasmid-mediated quinolone resistance determinants *qnrA*, *qnrB*, and *qnrS* among clinical isolates of Enterobacteriaceae in a Korean hospital. *Antimicrobial Agents and Chemotherapy*. 2008; 52(11): 4159-4162.
314. Park YJ, Yu JK, Kim SI, Lee K, Arakawa Y. Accumulation of plasmid-mediated fluoroquinolone resistance genes, *qepA* and *qnrS1*, in *Enterobacter aerogenes* co-producing RmtB and class A β-lactamase LAP-1. *Annals of Clinical and Laboratory Science*. 2009; 39: 55-59.
315. Li DX, Zhang SM, Hu GZ, Wang Y, Liu HB, Wu CM, *et al*. Tn*3*-associated *rmtB* together with *qnrS1*, *aac(6')-Ib-cr* and *bla*$_{CTX-M-15}$ are co-located on an F49:A-:B-plasmid in an *Escherichia coli* ST10 strain in China. *Journal of Antimicrobial Chemotherapy*. 2012; 67: 236-238.
316. Zenati K, Sahli F, Garcia V, Bakour S, Belhadi D, Rolain JM, *et al*. Occurrence and clonal diversity of multidrug-resistant *Klebsiella pneumoniae* recovered from inanimate surfaces in Algerian hospital environment: first report of *armA*, *qnrB* and *aac(6')-Ib-cr* genes. *Journal of Global Antimicrobial Resistance*. 2017; 10: 148-153.
317. Jiang Y, Yu D, Wei Z, Shen P, Zhou Z, Yu Y. Complete nucleotide sequence of *Klebsiella pneumoniae* multidrug resistance plasmid pKP048, carrying *bla*$_{KPC-2}$, *bla*$_{DHA-1}$, *qnrB4*, and *armA*. *Antimicrobial Agents and Chemotherapy*. 2010; 54(9): 3967-3969.
318. Seiffert SN, Marschall J, Perreten V, Carattoli A, Furrer H, Endimiani A. Emergence of *Klebsiella pneumoniae* co-producing NDM-1, OXA-48, CTX-M-15, CMY-16, QnrA and ArmA in Switzerland. *International Journal of Antimicrobial Agents*. 2014; 44(3): 260-262.
319. Guo Q, Spychala CN, McElheny CL, Doi Y. Comparative analysis of an IncR plasmid carrying *armA*, *bla*$_{DHA-1}$ and *qnrB4* from *Klebsiella pneumoniae* ST37 isolates. *Journal of Antimicrobial Chemotherapy*. 2016; 7(4): 882-886.
320. Kim ES, Jeong JY, Choi SH, Lee SO, Kim SH, Kim MN, *et al*. Plasmid-mediated fluoroquinolone efflux pump gene, *qepA*, in *Escherichia coli* clinical isolates in Korea. *Diagnostic Microbiology and Infectious Disease*. 2009; 65(3): 335-338.
321. Périchon B, Courvalin P, Galimand M. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-

mediated efflux in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. 2007; 51(7): 2464-2469.
322. Chen X, He L, Li Y, Zeng Z, Deng Y, Liu Y, *et al*. Complete sequence of a F2:A-:B- plasmid pHN3A11 carrying *rmtB* and *qepA*, and its dissemination in China. *Veterinary Microbiology*. 2014; 174(1-2): 267-271.
323. Mazei D. Integrons: agents of bacterial evolution. *Nature Reviews Microbiology*. 2006; 4: 608-620.
324. Bennett PM. Genome plasticity: insertion sequence elements, transposons and integrons, and DNA rearrangement. *Methods in Molecular Biology*. 2004; 266: 71-113.
325. Bennett PM. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*. 2008; 153(1): S347-S357.
326. Mahillon J, Chandler M. Insertion sequences. *Microbiology and Molecular Biology Reviews*. 1998; 62(3): 725-774.
327. Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology*. 2005; 3(9): 711-721.
328. Wagner A. Cooperation is fleeting in the world of transposable elements. *PLoS Computational Biology*. 2006; 2(12): e162. Available from: doi: 10.1371/journal.pcbi.0020162.
329. Siguier P, Gourbeyre E, Chandler M. Bacterial insertion sequences: their genomic impact and diversity. *FEMS Microbiology Reviews*. 2014; 38(5): 865-891.
330. Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiology Reviews*. 2011; 35(5): 912-935.
331. Szuplewska M, Ludwiczak M, Lyzwa K, Czarnecki J, Bartosik D. Mobility and generation of mosaic non-autonomous transposons by Tn*3*-derived inverted-repeat miniature elements (TIMEs). *PLoS One*. 2014; 9(8): e105010. Available from: doi: 10.1371/journal.pone.0105010.
332. Chen L, Chen ZL, Liu JH, Zeng ZL, Ma JY, Jiang HX. Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. *Journal of Antimicrobial Chemotherapy*. 2007; 59(5): 880-885.
333. Martins WMBS, Gales AC. Frequent Tn*2* misannotation in the genetic background of *rmtB*. *Antimicrobial Agents and Chemotherapy*. 2017; 61(8): e00811-17. Available from: doi: 10.1128/AAC.00811-17.
334. Toleman MA, Bennett PM, Walsh TR. IS*CR* elements: novel gene-capturing systems of the 21st century? *Microbiology and Molecular Biology Reviews*. 2006; 70(2): 296-316.
335. Partridge SR, Tsafnat G, Coiera E, Iredell JR. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiology Reviews*. 2009; 33(4): 757-784.
336. Domingues S, da Silva GJ, Nielsen KM. Integrons: vehicles and pathways for horizontal dissemination in bacteria. *Mobile Genetic Elements*. 2012; 2(5): 211-223.
337. Tavakoli N, Comanducci A, Dodd HM, Lett MC, Albiger B, Bennett P. IS*1294*, a DNA element that transposes by RC transposition. *Plasmid*. 2000; 44(1): 66-84.
338. Doi Y, Adams-Haduch JM, Paterson DL. Genetic environment of 16S rRNA methylase gene *rmtD*. *Antimicrobial Agents and Chemotherapy*. 2008; 52(6): 2270-2272.
339. Zong Z, Partridge SR, Iredell JR. RmtC 16S rRNA methyltransferase in Australia. *Antimicrobial Agents and Chemotherapy*. 2008; 52(2): 794-795.
340. Yu FY, Yao D, Pan JY, Chen C, Qin ZQ, Parsons C, *et al*. High prevalence of plasmid-mediated 16S rRNA methylase gene *rmtB* among *Escherichia coli* clinical isolates from a Chinese teaching hospital. *BMC Infectious Diseases.* 2010; 10: 184. Available from: doi: 10.1186/1471-2334-10-184.
341. Carattoli A. Resistance plasmid families in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy*. 2009; 53(6): 2227-2238.

342. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods*. 2005; 63(3): 219-228.
343. Ramsay JP, Firth N. Diverse mobilization strategies facilitate transfer of non-conjugative mobile genetic elements. *Current Opinion in Microbiology*. 2017; 38: 1-9.
344. Carattoli A. Plasmids in Gram negatives: molecular typing of resistance plasmids. *International Journal of Medical Microbiology*. 2011; 301(8): 654-658.
345. Datta N, Hedges RW. Compatibility groups among fi- R factors. *Nature*. 1971; 234(5326): 222-223.
346. Jain A, Srivastava P. Broad host range plasmids. *FEMS Microbiology Letters*. 2013; 348(2): 87-96.
347. Datta N, Hedges RW. Host ranges of R factors. *Journal of General Microbiology*. 1972; 70(3): 453-460.
348. Top EM, Van Daele P, De Saeyer N, Forney LJ. Enhancement of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in soil by dissemination of catabolic plasmids. *Antonie Van Leeuwenhoek*. 1998; 73: 87-94.
349. Sekizuka T, Matsui M, Yamane K, Takeuchi F, Ohnishi M, Hishinuma A, *et al*. Complete sequencing of the *bla*$_{NDM-1}$-positive IncA/C plasmid from *Escherichia coli* ST38 isolate suggests a possible origin from plant pathogens. *PLoS One*. 2011; 6(9): e25334. Available from: doi: 10.1371/journal.pone.0025334.
350. Pan YS, Liu JH, Hu H, Zhao JF, Yuan L, Wu H, *et al*. Novel arrangement of the *bla*$_{CTX-M-55}$ gene in an *Escherichia coli* isolate coproducing 16S rRNA methylase. *Journal of Basic Microbiology*. 2013; 53(11): 928-933.
351. Hou J, Huang X, Deng Y, He L, Yang T, Zeng Z, *et al*. Dissemination of the fosfomycin resistance gene *fosA3* with CTX-M β-lactamase genes and *rmtB* carried on IncFII plasmids among *Escherichia coli* isolates from pets in China. *Antimicrobial Agents and Chemotherapy.* 2012; 56(4): 2135-2138.
352. Hornsey M, Phee L, Wareham DW. A novel variant, NDM-5, of the New Delhi metallo-β-lactamase in a multidrug-resistant *Escherichia coli* ST648 isolate recovered from a patient in the United Kingdom. *Antimicrobial Agents and Chemotherapy*. 2011; 55(12): 5952-5954.
353. Turton JF, Payne Z, Coward A, Hopkins KL, Turton JA, Doumith M, *et al*. Virulence genes in isolates of *Klebsiella pneumoniae* from the UK during 2016, including among carbapenemase gene-positive hypervirulent K1-ST23 and 'non-hypervirulent' types ST147, ST15 and ST383. *Journal of Medical Microbiology*. 2018; 67(1): 118-128.
354. Yang X, Liu W, Liu Y, Wang J, Lv L, Chen X, *et al*. F33: A-: B-, IncHI2/ST3, and IncI1/ST71 plasmids drive the dissemination of *fosA3* and *bla*$_{CTX-M-55/-14/-65}$ in *Escherichia coli* from chickens in China. *Frontiers in Microbiology*. 2014; 5: 688.
355. Yang L, Yang L, Lü DH, Zhang WH, Ren SQ, Liu YH, *et al*. Co-prevalence of PMQR and 16S rRNA methylase genes in clinical *Escherichia coli* isolates with high diversity of CTX-M from diseased farmed pigeons. *Veterinary Microbiology*. 2015; 178 (3-4): 238-245.
356. Yang YX, Yang YQ, Lei CW, Liu BH, Jiang W, Wang HN, *et al*. Characterization of pHeBE7, an IncFII-type virulence-resistance plasmid carrying *bla*$_{CTX-M-98b}$, *bla*$_{TEM-1}$, and *rmtB* genes, detected in *Escherichia coli* from a chicken isolate in China. *Plasmid*. 2017; 92: 37-42.
357. Fu T, Du XD, Cheng PP, Li XR, Zhao XF, Pan YS. Characterization of an *rmtB*-carrying IncI1 ST136 plasmid in avian *Escherichia coli* isolates from chickens. *Journal of Medical Microbiology*. 2016; 65: 387-391.
358. Liao XP, Liu BT, Yang QE, Sun J, Li L, Fang LX, *et al*. Comparison of plasmids coharboring 16S rRNA methylase and extended-spectrum β-lactamase genes among

Escherichia coli isolates from pets and poultry. *Journal of Food Protection*. 2013; 76(12): 2018-2023.
359. Pan YS, Yuan L, Zong ZY, Liu JH, Wang LF, Hu GZ. A multidrug-resistance region containing $bla_{CTX-M-65}$, *fosA3* and *rmtB* on conjugative IncFII plasmids in *Escherichia coli* ST117 isolates from chicken. *Journal of Medical Microbiology*. 2014; 63: 485-488.
360. He T, Wei R, Zhang L, Sun L, Pang M, Wang R, *et al*. Characterization of NDM-5-positive extensively resistant *Escherichia coli* isolates from dairy cows. *Veterinary Microbiology*. 2017; 207: 153-158.
361. Yu T, He T, Yao H, Zhang JB, Li XN, Zhang RM, *et al*. Prevalence of 16S rRNA methylase gene *rmtB* among *Escherichia coli* isolated from bovine mastitis in Ningxia, China. *Foodborne Pathogens and Disease*. 2015; 12(9): 770-777.
362. One Health Commission. *What is one health?* Available from: https://www.onehealthcommission.org/en/why_one_health/what_is_one_health/ [Accessed 18th January 2019].
363. Petersen TN, Rasmussen S, Hasman H, Carøe C, Bælum J, Charlotte A, *et al*. Meta-genomic analysis of toilet waste from long distance flights; a step towards global surveillance of infectious diseases and antimicrobial resistance. *Scientific Reports*. 2015; 5: 11444. Available from: doi: 10.1038/srep11444.
364. Theuretzbacher U, Paul M. Developing a new antibiotic for extensively drug-resistant pathogens: the case of plazomicin. *Clinical Microbiology and Infection*. 2018; 24: 1231-1233.
365. Armstrong ES, Miller GH. Combating evolution with intelligent design: the neoglycoside ACHN-490. *Current Opinions in Microbiology*. 2010; 13(5): 565-573.
366. Shaeer KM, Zmarlicka MT, Chahine EB, Piccicacco N, Cho JC. Plazomicin: a next-generation aminoglycoside. *Pharmacotherapy*. 2019; 39(1): 77-93.
367. Castanheira M, Davis AP, Mendes RE, Serio AW, Krause KM, Flamm RK. In vitro activity of plazomicin against Gram-negative and Gram-positive isolates collected from U.S. hospitals and comparative activities of aminoglycosides against carbapenem-resistant Enterobacteriaceae and isolates carrying carbapenemase genes. *Antimicrobial Agents and Chemotherapy*. 2018; 62(8): e00313-18. Available from: doi: 10.1128/AAC.00313-18.
368. Livermore DM, Mushtaq S, Warner M, Zhang JC, Maharjan S, Doumith M, *et al*. Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant Enterobacteriaceae isolates. *Journal of Antimicrobial Chemotherapy*. 2011; 66: 48-53.
369. ZEMDRI™. *ZEMDRI™ (product package insert)*. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/210303Orig1s000lbl.pdf [Accessed 26th March 2019].
370. Sati GC, Shcherbakov D, Hobbie SN, Vasella A, Böttger EC, Crich D. N6', N6'", and O4' modifications to neomycin affect ribosomal selectivity without compromising antibacterial activity. *ACS Infectious Diseases*. 2017; 3(5): 368-377.
371. Matsushita T, Sati GC, Kondasinghe N, Pirrone MG, Kato T, Waduge P, *et al*. Design, multigram synthesis, and *in vitro* and *in vivo* evaluation of propylamycin: a semisynthetic 4,5-deoxystreptamine class aminoglycoside for the treatment of drug-resistant Enterobacteriaceae and other Gram-negative pathogens. *Journal of the American Chemical Society*. 2019; 141(12): 5051-5061.
372. Vinal K, Conn GL. Substrate recognition and modification by a pathogen-associated aminoglycoside resistance 16S rRNA methyltransferase. *Antimicrobial Agents and Chemotherapy*. 2017; 61(5): e00077-17. Available from: doi: 10.1128/AAC.00077-17.

373. Minamimura M, Taniyama Y, Inoue E, Mitsuhashi S. *In vitro* antibacterial activity and β-lactamase stability of CP-70,429 a new penem antibiotic, *Antimicrobial Agents and Chemotherapy*. 1993; 37(7): 1547-1551.
374. Koulenti D, Song A, Ellingboe A, Abdul-Aziz MH, Harris P, Gavey E, *et al*. Infections by multidrug-resistant Gram-negative bacteria: what's new in our arsenal and what's in the pipeline? *International Journal of Antimicrobial Agents*. 2019; 53(3): 211-224.
375. Ito A, Nishikawa T, Matsumoto S, Yoshizawa H, Sato T, Nakamura R, *et al.* Siderophore cephalosporin cefiderocol utilizes ferric iron transporter systems for antibacterial activity against *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. 2016; 60(12): 7396-7401.
376. Ito A, Sato T, Ota M, Takemura M, Nishikawa T, Toba S, *et al*. *In vitro* antibacterial properties of cefiderocol, a novel siderophore cephalosporin, against Gram-negative bacteria. *Antimicrobial Agents and Chemotherapy*. 2018; 62(1): e01454-17. Available from: doi: 10.1128/AAC.01454-17.
377. The Pew Charitable Trusts. *Antibiotics currently in global clinical development*. Available from: https://www.pewtrusts.org/-/media/assets/2019/03/antibiotics-currently-in-global-clinical-development.pdf?la=en&hash=078238EF15FACD9753ED2C4EBAB58F16B664B59E [Accessed 17th June 2019].
378. Andrews JM. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*. 2001; 48(1): 5-16.
379. EUCAST. *Clinical breakpoint for bacteria*. Available from: http://www.eucast.org/clinical_breakpoints/ [Accessed 22nd August 2018].
380. Shen Z, Qu W, Wang W, Lu Y, Wu Y, Li Z, *et al*. MPprimer: a program for reliable multiplex PCR primer design. *BMC Bioinformatics*. 2010; 11: 143. Available from: doi: 10.1186/1471-2105-11-143.
381. Bogaerts P, Rezende de Castro R, de Mendonça R, Huang TD, Denis O, Glupczynski Y. Validation of carbapenemase and extended-spectrum beta-lactamase multiplex endpoint PCR assays according to ISO 15189. *Journal of Antimicrobial Chemotherapy*. 2013; 68(7): 1576-1582.
382. Ellington MJ, Findlay J, Hopkins KL, Meunier D, Alvarez-Buylla A, Horner C, *et al*. Multicentre evaluation of a real-time PCR assay to detect genes encoding clinically relevant carbapenemases in cultured bacteria. *International Journal of Antimicrobial Agents*. 2016; 47(2): 151-154.
383. Turton JF, Perry C, Elgohari S, Hampton CV. PCR characterization and typing of *Klebsiella pneumoniae* using capsular type-specific, variable number tandem repeat and virulence gene targets. *Journal of Medical Microbiology*. 2010; 59(5): 541-547.
384. Turton JF, Turton SE, Yearwood L, Yarde S, Kaufmann ME, Pitt TL. Evaluation of a nine-locus variable-number tandem-repeat scheme for typing of *Pseudomonas aeruginosa*. *Clinical Microbiology and Infection*. 2010; 16(8): 1111-1116.
385. Onteniente L, Brisse S, Tassios PT, Vergnaud G. Evaluation of the polymorphisms associated with tandem repeats for *Pseudomonas aeruginosa* strain typing. *Journal of Clinical Microbiology*. 2003; 41(11): 4991-4997.
386. Vu-Thien H, Corbineau G, Hormigos K, Fauroux B, Corvol H, Clément A, *et al*. Multiple-locus variable-number tandem-repeat analysis for longitudinal survey of sources of *Pseudomonas aeruginosa* infection in cystic fibrosis patients. *Journal of Clinical Microbiology*. 2007; 45(10): 3175-3183.
387. Wright LL, Turton JF, Livermore DM, Hopkins KL, Woodford N. Dominance of international 'high-risk clones' among metallo-β-lactamase-producing *Pseudomonas aeruginosa* in the UK. *Journal of Antimicrobial Chemotherapy*. 2015; 70(1): 103-110.

388. Anjum MF, Zankari E, Hasman H. Molecular methods for detection of antimicrobial resistance. *Microbiology Spectrum*. 2017; 5(6). Available from: doi: 10.1128/microbiolspec.ARBA-0011-2017.
389. Woodford N, Fagan EJ, Ellington MJ. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *Journal of Antimicrobial Chemotherapy*. 2006; 57(1): 154-155.
390. Monstein HJ, Ostholm-Balkhed A, Nilsson MV, Nilsson M, Dornbusch K, Nilsson LE. Multiplex PCR amplification assay for the detection of *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes in Enterobacteriaceae. *APMIS*. 2007; 115(12): 1400-1408.
391. Cattoir V, Poirel L, Rotimi V, Soussy CJ, Nordmann P. Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing Enterobacterial isolates. *Journal of Antimicrobial Chemotherapy*. 2007; 60(2): 394-397.
392. Exner M, Bhattacharya S, Christiansen B, Gebel J, Goroncy-Bermes P, Hartemann P, *et al.* Antibiotic resistance: what is so special about multidrug-resistant Gram-negative bacteria? *GMS Hygiene and Infection Control*. 2017; 12: Doc05. Available from: doi: 10.3205/dgkh000290.
393. Berçot B, Poirel L, Nordmann P. Updated multiplex polymerase chain reaction for detection of 16S rRNA methylases: high prevalence among NDM-1 producers. *Diagnostic Microbiology and Infectious Disease*. 2011; 71(4): 442-445.
394. Corrêa LL, Montezzi LF, Bonelli RR, Moreira BM, Picão RC. Revised and updated multiplex PCR targeting acquired 16S rRNA methyltransferases. *International Journal of Antimicrobial Agents*. 2014; 43(5): 479-481.
395. Hu X, Xu B, Yang Y, Liu D, Yang M, Wang J, *et al*. A high throughput multiplex PCR assay for simultaneous detection of seven aminoglycoside-resistance genes in Enterobacteriaceae. *BMC Microbiology*. 2013; 13: 58. Available from: doi: 10.1186/1471-2180-13-58.
396. Tada T, Shimada K, Satou K, Hirano T, Pokhrel BM, Sherchand JB, *et al*. *Pseudomonas aeruginosa* clinical isolates in Nepal coproducing metallo-β-lactamases and 16S rRNA methyltransferases. *Antimicrobial Agents and Chemotherapy*. 2017; 61(9): e00694-17. Available from: doi: 10.1128/AAC.00694-17.
397. Oshiro S, Tada T, Kameoka Y, Suzuki K, Ohmagari N, Miyoshi-Akiyama T, *et al.* Development and evaluation of immunochromatography to detect Gram-negative bacteria producing *armA* 16S rRNA methylase responsible for aminoglycoside resistance. *Journal of Microbiological Methods.* 2015; 118: 159-163.
398. Nordmann P, Jayol A, Dobias J, Poirel L. Rapid aminoglycoside NP test for rapid detection of multiple aminoglycoside resistance in Enterobacteriaceae. *Journal of Clinical Microbiology*. 2017; 55(4): 1074-1079.
399. Nordmann P, Mazé A, Culebras E, Dobias J, Jayol A, Poirel L. A culture medium for screening 16S rRNA methylase-producing pan-aminoglycoside resistant Gram-negative bacteria. *Diagnostic Microbiology and Infectious Disease*. 2018; 91(2): 118-122.
400. Sheikhalizadeh V, Hasani A, Ahangarzadeh Rezaee M, Rahmati-Yamchi M, Hasani A, Ghotaslou R, *et al*. Comprehensive study to investigate the role of various aminoglycoside resistance mechanisms in clinical isolates of *Acinetobacter baumannii*. *Journal of Infection and Chemotherapy*. 2017; 23(2): 74-79.
401. Dally S, Lemuth K, Kaase M, Rupp S, Knabbe C, Weile J. DNA microarray for genotyping antibiotic resistance determinants in *Acinetobacter baumannii* clinical isolates. *Antimicrobial Agents and Chemotherapy*. 2013; 57(10): 4761-4768.
402. Findlay J, Hopkins KL, Alvarez-Buylla A, Meunier D, Mustafa N, Hill R, *et al*. Characterization of carbapenemase-producing Enterobacteriaceae in the West Midlands region of England: 2007-14. *Journal of Antimicrobial Chemotherapy*. 2017; 72(4): 1054-1062.

403. Doumith M, Day M, Ciesielczuk H, Hope R, Underwood A, Reynolds R, *et al*. Rapid identification of major *Escherichia coli* sequence types causing urinary tract and bloodstream infections. *Journal of Clinical Microbiology*. 2015; 53(1): 160-166.
404. Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, *et al*. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy.* 2001; 45: 1151-1161.
405. Turton JF, Hyde R, Martin K, Shah J. Genes encoding OXA-134-like enzymes are found in *Acinetobacter lwoffii* and *A. schindleri* and can be used for identification. *Journal of Clinical Microbiology*. 2012; 50(3): 1019-1022.
406. Poirel L, Le Thomas I, Naas T, Karim A, Nordmann P. Biochemical sequence analyses of GES-1, a novel class A extended-spectrum β-lactamase, and the class 1 integron In52 from *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy.* 2000; 44(3): 622-632.
407. Rasmussen BA, Bush K, Keeney D, Yang Y, Hare R, O'Gara C, *et al*. Characterization of IMI-1 β-lactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloacae*. *Antimicrobial Agents and Chemotherapy.* 1996; 40(9): 2080-2086.
408. Queenan AM, Torres-Viera C, Gold HS, Carmeli Y, Eliopoulos GM, Moellering RC Jr, *et al*. SME-type carbapenem-hydrolyzing class A β-lactamases from geographically diverse *Serratia marcescens* strains. *Antimicrobial Agents and Chemotherapy*. 2000; 44(11): 3035-3039.
409. Turton JF, Gabriel SN, Valderrey C, Kaufmann ME, Pitt TL: Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clinical Microbiology and Infection*. 2007; 13(8): 807-815.
410. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carriço JA. PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. *BMC Bioinformatics*. 2012; 13: 87. Available from: doi: 10.1186/1471-2105-13-87.
411. Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, *et al*. The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Research*. 2016; 44(D1): D279-D285. Available from: doi: 10.1093/nar/gkv1344.
412. Hasman H, Saputra D, Sicheritz-Pontén T, Lund O, Svendsen CA, Frimodt-Møller N, *et al.* Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. *Journal of Clinical Microbiology*. 2014; 52(1): 139-146.
413. Larsen MV, Cosentino S, Lukjancenko O, Saputra D, Rasmussen S, Hasman H, *et al*. Benchmarking of methods for genomic taxonomy. *Journal of Clinical Microbiology*. 2014; 52(5): 1529-1539.
414. Clausen PTLC, Aarestrup FM, Lund O. Rapid and precise alignment of raw reads against redundant databases with KMA. *BMC Bioinformatics*. 2018; 19(1): 307. Available from: doi: 10.1186/s12859-018-2336-6.
415. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, *et al*. Multilocus sequence typing of total genome sequenced bacteria. *Journal of Clinical Microbiology*. 2012; 50(4): 1355-1361.
416. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, *et al*. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*. 2011; 7: 539. Available from: doi: 10.1038/msb.2011.75.
417. Doi Y, Wachino J, Arakawa Y. Nomenclature of plasmid-mediated 16S rRNA methylases responsible for panaminoglycoside resistance. *Antimicrobial Agents and Chemotherapy*. 2008; 52(6): 2287-2288.
418. Otter JA, Doumith M, Davies F, Mookerjee S, Dyakova E, Gilchrist M, *et al*. Emergence and clonal spread of colistin resistance due to multiple mutational mechanisms in

carbapenemase-producing *Klebsiella pneumoniae* in London. *Scientific Reports*. 2017; 7: 12711. Available from: doi: 10.1038/s41598-017-12637-4.
419. Kim JY, Park YJ, Kwon HJ, Han K, Kang MW, Woo GJ. Occurrence and mechanisms of amikacin resistance and its association with beta-lactamases in *Pseudomonas aeruginosa*: a Korean nationwide study. *Journal of Antimicrobial Chemotherapy*. 2008; 62(3): 479-483.
420. Michalska AD, Sacha PT, Ojdana D, Wieczorek A, Tryniszewska E. Prevalence of resistance to aminoglycosides and fluoroquinolones among *Pseudomonas aeruginosa* strains in a University Hospital in Northeastern Poland. *Brazilian Journal of Microbiology*. 2015; 45(4): 1455-1458.
421. Poole K. *Pseudomonas aeruginosa*: resistance to the max. *Frontiers in Microbiology*. 2011; 2: 65. Available from: doi: 10.3389/fmicb.2011.00065.
422. Yu F, Wang L, Pan J, Yao D, Chen C, Zhu T, *et al*. Prevalence of 16S rRNA methylase genes in *Klebsiella pneumoniae* isolates from a Chinese teaching hospital: coexistence of *rmtB* and *armA* genes in the same isolate. *Diagnostic Microbiology and Infectious Disease*. 2009; 64(1): 57-63.
423. Ma L, Lin CJ, Chen JH, Fung CP, Chang FY, Lai YK, *et al*. Widespread dissemination of aminoglycoside resistance genes *armA* and *rmtB* in *Klebsiella pneumoniae* isolates in Taiwan producing CTX-M-type extended-spectrum beta-lactamases. *Antimicrobial Agents and Chemotherapy*. 2009; 53(1): 104-111.
424. Pendleton JN, Gorman SP, Gilmore BF. Clinical relevance of the ESKAPE pathogens. *Expert Review of Anti-infective Therapy*. 2014; 11(3): 297-308.
425. Gholami M, Haghshenas M, Moshiri M, Razavi S, Pournajaf A, Irajian G, *et al.* Frequency of 16S rRNA methylase and aminoglycoside-modifying enzyme genes among clinical isolates of *Acinetobacter baumannii* in Iran. *Iranian Journal of Pathology*. 2017; 12(4): 329-338.
426. Livermore DM. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clinical Infectious Diseases*. 2002; 34(5): 634-640.
427. Strateva T, Yordanov D. *Pseudomonas aeruginosa* – a phenomenon of bacterial resistance. *Journal of Medical Microbiology*. 2009; 58(9): 1133-1148.
428. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Contribution of the MexX-MexY-oprM system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy.* 2000; 44(9): 2242-2246.
429. Gurung M, Moon DC, Tamang MD, Kim J, Lee YC, Seol SY, *et al*. Emergence of 16S rRNA methylase gene *armA* and cocarriage of bla_{IMP-1} in *Pseudomonas aeruginosa* isolates from South Korea. *Diagnostic Microbiology and Infectious Disease*. 2010; 68: 468-470.
430. Kiiru J, Kariuki S, Goddeeris BM, Revathi G, Maina TW, Ndegwa DW, *et al*. *Escherichia coli* strains from Kenyan patients carrying conjugatively transferable broad-spectrum β-lactamase, *qnr*, *aac(6')-Ib-cr* and 16S rRNA methyltransferase genes. *Journal of Antimicrobial Chemotherapy*. 2011; 66(7): 1639-1642.
431. Wei DD, Wan LG, Yu Y, Xu QF, Deng Q, Cao XW, *et al.* Characterization of extended-spectrum β-lactamase, carbapenemase, and plasmid quinolone determinants in *Klebsiella pneumoniae* isolates carrying distinct types of 16S rRNA methylase genes, and their association with mobile genetic elements. *Microbial Drug Resistance*. 2015; 21(2): 186-193.
432. Durante-Mangoni E, Zarrilli R. Global spread of drug-resistant *Acinetobacter baumannii*: molecular epidemiology and management of antimicrobial resistance. *Future Microbiology*. 2011; 6(4): 407-422.
433. Milan A, Furlanis L, Cian F, Bressan R, Luzzati R, Lagatolla C, *et al*. Epidemic dissemination of a carbapenem-resistant *Acinetobacter baumannii* clone carrying *armA*

two years after its first isolation in an Italian hospital. *Microbial Drug Resistance*. 2016; 22(8): 668-674.
434. Giske CG, Fröding I, Hasan CM, Turlej-Rogacka A, Toleman M, Livermore D, *et al*. Diverse sequence types of *Klebsiella pneumoniae* contribute to the dissemination of bla_{NDM-1} in India, Sweden, and the United Kingdom. *Antimicrobial Agents and Chemotherapy*. 2012; 56(5): 2735-2738.
435. Khan AU, Nordmann P. NDM-1-producing *Enterobacter cloacae* and *Klebsiella pneumoniae* from diabetic foot ulcers in India. *Journal of Medical Microbiology*. 2012; 61(3): 454-456.
436. Poirel L, Al Maskari Z, Al Rashdi F, Bernabeu S, Nordmann P. NDM-1-producing *Klebsiella pneumoniae* isolated in the Sultanate of Oman. *Journal of Antimicrobial Chemotherapy*. 2011; 66(2): 304-306.
437. Lomonaco S, Crawford MA, Lascols C, Timme RE, Anderson K, Hodge DR, *et al*. Resistome of carbapenem- and colistin-resistant *Klebsiella pneumoniae* clinical isolates. *PloS One*. 2018; 13(6): e0198526. Available from: doi: 10.1371/journal.pone.0198526.
438. Zhu YQ, Zhao JY, Xu C, Zhao H, Jia N, Li Y. Identification of an NDM-5 producing *Escherichia coli* sequence type 167 in a neonatal patient in China. *Scientific Reports*. 2016; 6: 29934. Available from: doi: 10.1038/srep29934.
439. Zhang LP, Xue WC, Meng DY. First report of New Delhi metallo-β-lactamase 5 (NDM-5)-producing *Escherichia coli* from blood cultures of three leukaemia patients. *International Journal of Infectious Diseases*. 2016; 42: 45-46.
440. Coque TM, Novais A, Carattoli A, Poirel L, Pitout J, Peixe L, *et al*. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum β-lactamase CTX-M-15. *Emerging Infectious Diseases.* 2008; 14(2): 195-200.
441. Barrado L, Pérez-Vázquez M, Del Pozo JL, Martín-Salas C, Leiva J, Mazón A, *et al*. Clonal transmission of NDM-5-producing *Escherichia coli* belonging to high-risk sequence type ST405*. International Journal of Antimicrobial Agents*. 2018; 52(1): 123-124.
442. Sun L, Xu J, He F. Draft genome sequence of an NDM-5, CTX-M-15 and OXA-1 co-producing *Escherichia coli* ST167 clinical strain isolated from a urine sample. *Journal of Global Antimicrobial Resistance*. 2018; 14: 284-228.
443. Bitar I, Piazza A, Gaiarsa S, Villa L, Pedroni P, Oliva E, *et al*. ST405 NDM-5 producing *Escherichia coli* in Northern Italy: the first two clinical cases. *Clinical Microbiology and Infection*. 2017; 23(7): 489-490.
444. Papagiannitsis CC, Studentova V, Ruzicka F, Tejkalova R, Hrabak J. Molecular characterization of metallo-β-lactamase-producing *Pseudomonas aeruginosa* in a Czech hospital (2009-2011). *Journal of Medical Microbiology*. 2013; 62(6): 945-947.
445. Hrabák J, Cervená D, Izdebski R, Duljasz W, Gniadkowski M, Fridrichová M, *et al*. Regional spread of *Pseudomonas aeruginosa* ST357 producing IMP-7 metallo-β-lactamase in Central Europe. *Journal of Clinical Microbiology*. 2011; 49(1): 474-475.
446. Mathers AJ, Peirano G, Pitout JD. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. *Clinical Microbiology Reviews*. 2015; 28(3): 565-591.
447. Souli M, Galani I, Giamarellou H. Emergence of extensively drug-resistant and pandrug-resistant Gram-negative bacilli in Europe. *Euro Surveillance*. 2008; 13(47): 19045. Available from: doi: 10.2807/ese.13.47.19045-en.
448. Livermore DM, Warner M, Mushtaq S, Doumith M, Zhang J, Woodford N. What remains against carbapenem-resistant Enterobacteriaceae? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline. *International Journal of Antimicrobial Agents*. 2011; 37(5): 415-419.

449. Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. Colistin: an update on the antibiotic of the 21st century. *Expert Review of Anti-Infective Therapy*. 2012; 10(8): 917-934.
450. When the drugs don't work. *Nature Microbiology*. 2016; 1: 16003.
451. Wilson ME, Chen LH. NDM-1 and the role of travel in its dissemination. *Current Infectious Disease Reports*. 2012; 14(3): 213-226.
452. Nordmann P, Poirel L, Walsh TR, Livermore DM. The emerging NDM carbapenemases. *Trends in Microbiology*. 2011; 19(12): 588-595.
453. Wailan AM, Paterson DL. The spread and acquisition of NDM-1: a multifactorial problem. *Expert Reviews of Anti-Infective Therapy*. 2014; 12(1): 91-115.
454. SMAC Sub-group on Antimicrobial Resistance. *The path of least resistance*. Available from: http://antibiotic-action.com/wp-content/uploads/2011/07/Standing-Medical-Advisory-Committee-The-path-of-least-resistance-1998.pdf [Accessed 2nd November 2018].
455. Reynolds R, Hope R, Williams R. Survey, laboratory and statistical methods for the BSAC Resistance Surveillance Programmes. *Journal of Antimicrobial Chemotherapy*. 2008; 62(2): 15-28.
456. White AR. The British Society for Antimicrobial Chemotherapy Resistance Surveillance Project: a successful collaborative model. *Journal of Antimicrobial Chemotherapy*. 2008; 62(2): 3-14.
457. BSAC. *Isolate collection*. Available from: http://www.bsacsurv.org/isolate-collection/ [Accessed 22nd November 2018].
458. Kallonen T, Brodrick HJ, Harris SR, Corander J, Brown NM, Martin V, *et al*. Systematic longitudinal survey of invasive *Escherichia coli* in England demonstrates a stable population structure only transiently disturbed by the emergence of ST131. *Genome Research*. 2017; 18. Available from: doi: 10.1101/gr.216606.116.
459. Moradigaravand D, Reuter S, Martin V, Peacock SJ, Parkhill J. The dissemination of multidrug-resistant *Enterobacter cloacae* throughout the UK and Ireland. *Nature Microbiology*. 2016; 1: 16173. Available from: doi: 10.1038/nmicrobiol.2016.173.
460. Moradigaravand D, Martin V, Peacock SJ, Parkhill J. Evolution and epidemiology of multidrug-resistant *Klebsiella pneumoniae* in the United Kingdom and Ireland. *mBio*. 2017; 8(1): e01976-16. Available from: doi: 10.1128/mBio.01976-16.
461. Moradigaravand D, Boinett CJ, Martin V, Peacock SJ, Parkhill J. Recent independent emergence of multiple multidrug-resistant *Serratia marcescens* clones within the United Kingdom and Ireland. *Genome Research*. 2016; 26(8): 1101-1109.
462. Bhattacharya M, Parakh A, Narang M. Tigecycline. *Journal of Postgraduate Medicine*. 2009; 55(1): 65-68.
463. Navon-Venezia S, Kondratyeva K, Carattoli A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiology Reviews*. 2017; 41(3): 252-275.
464. Arpin C, Noury P, Boraud D, Coulange L, Manetti A, André C, *et al*. NDM-1-producing *Klebsiella pneumoniae* resistant to colistin in a French community patient without history of foreign travel. *Antimicrobial Agents and Chemotherapy*. 2012; 56(6): 3432-3434.
465. Abdul Momin MHF, Liakopoulos A, Phee LM, Wareham DW. Emergence and nosocomial spread of carbapenem-resistant OXA-232-producing *Klebsiella pneumoniae* in Brunei Darussalam. *Journal of Global Antimicrobial Resistance*. 2017; 9: 96-99.
466. Teo JWP, Kurup A, Lin RTP, Hsien KT. Emergence of clinical *Klebsiella pneumoniae* producing OXA-232 carbapenemase in Singapore. *New Microbes New Infections*. 2013; 1(1): 13-15.

467. Lascols C, Peirano G, Hackel M, Laupland KB, Pitout JD. Surveillance and molecular epidemiology of *Klebsiella pneumoniae* isolates that produce carbapenemases: first report of OXA-48-like enzymes in North America. *Antimicrobial Agents and Chemotherapy*. 2013; 57(1): 130-136.
468. Fischer S, Klockgether J, Morán Losada P, Chouvarine P, Cramer N, Davenport CF, *et al*. Intraclonal genome diversity of the major *Pseudomonas aeruginosa* clones C and PA14. *Environmental Microbiology Reports*. 2016; 8(2): 227-234.
469. Kidd TJ, Ritchie SR, Ramsay KA, Grimwood K, Bell SC, Rainey PB. *Pseudomonas aeruginosa* exhibits frequent recombination, but only a limited association between genotype and ecological setting. *PLoS One*. 2012; 7(9): e44199. Available from: doi: 10.1371/journal.pone.0044199.
470. BSAC. *Bacteraemia data*. Available from: http://www.bsacsurv.org/reports/bacteraemia/ [Accessed 22nd November 2018].
471. Galani I, Souli M, Panagea T, Poulakou G, Kanellakopoulou K, Giamarellou H. Prevalence of 16S rRNA methylase genes in Enterobacteriaceae isolates from a Greek university hospital. *Clinical Microbiology and Infection*. 2012; 18(3): E52-E54. Available from: doi: 10.1111/j.1469-0691.2011.03738.x.
472. Wu Q, Zhang Y, Han L, Sun J, Ni Y. Plasmid-mediated 16S rRNA methylases in aminoglycoside-resistant Enterobacteriaceae isolates in Shanghai, China. *Antimicrobial Agents and Chemotherapy*. 2009; 53(1): 271-272.
473. Cheng L, Cao XL, Zhang ZF, Ning MZ, Xu XJ, Zhou W, *et al*. Clonal dissemination of KPC-2 producing *Klebsiella pneumoniae* ST11 clone with high prevalence of *oqxAB* and *rmtB* in a tertiary hospital in China: results from a 3-year period. *Annals of Clinical Microbiology and Antimicrobials.* 2016; 15: 1. Available from: doi: 10.1186/s12941-015-0109-x.
474. Johnson AP. Surveillance of antibiotic resistance. *Philosophical Transactions of the Royal Society of London.* 2015; 370(1670): 20140080. Available from: doi: 10.1098/rstb.2014.0080.
475. Santajit S, Indrawattana N. Mechanisms of antimicrobial resistance in ESKAPE pathogens. *BioMed Research International*. 2016; 2016: 2475067. Available from: doi: 10.1155/2016/2475067.
476. Peirano G, Pillai DR, Pitondo-Silva A, Richardson D, Pitout JD. The characteristics of NDM-producing *Klebsiella pneumoniae* from Canada. *Diagnostic Microbiology and Infectious Disease*. 2011; 71(2): 106-109.
477. Pitout JD, Nordmann P, Poirel L. Carbapenemase-producing *Klebsiella pneumoniae*, a key pathogen set for global nosocomial dominance. *Antimicrobial Agents and Chemotherapy*. 2015; 59(10): 5873-5884.
478. Nahid F, Zahra R, Sandegren L. A *bla*$_{OXA-181}$-harbouring multi-resistant ST147 *Klebsiella pneumoniae* isolate from Pakistan that represent an intermediate stage towards pan-drug resistance. *PLoS One*. 2017; 12(12): e0189438. Available from: doi: 10.1371/journal.pone.0189438.
479. Zowawi HM, Forde BM, Alfaresi M, Alzarouni A, Farahat Y, Chong TM, *et al*. Stepwise evolution of pandrug-resistance in *Klebsiella pneumoniae*. *Scientific Reports*. 19; 5: 15082. Available from: doi: 10.1038/srep15082.
480. Kim MJ, Bae IK, Jeong SH, Kim SH, Song JH, Choi JY, *et al*. Dissemination of metallo-β-lactamase-producing *Pseudomonas aeruginosa* of sequence type 235 in Asian countries. *Journal of Antimicrobial Chemotherapy*. 2013; 68(12): 2820-2824.
481. Yousefi S, Nahaei MR, Farajnia S, Aghazadeh M, Iversen A, Edquist P, *et al*. A multiresistant clone of *Pseudomonas aeruginosa* sequence type 773 spreading in a burn unit in Orumieh, Iran. *APMIS*. 2013; 121(2): 146-152.
482. Norman DC. Fever in the elderly. *Clinical Infectious Diseases*. 2000; 31(1): 148-151.

483. Oksuzyan A, Juel K, Vaupel JW, Christensen K. Men: good health and high mortality. Sex differences in health and aging. *Aging Clinical and Experimental Research.* 2008; 20(2): 91-102.
484. Ferreira E, Pina E, Sousa-Uva M, Sousa-Uva A. Risk factors for health care-associated infections: from better knowledge to better prevention. *American Journal of Infection Control*. 2017; 45(10): e103-e107. Available from: doi: 10.1016/j.ajic.2017.03.036.
485. Ayukekbong JA, Ntemgwa M, Atabe AN. The threat of antimicrobial resistance in developing countries: causes and control strategies. *Antimicrobial Resistance and Infection Control*. 2017; 6: 47. Available from: doi:10.1186/s13756-017-0208-x.
486. ECDC. *Antimicrobial resistance surveillance in Europe 2016 report*. Available from: https://ecdc.europa.eu/en/publications-data/antimicrobial-resistance-surveillance-europe-2016 [Accessed 22nd August 2018].
487. Darling AC, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Research*. 2004; 14(7): 1394-1403.
488. Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. *Bioinformatics*. 2011; 27(7): 1009-1010.
489. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, *et al*. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrobial Agents and Chemotherapy*. 2014; 58(7): 3895-3903.
490. Xie Y, Wei Y, Shen Y, Li X, Zhou H, Tai C, *et al*. TADB 2.0: an updated database of bacterial type II toxin-antitoxin loci. *Nucleic Acids Research*. 2018; 46(D1): D749-D753. Available from: doi: 10.1093/nar/gkx1033.
491. Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics*. 2011; 12: 402. Available from: doi: 10.1186/1471-2164-12-402.
492. Chan KH, Lee KM, Wong KB. Interaction between hydrogenase maturation factors HypA and HypB is required for [NiFe]-hydrogenase maturation. *PLoS One*. 2012; 7(2): e32592. Available from: doi: 10.1371/journal.pone.0032592.
493. Ingti B, Paul D, Maurya AP, Bora D, Chanda DD, Chakravarty A, *et al*. Occurrence of bla_{DHA-1} mediated cephalosporin resistance in *Escherichia coli* and their transcriptional response against cephalosporin stress: a report from India. *Annals of Clinical Microbiology and Antimicrobials*. 2017; 16(1): 13. Available from: doi: 10.1186/s12941-017-0189-x.
494. Henn-Sax M, Thoma R, Schmidt S, Hennig M, Kirschner K, Sterner R. Two (betaalpha)(8)-barrel enzymes of histidine and tryptophan biosynthesis have similar reaction mechanisms and common strategies for protecting their labile substrates. *Biochemistry*. 2002; 41(40): 12032-12042.
495. Lin Z, Rye HS. GroEL-mediated protein folding: making the impossible, possible. *Critical Reviews in Biochemistry and Molecular Biology*. 2006; 41(4): 211-239.
496. Padan E, Venturi M, Gerchman Y, Dover N. Na^+/H^+ antiporters. *Biochimica et Biophysica Acta*. 2001; 1505(1): 144-157.
497. Wank H, SanFilippo J, Singh RN, Matsuura M, Lambowitz AM. A reverse transcriptase/maturase promotes splicing by binding at its own coding segment in a group II intron RNA. *Molecular cell*. 1999; 4(2): 239-250.
498. Favrot L, Blanchard JS, Vergnolle O. Bacterial GCN5-related N-acetyltransferases: from resistance to regulation. *Biochemistry*. 2016; 55(7): 989-1002.
499. Allocati N, Federici L, Masulli M, Di Ilio C. Glutathione transferases in bacteria. *The FEBS Journal*. 2009; 276(1): 58-75.
500. Natale P, Brüser T, Driessen AJ. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane-distinct translocases and mechanisms. *Biochimica et Biophysica Acta*. 2008; 1778(9): 1735-1756.

501. Khursigara CM, Wu X, Zhang P, Lefman J, Subramaniam S. Role of HAMP domains in chemotaxis signalling by bacterial chemoreceptors. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(43): 16555-16560.
502. Dunwell JM, Culham A, Carter CE, Sosa-Aguirre CR, Goodenough PW. Evolution of functional diversity in the cupin superfamily. *Trends in Biochemical Sciences*. 2001; 26(12): 740-746.
503. Patoli AA, Winter JA, Bunting KA. The UmuC subunit of the *E. coli* DNA polymerase V shows a unique interaction with the β-clamp processivity factor. *BMC Structural Biology*. 2013; 13: 12. Available from: doi: 10.1186/1472-6807-13-12.
504. Janvier F, Otto MP, Jové T, Mille A, Contargyris C, Meaudre E, *et al*. A case of multiple contamination with methylase ArmA-producing pathogens. *Journal of Antimicrobial Chemotherapy*. 2017; 72(2): 618-620.
505. Kashef MT, Helmy OM. Genetic Characterization of a novel composite transposon carrying *armA* and *aac(6)-Ib* genes in an *Escherichia coli* isolate from Egypt. *Polish Journal of Microbiology.* 2017; 66(2): 163-169.
506. Doi Y, Adams JM, Yamane K, Paterson DL. Identification of 16S rRNA methylase-producing *Acinetobacter baumannii* clinical strains in North America. *Antimicrobial Agents and Chemotherapy.* 2007; 51(11): 4209-4210.
507. Yamane K, Wachino J, Suzuki S, Kimura K, Shibata N, Kato H, *et al*. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. *Antimicrobial Agents and Chemotherapy*. 2007; 51(9): 3354-3360.
508. Périchon B, Bogaerts P, Lambert T, Frangeul L, Courvalin P, Galimand M. Sequence of conjugative plasmid pIP1206 mediating resistance to aminoglycosides by 16S rRNA methylation and to hydrophilic fluoroquinolones by efflux. *Antimicrobial Agents and Chemotherapy*. 2008; 52(7): 2581-2592.
509. Cattoir V, Poirel L, Nordmann P. Plasmid-mediated quinolone resistance pump QepA2 in an *Escherichia coli* isolate from France. *Antimicrobial Agents and Chemotherapy*. 2008; 52(10): 3801-3804.
510. Deng Y, He L, Chen S, Zheng H, Zeng Z, Liu Y, *et al*. F33:A-:B- and F2:A-:B- plasmids mediate dissemination of *rmtB*-$bla_{\text{CTX-M-9}}$ group genes and *rmtB-qepA* in Enterobacteriaceae isolates from pets in China. *Antimicrobial Agents and Chemotherapy*. 2011; 55(10): 4926-4929.
511. Xia J, Sun J, Cheng K, Li L, Fang LX, Zou MT, *et al*. Persistent spread of the *rmtB* 16S rRNA methyltransferase gene among *Escherichia coli* isolates from diseased food-producing animals in China. *Veterinary Microbiology*. 2016; 188: 41-46.
512. Khan A, Shropshire WC, Hanson B, Dinh AQ, Wanger A, Ostrosky-Zeichner L, *et al*. Simultaneous infection with *Enterobacteriaceae* and *Pseudomonas aeruginosa* harboring multiple carbapenemases in a returning traveller colonized with *Candida auris*. *Antimicrobial Agents and Chemotherapy*. 2020; 64(2): e01466-19. Available from: doi: 10.1128/AAC.01466-19.
513. Wailan AM, Sidjabat HE, Yam WK, Alikhan NF, Petty NK, Sartor AL. Mechanisms involved in acquisition of bla_{NDM} genes by IncA/C2 and IncFIIY plasmids. *Antimicrobial Agents and Chemotherapy*. 2016; 60(7): 4082-4088.
514. Karim A, Poirel L, Nagarajan S, Nordmann P. Plasmid-mediated extended-spectrum beta-lactamase (CTX-M-3 like) from India and gene association with insertion sequence IS*Ecp1*. *FEMS Microbiology Letters*. 2001; 201(2): 237-241.
515. Phan HTT, Stoesser N, Maciuca IE, Toma F, Szekely E, Flonta M, *et al*. Illumina short-read and MinION long-read WGS to characterize the molecular epidemiology of an NDM-1 *Serratia marcescens* outbreak in Romania. *Journal of Antimicrobial Chemotherapy*. 2018; 73(3): 672-679.

516. Huang TW, Wang JT, Lauderdale TL, Liao TL, Lai JF, Tan MC, *et al*. Complete sequences of two plasmids in a *bla*$_{NDM-1}$-positive *Klebsiella oxytoca* isolate from Taiwan. *Antimicrobial Agents and Chemotherapy*. 2013; 57(8): 4072-4076.
517. Yang S, Hemarajata P, Hindler J, Li F, Adisetiyo H, Aldrovandi G, *et al*. Evolution and transmission of carbapenem-resistant *Klebsiella pneumoniae* expressing the *bla*$_{OXA-232}$ gene during an institutional outbreak associated with endoscopic retrograde cholangiopancreatography. *Clinical Infectious Diseases*. 2017; 64(7): 894-901.
518. Habeeb MA, Haque A, Iversen A, Giske CG. Occurrence of virulence genes, 16S rRNA methylases, and plasmid-mediated quinolone resistance genes in CTX-M-producing *Escherichia coli* from Pakistan. *European Journal of Clinical Microbiology & Infectious Diseases.* 2014; 33(3): 399-409.
519. Borgia S, Lastovetska O, Richardson D, Eshaghi A, Xiong J, Chung C, *et al*. Outbreak of carbapenem-resistant Enterobacteriaceae containing *bla*$_{NDM-1}$, Ontario, Canada. *Clinical Infectious Diseases*. 2012; 55(11): e109-17. Available from: doi: 10.1093/cid/cis737.
520. Poirel L, Lascols C, Bernabeu S, Nordmann P. NDM-1-Producing *Klebsiella pneumoniae* in Mauritius. *Antimicrobial Agents and Chemotherapy*. 2012; 56(1): 598-599.
521. Teo J, Ngan G, Balm M, Jureen R, Krishnan P, Lin R. Molecular characterization of NDM-1 producing Enterobacteriaceae isolates in Singapore hospitals. *Western Pacific Surveillance and Response Journal.* 2012; 3(1): 19-24.
522. Sonnevend A, Al Baloushi A, Ghazawi A, Hashmey R, Girgis S, Hamadeh MB, *et al*. Emergence and spread of NDM-1 producer Enterobacteriaceae with contribution of IncX3 plasmids in the United Arab Emirates. *Journal of Medical Microbiology*. 2013; 62(7): 1044-1050.
523. Suzuki H, Yano H, Brown CJ, Top EM. Predicting plasmid promiscuity based on genomic signature. *Journal of Bacteriology*. 2010; 192(22): 6045-6055.
524. Johnson TJ, Lang KS. IncA/C plasmids; an emerging threat to human and animal health? *Mobile Genetic Elements*. 2012; 2(1): 55-58.
525. Carattoli A. Plasmids and the spread of resistance. *International Journal of Medical Microbiology*. 2013; 303(6-7): 298-304.
526. Shin J, Baek JY, Cho SY, Huh HJ, Lee NY, Song JH, *et al. bla*$_{NDM-5}$-bearing IncFII-type plasmids of *Klebsiella pneumoniae* sequence type 147 transmitted by cross-border transfer of a patient. *Antimicrobial Agents and Chemotherapy*. 2016; 60(3): 1932-1934.
527. Rojas LJ, Hujer AM, Rudin SD, Wright MS, Domitrovic TN, Marshall SH, *et al*. NDM-5 and OXA-181 beta-lactamases, a significant threat continues to spread in the Americas. *Antimicrobial Agents and Chemotherapy*. 2017; 61(7): e00454-17. Available from: doi: 10.1128/AAC.00454-17.
528. Feng Y, Liu L, McNally A, Zong Z. Coexistence of two *bla*$_{NDM-5}$ genes on an IncF plasmid as revealed by nanopore sequencing. *Antimicrobial Agents and Chemotherapy*. 2018; 62(5): e00110-18. Available from: doi: 10.1128/AAC.00110-18.
529. Mataseje LF, Boyd DA, Lefebvre B, Bryce E, Embree J, Gravel D, *et al*. Complete sequences of a novel *bla*$_{NDM-1}$-harbouring plasmid from *Providencia rettgeri* and an FII-type plasmid from *Klebsiella pneumoniae* identified in Canada. *Journal of Antimicrobial Chemotherapy*. 2014; 69(3): 637-642.
530. Doi Y, O'Hara JA, Lando JF, Querry AM, Townsend BM, Pasculle AW, *et al*. Co-production of NDM-1 and OXA-232 by *Klebsiella pneumoniae*. *Emerging Infectious Diseases*. 2014; 20(1): 163-165.
531. Balm MND, Ngan G, Jureen R, Lin RT, Teo JW. OXA-181-producing *Klebsiella pneumoniae* establishing in Singapore. *BMC Infectious Diseases*. 2013; 13: 58. Available from: doi: 10.1186/1471-2334-13-58.

532. Sheng JF, Li JJ, Tu S, Sheng ZK, Bi S, Zhu MH, *et al*. *bla*$_{KPC}$ and *rmtB* on a single plasmid in *Enterobacter amnigenus* and *Klebsiella pneumoniae* isolates from the same patient. *European Journal of Clinical Microbiology & Infectious Diseases*. 2012; 31(7): 1585-1591.
533. Frasson I, Lavezzo E, Franchin E, Toppo S, Barzon L, Cavallaro A, *et al*. Antimicrobial treatment and containment measures for an extremely drug-resistant *Klebsiella pneumoniae* ST101 isolate carrying pKPN101-IT, a novel fully sequenced blaKPC-2 plasmid. *Journal of Clinical Microbiology*. 2012; 50: 3768-3772.
534. Cassu-Corsi D, Martin WMBS, Nicoletti AG, Almeida LGP, Vasconcelos ATR, Gales AC. Characterisation of plasmid-mediated *rmtB-1* in Enterobacteriaceae clinical isolates from São Paulo, Brazil. *Memórias do Instituto Oswaldo Cruz.* 2018; 113(12): e180392. Available from: doi: 10.1590/0074-02760180392.
535. Wu Q, Liu Q, Han L, Sun J, Ni Y. Plasmid-mediated carbapenem-hydrolyzing enzyme KPC-2 and ArmA 16S rRNA methylase conferring high-level aminoglycoside resistance in carbapenem-resistant *Enterobacter cloacae* in China. *Diagnostic Microbiology and Infectious Disease.* 2010; 66(3): 326-328.
536. Kumarasamy K, Kalyanasundaram A. Emergence of *Klebsiella pneumoniae* isolate co-producing NDM-1 with KPC-2 from India. *Journal of Antimicrobial Chemotherapy*. 2012; 67(1): 243-244.
537. Zacharczuk K, Piekarska K, Szych J, Zawidzka E, Sulikowska A, Wardak S, *et al.* Emergence of *Klebsiella pneumoniae* coproducing KPC-2 and 16S rRNA methylase ArmA in Poland. *Antimicrobial Agents and Chemotherapy*. 2011; 55(1): 443-446.
538. Sugawara Y, Akeda Y, Sakamoto N, Takeuchi D, Motooka D, Nakamura S, *et al*. Genetic characterization of *bla*$_{NDM}$-harboring plasmids in carbapenem-resistant *Escherichia coli* from Myanmar. *PLoS One*. 2017; 12(9): e0184720. Available from: doi: 10.1371/journal.pone.0184720.
539. Huang J, Wang M, Ding H, Ye M, Hu F, Guo Q*, et al*. New Delhi metallo-β-lactamase-1 in carbapenem-resistant *Salmonella* strain, China. *Emerging Infectious Diseases*. 2013; 19(12): 2049-2051.
540. DeNap JC, Hergenrother PJ. Bacterial death comes full circle: targeting plasmid replication in drug-resistant bacteria. *Organic & Biomolecular Chemistry*. 2005; 3(6): 959-966.
541. Orlek A, Stoesser N, Anjum MF, Doumith M, Ellington MJ, Peto T, *et al*. Plasmid classification in an era of whole-genome sequencing: application in studies of antibiotic resistance epidemiology. *Frontiers in Microbiology*. 2017; 8: 182. Available from: doi: 10.3389/fmicb.2017.00182.
542. Schmid M, Frei D, Patrignani A, Schlapbach R, Frey JE, Remus-Emsermann MNP, *et al*. Pushing the limits of *de novo* genome assembly for complex prokaryotic genomes harboring very long, near identical repeats. *Nucleic Acids Research*. 2018; 46(17): 8953-8965.
543. Codjoe FS, Donkor ES. Carbapenem resistance: a review. *Medical Sciences*. 2017; 6(1): E1. Available from: doi: 10.3390/medsci6010001.
544. PHE. *Acute trust toolkit for the early detection, management and control of carbapenemase-producing Enterobacteriaceae*. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/329227/Acute_trust_toolkit_for_the_early_detection.pdf [Accessed 25th July 2019].
545. Savic M, Lovric J, Tomic TI, Vasiljevic B, Conn GL. Determination of the target nucleosides for members of two families of 16S rRNA methyltransferases that confer resistance to partially overlapping groups of aminoglycoside antibiotics. *Nucleic Acids Research*. 2009; 37(16): 5420-5431.

546. Doi Y, Wachino J, Yamane K, Shibata N, Yagi T, Shibayama K, *et al*. Spread of novel aminoglycoside resistance gene *aac(6')-Iad* among *Acinetobacter* clinical isolates in Japan. *Antimicrobial Agents and Chemotherapy*. 2004; 48(6): 2075-2080.
547. Lioy VS, Goussard S, Guerineau V, Yoon EJ, Courvalin P, Galimand M, *et al*. Aminoglycoside resistance 16S rRNA methyltransferases block endogenous methylation, affect translation efficiency and fitness of the host. *RNA*. 2014; 20(3): 382-391.
548. Ou B, Chen L, Song Y, Yang Y, Zhang Q, Yang Y, *et al*. Impact of acquisition of 16S rRNA methylase RmtB on the fitness of *Escherichia coli*. *Journal of Global Antimicrobial Resistance*. 2016; 6: 32-38.
549. Gutierrez B, Escudero JA, San Millan A, Hidalgo L, Carrilero L, Ovejero CM, *et al*. Fitness cost and interference of Arm/Rmt aminoglycoside resistance with the RsmF housekeeping methyltransferases. *Antimicrobial Agents and Chemotherapy*. 2012; 56(5): 2335-2341.
550. Hidalgo L, Gutierrez B, Ovejero CM, Carrilero L, Matrat S, Saba CK, *et al*. *Klebsiella pneumoniae* sequence type 11 from companion animals bearing ArmA methyltransferase, DHA-1 β-lactamase, and QnrB4. *Antimicrobial Agents and Chemotherapy*. 2013; 57(9): 4532-4534.

Appendix

Appendix 1: Figures and tables

Table 1: MIC values for 16S RMTase-positive (n=531) and -negative (n=19) *A. baumannii* isolates from AMRHAI's strain collection, 2003-2015. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	%R
piperacillin-tazobactam (1-64)	-	Positive										531[a]		-	-
		Negative										19[a]		-	-
minocycline (0.125-32)	-	Positive	4	4	7	22	86	175	120	77	13[a]			-	-
		Negative	4	3	3		3	2	3		1			-	-

[a] = MIC greater than or equal to indicated value. ND = not determined; R = resistance.

Table 2: MIC values for 16S RMTase-positive (n=762) and -negative (n=44) Enterobacterales isolates from AMRHAI's strain collection, 2003-2015. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	%R
ampicillin (0.5-64)	≤8/>8	Positive									1	723	1	37	100
		Negative										41[a]		3	100
augmentin (0.125-64)	≤8/>8	Positive							1	8	27	635	2	89	99.9
		Negative								2	2	35		5	100
temocillin (1-128)	-	Positive	2				3	2	6	16	48	93	410	182	-
		Negative							2	2	3	4	9	22	-
piperacillin-tazobactam (1-64)	≤8/>16	Positive	1					1	3	8	6	719	1	23	98.2
		Negative				1			1	1	2	39		-	93.2
ceftazidime + avibactam (0.06-32)	≤8/>8	Positive	1				1	1			4[a]			755	57.1
		Negative												44	-
cefepime (0.125-64)	≤1/>4	Positive				2	3	2	5	10	39	471	1	229	98.7
		Negative	1			1		1		2	1	18		20	87.5
ceftolozane + tazobactam (0.25-16)	≤1/>1	Positive			1	2	2	1		101[a]				655	97.2
		Negative								3[a]				41	100
cefoxitin (1-64)	-	Positive					1	1		8	10	715[a]		27	-
		Negative						1	2		1	40[a]		-	-
aztreonam (0.125-64)	≤1/>4	Positive	31	11	13	9	7	9	13	15	30	595[a]		29	89.1
		Negative	2			1				1	1	39[a]		-	93.2
minocycline (0.125-32)	-	Positive			1	6	44	117	145	137	192	642		120	-
		Negative				1	4	3	12	7	12			5	-

[a] = MIC greater than or equal to indicated value. ND = not determined; R = resistance. Dark blue indicates resistance and no colour indicates susceptibility.

Table 3: MIC values for 16S RMTase-positive (n=19) and -negative (n=201) *P. aeruginosa* isolates from AMRHAI's strain collection, 2003-2015. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	%R
carbenicillin (16-512)	-	Positive											17[a]	2	-
		Negative									1	2	192[a]	6	-
piperacillin-tazobactam (1-64)	≤16/>16	Positive										19[a]		-	100
		Negative				4		3	6	14	23	148[a]	1	2	86.4
ceftolozane + tazobactam (0.25-16)	≤4/>4	Positive								3[a]				16	100
		Negative				1				24[a]				176	96.0
aztreonam (0.125-64)	≤1/>16	Positive						2	1	2	4	9[a]	1	-	73.7
		Negative		1	1		4	9	59	41	30	50[a]	1	5	41.3

[a] = MIC greater than or equal to indicated value. ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 4: VNTR profiles found in ≥5 16S RMTase-positive *K. pneumoniae* isolates (n=319) from AMRHAI's strain collection, 2003-2015.

Number of isolates	VNTR profile (A,E,H,J,K,D,N1,N2)	Inferred ST	16S RMTase(s)	Carbapenemase(s)	Geographical distribution
4	4,4,4,5,2,1,1,1	307	ArmA (3)	NDM-1 (3)	London (3)
			RmtB (1)	NDM-5 (1)	East (1)
3	6,3,6,4,-,2,2,2	Unknown	ArmA (2)	NDM (2)	London (2)
		Unknown	RmtC (1)	NDM-1 (1)	East (1)
3	5,5,-,1,2,1,1,3	Unknown	ArmA (1)	NDM (1)	London (1)
		45	RmtB (1)	OXA-48 (1)	London (1)
		45	RmtC (1)	NDM-4 + OXA-48 (1)	East (1)
3	3,5,5,20,2,1,2,4	Unknown	ArmA (1)	OXA-48-like (1)	South East (1)
			RmtF (1)	OXA-232 (1)	North East (1)
			ArmA + RmtF (1)	OXA-181 (1)	East Midlands (1)
2	14,5,2,13,2,1,3,4	1680	RmtC (2)	NDM-1 (2)	Scotland (1) and West Midlands (1)
2	4,5,6,0,1,2,4,4	152	RmtC (1)	NDM-1 (1)	North East (1)
			RmtF (1)	NDM + OXA-48-like (1)	East (1)
2	3,3,2,13,2,1,3,1	859	ArmA (2)	NDM (2)	East (2)
2	-,5,4,5,-,2,3,3	16	RmtF (2)	OXA-48-like (2)	London (2)

Table 5: MIC values for 16S RMTase-positive (n=1) and -negative (n=12) *A. baumannii* isolates from the Bacteraemia Programme of the BSAC Resistance Surveillance Project, 2001-2013. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase	MIC (mg/L)												
			≤0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**≥128**	**ND**	**% R**
gentamicin (0.25-256)	≤4/>4	Positive											1	-	100
		Negative			1				2	3	2	2	2	-	91.7
piperacillin-tazobactam (0.5-512)	-	Positive											1	-	-
		Negative								1	2	1	8	-	-
minocycline (0.12-64)	-	Positive						1						-	-
		Negative	1		1		1	2	1					6	-

ND = not determined; R = resistance. Dark blue indicates resistance and no colour indicates susceptibility.

Table 6: MIC values for 16S RMTase-positive (n=3) and -negative (n=1,257) Enterobacterales isolates from the Bacteraemia Programme of the BSAC Resistance Surveillance Project, 2001-2013. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTases	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	% R
gentamicin (0.25-256)	≤2/>4	Positive											3	-	100
		Negative	5	90	394	220	37	31	51	56	73	123	177	-	38.2
amoxicillin (4-512)	≤8/>8	Positive											3	-	100
		Negative						2		4	4	17	1,230	-	99.8
amoxicillin + clavulanic acid (0.5-128)	≤8/>8	Positive								1	1	1		-	100
		Negative			1	5	11	49	127	310	193	364	197	-	84.6
piperacillin-tazobactam (0.5-512)	≤8/>16	Positive								1			2	-	66.7
		Negative			1	6	60	108	139	259	186	204	294	-	54.4
cefoxitin (0.5-256)	-	Positive						1		1			1	-	-
		Negative			1		15	105	210	143	169	144	470	-	-
cefuroxime (1-256)	≤8/>8	Positive											3	-	100
		Negative				1	2	24	50	91	91	70	928	-	93.9
minocycline (0.12-64)	-	Positive						1	1		1			-	-
		Negative		1	4	51	204	355	227	152	134	81		48	-
tetracycline (1-256)	-	Positive					1	1					1	-	-
		Negative				15	160	206	204	157	65	18	384	48	-

ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 7: MIC values for 16S RMTase-negative *P. aeruginosa* isolates (n=293) from the Bacteraemia Programme of the BSAC Resistance Surveillance Project, 2001-2013. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTases	MIC (mg/L)												
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	ND	% R
amikacin (2-64)	≤8/>16	Negative					223	28	15	11	3	5		8	2.8
gentamicin (1-16)	≤4/>4	Negative				194	18	22	7	42				10	17.3
tobramycin (1-16)	≤4/>4	Negative				240	2		2	41				8	15.1
piperacillin-tazobactam (4-128)	≤16/>16	Negative						67	107	12	21	5	74	7	39.1
ticarcillin-clavulanate (8-128)	≤16/>16	Negative							13	100	44	14	113	9	95.4
aztreonam (1-64)	≤1/>16	Negative				11	41	69	16	76	12	58		10	51.6

ND = not determined; R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 8: Mutations in QRDRs in GyrA and ParC found in 16S RMTase-negative *E. coli* isolates (n=260) from the BSAC Resistance Surveillance Project's Bacteraemia Programme between 2001-2013.

Species	GyrA position		ParC positions		Total
E. coli			S57T		1
	S83L				9
	S83L		S80I		2
	S83L		S57T		1
	S83L		E84G		1
	S83L	D87N	S80I		42
	S83L	D87N	S80I	S57T	11
	S83L	D87N	S80I	E84G	10
	S83L	D87N	S80I	E84V	164
	S83L	D87N	S80R		3
	S83L	D87N	S80R	G78C	1
	S83L	D87N	S80R	E84K	1
	S83L	D87N	E84K		2
	S83L	D87Y	S80I		2
	S83L	D87Y	S80R	E84V	9
	S83L	D87Y	S80W		1



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14th March 2015

Katie Hopkins
PHE
61 Colindale Avenue
London NW9 5EQ

Dear Katie

PROJECT TITLE: PHE enhanced surveillance of Aminoglycoside resistance
Approval date: 14th March 2016

Thank you for submitting your project to the PHE R&D Office.

Following an internal review it has been decided that this project is not research, but is an enhanced surveillance study. As this study involves only the usual work of your department it will not require an ethical review. This decision has been made based on the protocol provided on 22nd February 2016 and the information you gave me by phone on 11th March.

Based on this information the R&D office approves this investigation. This approval is given on the understanding that the surveillance is conducted in accordance with your protocol and is compliant with all relevant regulatory requirements. If any aspect of the study should change you will need to inform this office to ensure this approval remains in place.

If you need any further support or information, please do not hesitate to contact the PHE R&D Office.

Wishing you every success with your enhanced surveillance

Yours sincerely,

Elizabeth Coates
Head of Research Governance
Public Health England

cc Emma Taylor

Figure 1: Study approval letter written by Dr Elizabeth Coates, Head of Research Governance, PHE.

Table 9: MIC values for 16S RMTase-positive (n=11) and -negative (n=5) *A. baumannii* isolates submitted by hospital laboratories during the study period. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)											
			≤0.125	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**≥128**	**%R**
piperacillin-tazobactam (1-64)	-	Positive											11	-
		Negative											5	-
minocycline (0.125-32)	-	Positive		1		2	4	1	3					-
		Negative			1		1	3						-

R = resistance.

Table 10: MIC values for 16S RMTase-positive (n=66) and -negative (n=69) Enterobacterales isolates submitted by hospital laboratories during the study period. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)											%R
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	
ampicillin (0.5-64)	≤8/>8	Positive										66[a]		100
		Negative										69[a]		100
augmentin (0.125-64)	≤8/>8	Positive									1	1	64	100
		Negative								1	2		66	100
temocillin (1-128)	-	Positive							1	6	5	9	45	-
		Negative					1	2	7	11	8	9	31	-
piperacillin-tazobactam (1-64)	≤8/>16	Positive								2		64[a]		97.0
		Negative				1	1	1	1	1	4	60[a]		92.8
ceftazidime + avibactam (0.06-32)	≤8/>8	Positive	2	1	6	8	3		1	1	44[a]			68.2
		Negative	12	9	18	13	1	5	2		9[a]			13.0
cefepime (0.125-64)	≤1/>4	Positive			1	2	1	1	3	2	6	50[a]		92.4
		Negative	2		1	1	2	4	11	6	10	32[a]		85.5
ceftolozane + tazobactam (0.25-16)	≤1/>1	Positive		1[b]			1	1	1	62[a]				98.5
		Negative		7[b]	13	5	2	2	5	35[a]				63.8
cefoxitin (1-64)	-	Positive							1	1	1	63[a]		-
		Negative					1	3	7	8	5	45[a]		-
aztreonam (0.125-64)	≤1/>4	Positive	4		1			1		1	3	56[a]		90.9
		Negative	6	2	2	3		3	3	3	10	38[a]		78.3
minocycline (0.125-32)	-	Positive				2	7	10	12	12	23[a]			-
		Negative				3	5	18	15	9	19[a]			-

[a] = MIC greater than or equal to indicated value. R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 11: MIC values for 16S RMTase-positive (n=2) and -negative (n=35) *P. aeruginosa* isolates submitted by hospital laboratories during the study period. MICs were interpreted according to EUCAST clinical breakpoints (version 8.1) [379].

Antibiotic (range tested, mg/L)	EUCAST breakpoints (≤S/>R, mg/L)	16S RMTase gene	MIC (mg/L)											%R
			≤0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	
carbenicillin (16-512)	-	Positive											2	-
		Negative								4[b]		8	23	-
piperacillin-tazobactam (1-64)	≤16/>16	Positive										2[a]		100
		Negative				1[b]	3	7	5	7	4	8[a]		34.3
ceftazidime + avibactam (0.06-32)	≤8/>8	Positive									2[a]			100
		Negative			2	6	9	7	3	2	6[a]			22.9
ceftolozane + tazobactam (0.25-16)	≤4/>4	Positive								2[a]				100
		Negative			9	13	2	2	4	5[a]				25.7
aztreonam (0.125-64)	≤1/>16	Positive					1		1					0
		Negative			3	2	2	8	7	4	4	5[a]		37.1

[a] = MIC greater than or equal to indicated value. R = resistance. Dark blue indicates resistance, light blue indicates susceptible with increased exposure and no colour indicates susceptibility.

Table 12: Plasmid DNA sequences identified in *armA* plasmids, where regions of the plasmids shared identity to different plasmids.

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
E. cloacae complex ST265 (Scotland)	pEC224_8	CP018940.1	2,883	2,883	100	1-2,883	-
	unitig_1	CP022612.1	334,957	23,268	6.9	115,643-131,224, 131,299-133,493, 148,873-151,321, 178,300-179,421, 190,161-191,879 and 226,985-227,191	*armA*, *aadA2*, bla_{TEM-1}, *mphE*, *msrE*, *sul1* and *dfrA12*
	unnamed6	CP032212.1	83,015	3,258	3.9	44,065-47,323	-
	pIMP-4-EC62	MH829594.1	314,351	1,136	0.4	124,026-125,162	-
	pV046-a DNA	LC056194.1	4,067	220	0.1	825-1,045	-
K. oyxtoca (London)	unnamed	CP023878.1	108,606	44,600	41.1	3,987-23,650, 37,717-38,331, 63,122-63,738, 67,527-70,378 and 74,311-95,167	*dfrA14*
	unnamed2	CP032174.1	63,589	16,114	25.3	18,554-34,668	-
	tig00000002_u	CP021958.1	128,920	12,955	10.0	8,208-21,001 and 68,635-68,797	*armA*, *msrE* and *mphE*
	p19051-IMP	MF344565.1	316,843	21,350	6.7	115,379-119,045, 113,794-131,311 and 165,209-165,376	-
	unnamed1	CP023917.1	164,607	4,539	2.8	142,746-147,285	-
	pM629-2-NDM5	AP019191.1	110,735	2,339	2.1	95,575-97,914	*aac(6')-Ib-cr*, bla_{OXA-1} and *catB4*
	unnamed1	CP033847.1	143,865	154	0.1	60,822-60,976	-

Table 12 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST14 (East)	tig00000200	CP021947.1	54,064	23,782	44.0	10,480-14,854 and 16,798-36,206	*strA* and *strB*
	pGMI16-006_1	CP028177.1	116,768	38,843	33.3	65,261-99,790, 99,873-100,961, 101,495-102,239 and 102,285-104,767	-
	unnamed1	CP029000.1	125,913	13,954	11.1	52,976-66,930	-
	pPMK1-NDM	CP008933.1	304,526	13,822	4.5	38,162-38,297, 193,832-205,811 and 208,732-210,440	*armA*, *aac(6')-Ib-cr*, *mphE*, *msrE* and *sul1*
	pGMI14-002_1	CP028197.1	444,417	6,661	1.5	184,527-191,069 and 267,450-267,569	-
	pRo24724	CP021328.1	446,611	4,337	1.0	40,723-41,277, 138,063-141,727 and 199,089-199,208	-
K. pneumoniae ST15 (London)	unitig_1	CP022612.1	334,957	49,383	14.7	115,644-129,020, 131,299-133,060, 148,258-151,319, 167,670-171,070, 171,482-175,201, 178,937-187,108, 188,421-197,168, 197,862-199,090, 200,286-202,094 and 201,124-205,236	*armA*, *aac(3)-IId*, *aac(6')-Ib-cr*, *aadA1*, *aadA2*, *bla*$_{CTX-M-15}$, *bla*$_{OXA-9}$, *bla*$_{SHV-11}$, *mphE*, *msrE*, *sul1* and *dfrA12*
	81	LT968767.1	46,254	4,158	9.0	13,472-17,630	-
	pKpN06-CTX	CP012993.2	190,072	7,448	3.9	99,959-100,736 and 131,088-137,759	-
	pKPN-10f7	CP026397.1	220,406	5,770	2.6	119,021-124,791	-
	unnamed2	CP024508.1	228,353	2,534	1.1	55,128-57,230, 128,012-128,218 and 146,945-147,171	-

Table 12 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST37 (London)	pKO_JKo3_4 DNA	AP014955.1	3,897	3,897	100	1-3,897	-
	pEC867_KPC	CP018981.1	14,029	4,023	28.7	6,816-10,839	-
	tig00001069_pilon	CP024857.1	76,680	18,416	24.0	25,454-43,527 and 67,373-67,716	-
	pKP048	FJ628167.2	151,188	21,096	14.0	3,565-3,924, 32,552-52,521 and 65,909-66,677	*armA*, bla_{DHA-1}, *qnrB4*, *mphE*, *msrE* and *sul1*
	pN1863-HI2	MF344583.1	349,834	11,992	3.4	203,557-215,377 and 242,886-243,058	*ant(2'')-Ia* and *aph(3')-Ia*
	pNDM5_020046	CP028781.1	159394	2,998	1.9	145,681-148,679	-
	pR31014-IMP	MF344571.1	374,000	1,208	0.3	236,925-238,133	-
	pSIM-1-BJ0	MH681289.1	316,557	955	0.3	74,289-75,243	-
K. pneumoniae ST307 (London)	pKOX105	HM126016.1	54,641	23,186	42.4	18,146-18,788, 19,699-22,458, 22,626-39,620 and 43,595-46,386	*armA*, *aac(6')-Ib-cr*, *aadA16*, *sul1* *dfrA27*, and *arr-3*
	unnamed3	CP023894.1	71,267	17,487	24.5	18,806-32,057 and 62,839-67,075	-
	unnamed2	CP032193.1	87,450	5,003	5.7	3,499-8,502	-
	pNDM-BJ03	MF415608.1	60,125	2,159	3.6	30,792-32,951	-
	pOW16C2	KF977034.1	59,228	1,316	2.2	34,545-35,861	-
	15	LT968701.1	53,106	961	1.8	41,055-42,016	-

Table 5: Plasmid DNA sequences identified in *armA* plasmids that had <90.0% identity to published plasmids on GenBank.

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
E. cloacae complex ST265 (Scotland)	pCTX-M3	AF550415.2	89,468	26,729	28.9	3,343-5,248, 56,788-62,968, 63,800-64,411, 64,414-80,206 and 80,829-83,070	*armA*, *aadA2*, bla_{TEM-1}, *mphE*, *msrE*, *sul1* and *dfrA12*
E. coli ST405 (London)	unnamed1	CP029729.1	250,887	126,437	50.4	1-21,024, 22,494-30,676, 31,225-31,348, 72,721-89,959, 91,291-104,152, 106,590-115,021, 142,782-187,668, 190,743-191,079, 191,440-203,226 and 249,317-250,887	*armA*, bla_{NDM-1}, *mphE*, *msrE* and *sul1*

Table 6: Plasmid DNA sequences identified in *rmtB* plasmids, where regions of the plasmids shared identity to different plasmids.

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
E. coli ST167 (London)	p4_2_1.1	CP023835.1	152,425	107,288	70.4	1-9,402, 19,772-38,901, 40,218-60,470, 60,734-81,872, 114,209-129,393, 129,823-145,707 and 146,126-152,425	-
	pHN3A11	JX997935.2	76,626	7,167	9.4	7,075-14,036 and 14,433-14,639	*rmtB*, *bla*$_{TEM-1}$ and *qepA*
	pLAP2_020009	CP038004.1	228,706	4,043	7.8	77,439-81,482	*aac(3)-IId*
	pLZ135-CTX	MF353155.1	128,976	10,060	7.8	30,223-40,283	*aadA5*, *mphA*, *sul1* and *dfra17*
	p675SK2_B	CP027703.1	173,882	9,449	5.4	137,000-140,134, 142,795-143,441 and 145,784-151,453	-
E. coli ST405 (London)	pMS6198D	CP015838.1	50,899	33,460	65.7	1-7,044, 7,532-32,262 and 49,213-50,899	*bla*$_{CMY-42}$
	pMH13-051M_3	AP018574.1	74,444	36,795	49.4	3,246-24,976, 30,524-33,474, 34,706-34,868, 34,980-39,312, 39,827-44,248 and 44,925-48,125	*rmtB*, *bla*$_{TEM-1}$ and *mphA*
	pNDM-d2e9	CP026201.1	100,989	10,499	10.4	32,840-33,001; 43,423-43,628; 46,967-57,100	*aadA2*, *bla*$_{NDM-5}$, *sul1* and *dfrA12*
	pNDM5_020007	CP025626.1	144,225	3,139	2.2	84,445-87,584	-

Table 14 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
E. coli ST405 (North West)	pM309-NDM5	AP018833.1	136,947	81,827	59.8	1-3,797, 13,439-13,896, 14,053-16,650, 17,084-21,503, 21,993-22,143, 22,263-23,155, 24,850-31,997, 32,431-40,912, 41,346-56,177, 78,151-82,478, 92,395-93,158, 102,545 - 111,938 and 112,373-136,947	*rmtB*, *aac(6')-Ib-cr*, *aadA5*, bla_{NDM-5}, $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1}, *mphA*, *sul1*, *dfra17* and *catB4*
	pDA33135-139	CP029577.1	139,191	13,415	9.6	32,017-32,197 and 83,141-96,649	-
	unitig_2	CP021536.1	244,955	17,940	7.3	200,155-218,095	-
	pCTXM15_005784	CP028576.1	112,422	6,549	5.8	7,456-7,663 and 96,968-103,310	*strA*, *strB*, *sul2* and *tet*(A)
	unnamed3,	CP023950.1	99,251	1,545	1.6	60,472-61,811 and 71,206-71,412	-
	pNMEC-O75A	CP030112.1	88,421	261	0.3	3,399-3,660	-
E. coli ST448 (London)	pEC1188-NDM16	MH213345.1	80,215	4,779	5.6	8,990-13,116 and 13,118-13,770	*rmtB* and bla_{TEM-1}
	RCS46_p	LT985249.1	216,620	10,895	5.0	30,418-41,308	-
	pKP70-2	MF398271.1	238,153	5,325	2.2	18,374-21,668 and 94,766-96,868	-
	pOSUKPC4	CP024910.1	351,806	3,344	1.0	3,976-7,320	-

Table 14 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
E. coli ST448 (London)	pV085-c	AP014877.1	65,140	26,513	40.7	38,106-64,619	-
	p66	CP023369.1	66,332	23,865	36.0	42,461-66,326	-
	pM214_FII	AP018144.1	94,643	29,475	31.1	5,080-5,212, 6,498-9,412, 9,950-28,806 and 29,352-45,830	*rmtB*, *aadA2*, bla_{NDM-5}, bla_{TEM-1}, *ermB*, *mphA*, *sul1* and *dfrA12*
	III	LT795505.1	71,544	6,694	9.4	38,978-45,672	-
	unnamed2	CP024475.1	82,833	6,909	8.3	30,080-36,989	-
	pCRKP-1215_2	CP024840.1	96,185	4,804	5.0	88,357-93,161	-
E. coli ST1702 (West Midlands)	pMR0617ndm	CP024039.1	125,285	65,559	52.3	15,662-32,937, 33,037-33,688, 34,448-39,225; 39,621-67,280 and 96,554-106,410	*aadA2*, bla_{NDM-5}, *sul1* and *tet*(B)
	unnamed1	CP029113.1	114,412	36,116	31.7	11,943-48,059	-
	pEC881_1	CP019028.1	147,346	27,403	18.6	103,641-131,044	-
	pM214_FII	AP018144.1	94,643	8,907	7.1	9,421-18,328	*rmtB*, bla_{TEM-1}, *ermB* and *mphA*
	pCTXM15_000533	CP028587.1	150,853	10,405	6.9	76,175-86,579	-
	pDA33133-157	CP029575.1	156,518	5,466	3.5	115,037-120,503	-

Table 14 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
E. coli ST2450 (London)	unitig_1_pilon	CP024863.1	139,059	58,871	42.3	26,663-67,009, 69,061-71,542, 75,438-76,258, 78,499-80,980, 83,024-83,844, 85,564-88,020, 88,558-90,948, 93,293-94,821 and 120,890-126,439	*rmtB*, *aac(6')-Ib-cr*, *aadA5*, *bla*$_{NDM-5}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *sul1*, *catB4* and *tet*(B)
	pM629-2-NDM5	AP019191.1	110,735	8,050	7.3	78,499-80,980	-
	pNDM5_020007	CP025626.1	144,225	2,694	1.9	86,841-87,432 and 88,959-91,062	-
E. coli ST4108 (London)	unnamed8	CP029121.1	3,174	3,174	100	1-3,174	-
	pMC-NDM	HG003695.1	87,619	58,248	66.5	1-802, 11,962-29,159, 31,082-47,450 and 63,738-87,619	*rmtB*, *bla*$_{TEM-1}$, *bla*$_{SFO-1}$ and *mphA*
	unnamed1	CP033761.1	111,938	10,831	9.7	90,425-90,769 and 175,696-186,183	-
	pKP1814-1	KX839207.1	299,858	23,253	7.8	166,661-182,245, 205,543-213,031 and 213,491-213,672	-

Table 14 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST147 (East)	unnamed5	CP028996.1	176,325	138,597	78.6	1,999-2,179, 2,613-2,819, 6,166-7,468, 8,113-13,904, 13,895-24,265, 33,036-98,517, 99,747-121,653 and 133,161-166,522	*rmtB*, *aadA2*, *strA*, *strB*, bla_{TEM-1}, *sul1*, *sul2*, *dfrA12*, *tet*(A) and *tet*(G)
	pG06-VIM-1	KU665641.1	53,618	14,498	27.0	5156-18,558, 41,148-41,354 and 46,408-47,296	-
	p2741	JN406319.1	16,012	8,511	53.2	607-1,426 and 2,077-9,763	*aadA1*, *ant(2")-Ia*, bla_{OXA-10}, bla_{VEB-1}, *cmlA1* and *arr-2*
	unnamed3	CP023950.1	99,251	6,108	6.2	56,232-56,413 and 71,846-77,772	-
	pConj83k	MK033501.1	83,425	1,916	2.3	36,098-37,379 and 37,397-38,030	-
	unnamed1	CP029979.1	36,564	695	1.9	6,131-6,480 and 6,609-6,953	-
	unnamed3	CP022063.2	173,817	2,991	1.7	90,616-93,606	-
	unnamed2	CP031802.1	128,238	1,301	1.0	41,849-43,149	-
	unnamed1	CP030343.1	207,546	1,427	0.7	24,777-26,203	*aph(3')-Ia*

Table 7: Plasmid DNA sequences identified in *rmtB* plasmids that had <90.0% identity to published plasmids on GenBank.

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
E. coli ST410 (East)	pM109_FII	AP018139.1	90,294	70,730	78.3	1-11,827, 12,373-22,911, 23,446-27,251, 27,793-63,156 and 81,095-90,294	*rmtB, aadA2, aac(3)-IIa,* bla_{NDM-4}, bla_{TEM-1}, *sul1* and *dfrA12*
E. coli ST410 (London)	pNDM-d2e9	CP026201.1	100,989	78,904	78.1	1-32,839, 43,039-57,614, 58,132-82,274, 84,520-84,988 and 94,108-100,989	*rmtB*, *aadA2*, bla_{NDM-5}, bla_{TEM-1}, *sul1* and *dfrA12*
E. coli ST46 (London)	unnamed2	CP023895.1	91,648	69,732	76.1	1-4,383, 18,446-35,087, 35,597-35,756, 41,782-45,863, 46733-56,357, 56,867-76,239 and 76,536-91,648	*rmtB*, *aadA2*, bla_{NDM-5}, bla_{TEM-1}, *sul1* and *dfrA12*

Table 8: Plasmid DNA sequences identified in *rmtC* plasmids that had <90.0% identity to published plasmids on GenBank.

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
C. freundii ST18 (London)	p1605752AC2	CP022126.1	140,133	68,466	48.9	1-4,840 and 76,624-140,133	*rmtC* and bla_{NDM-1}
K. pneumoniae ST11 (London)	unitig_2	CP021936.1	138,340	62,923	45.5	50,623-60,395 and 68,762-121,913	*rmtC*, *aac(6')-Ib-cr*, bla_{NDM-1}, bla_{DHA-1} and *sul1*

Table 9: Plasmid DNA sequences identified in *rmtF* plasmids, where regions of the plasmids shared identity to different plasmids.

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST16 (North West)	pUCLAOXA232-2	CP012563.1	36,889	36,889	100	1-36,889	-
	tig00000001	CP021758.1	121,057	16,838	13.9	10,022-26,860	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
K. pneumoniae ST11 (London)	pKp_Goe_917-7	CP018446.1	3,559	3,559	100	1-3,559	-
	unnamed3	CP027162.1	42,420	15,719	37.1	8,917-24,636	*mphA*
	tig00000001	CP021758.1	121,057	13,451	11.1	13,409-26,860	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
	tig00000856	CP021711.1	78,638	6,100	7.8	27,566-33,094; 35,392-35,597 and 46,115-46,482	-
	pKPX-1	AP012055.1	250,444	10,663	4.3	56,862-56,985; 167,039-168,608 and 173,218-182,189	*aadA2*, *aph(3')-Ia*, *sul1* and *dfrA12*
	pPMK1-NDM	CP008933.1	304,526	10,069	3.3	43,523-43,686, 61,525-70,255 and 73,358-74,534	bla_{NDM-1} and *qnrB1*
K. pneumoniae ST11 (North West)	tig00000005_pilon	CP021858.1	47,277	7,354	15.6	13,226-20,006, 33,429-33,599 and 33,845-34,249	-
	pKPN1482-1	CP020842.1	180,210	12,251	6.8	91,814-103,244 and 107,776-108,597	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
	tig00000856	CP021711.1	78,638	5,004	6.4	27,566-30,399, 33,087-35,087 and 36,042-36,213	-

Table 17 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST147 (East Midlands)	pKp81_2	CP025818.1	30,492	3,429	11.2	20,162-23,591	-
	pKPN1482-1	CP020842.1	180,210	16,738	9.3	87,852-104,590	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
	pNDM-BJ03	MF415608.1	60,125	1,251	2.1	22,821-24,072	-
K. pneumoniae ST147 (Yorkshire and the Humber)	pCRKP-1215_1	CP024839.1	130,922	117,580	89.8	1-1,735, 2,632-3,207, 6,883-7,244, 7,470-7,675, 8,022-8,289, 9,689-10,509, 11,228-26,262, 26,696-30,869, 32,119-45,354, 48,312-74,676, 74,949-124,445, 125,173-128,887 and 129,321-130,922	*rmtF*, *aac(6')-Ib-cr*, *qnrB1* and *arr-2*
	pSa128	MG870194.1	111,940	6,190	5.5	5,458-5,663; 34,353-34,661 and 72,284-77,961	-
	p48896_1	CP024430.1	131,243	3,207	2.4	30,740-32,248 and 55,980-57,679	-
	pKPN1482-1	CP020842.1	180,210	2,989	1.7	72,003-74,992	-
	pKp11-42	KF295829.1	146,695	1,848	1.3	113,238-115,086	-
	unnamed6	CP032212.1	83,015	485	0.6	3,769-4,254	*dfrA14*
	pCP53-92k	CP033095.1	92,168	375	0.4	62,179-62,554	-
	unnamed3	CP032188.1	93,870	270	0.3	17,588-17,858	-

Table 17 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST147 (London)	pCRKP-1215_1	CP024839.1	130,922	90,386	69.0	1-1,725, 7,469-7,675, 10,358-10,508, 11,228-16,275, 16,153-26,232, 26,696-32,768, 59,988-124,445, 127,743-129,164 and 129,693-130,922	*rmtF*, *aac(6')-Ib-cr*, *qnrB1* and *arr-2*
	pKpn70742_1	CP023250.1	65,276	1,200	1.8	44,740-45,940	*dfra14*
	pKPN1482-1	CP020842.1	180,210	751	0.4	102,292-102,837 and 108,391-108,597	-
K. pneumoniae ST231 (London)	p1	CP033947.1	142,764	101,064	70.8	1-17,292, 43,684-45,233, 58,641-69,726 and 71,626-142,764	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
	pRSB225	JX127248.1	164,550	15,769	9.6	136,263-152,032	-
K. pneumoniae ST231 (London)	pV323-a	LC056638.1	5,544	5,544	100	1-4,592	-
	pDA33140-9	CP029585.1	8,809	1,783	20.2	608-2,391	-
	tig00000001	CP021758.1	121,057	16,575	13.7	10,019-16,973 and 17,239-26,860	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
K. pneumoniae ST231 (London)	pKp_Goe_917-7	CP018446.1	3,559	3,559	100	1-3,559	-
	unnamed4	CP017989.1	61,011	13,987	22.9	4,287-14,767 and 16,477-19,984	$bla_{CTX-M-15}$, bla_{TEM-1} and *qnrS1*
	pKPN1482-1	CP020842.1	180,210	16,972	9.4	87,849-104,485, 107,776-107,963 and 108,446-108,596	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
	pMRVIM0912	KP975074.1	168,041	920	0.5	113,337-114,107 and 118,150-118,300	-

Table 17 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST231 (London)	p2_045523	CP032894.1	3,994	3,994	100	1-3,994	-
	pEC648_5	CP008719.1	2,101	2,101	100	1-2,101	-
	pCREC-544_2	CP024828.1	51,455	6,501	12.6	21,357-23,048, 30,698-30,889 and 30,934-35,553	bla_{SHV-12}
	pCRKP-1215_1	CP024839.1	130,922	9,029	6.9	15,282-23,930, 26,081-26,271 and 129,335-129,526	*rmtF* and *aac(6')-Ib-cr*
	p1	CP028484.1	128,911	438	0.3	71,467-71,905	-
	p14EC020b	CP024140.1	166,233	381	0.2	37,605-37,796 and 39,803-39,993	-
K. pneumoniae ST231 (North West)	unnamed4	CP017989.1	61,011	5,121	8.4	4,896-5,110 and 11,577-16,484	bla_{TEM-1}
	pCRKP-1215_1	CP024839.1	130,922	7,712	5.9	8,066-8,289 and 11,225-18,714	-
	pKPN1482-1	CP020842.1	180,210	8,160	4.5	95,141-103,077 and 107,776-108,000	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
	pDA33144-220	CP029591.1	219,996	4,366	2.0	50,681-51,500 and 69,531-73,078	$bla_{CTX-M-15}$
	unnamed1	CP023937.1	279,104	3,365	1.2	157,370-158,920 and 168,724-170,539	-
K. pneumoniae ST336 (London)	pKPN7	CP000652.1	3,478	3,478	100	1-3,478	-
	pKPN1482-1	CP020842.1	180,210	16,847	9.3	87,852-104,699	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
K. pneumoniae ST336 (London)	pKPN7	CP000652.1	3,478	3,478	100	1-3,478	-
	tig00000001	CP021758.1	121,057	16,840	13.9	10,020-26,860	*rmtF*, *aac(6')-Ib-cr* and *arr-2*

Table 17 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST231 (West Midlands)	pIncFIBpQil	CP036321.1	127,300	97,785	76.8	1-3,694, 4,010-9,473, 33,319-43,255, 44,088-48,698, 52,231-53,065 and 54,052-127,300	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
	_Plasmid_C_Kpneumoniae_MS6671	LN824136.1	34,604	23,388	67.6	9,500-32,887	bla_{TEM-1}
	tig00000793	CP021720.1	216,378	7,160	3.3	29,502-36,662	bla_{NDM-1}
	unnamed2	CP023922.1	129,106	1,177	0.9	118,057-119,234	*qnrB1*
	p0716-KPC	KY270849.1	143,538	356	0.2	51,239-51,595	-
K. pneumoniae ST395 (East)	pG-09EL50	CP003300.1	1,549	1,549	100	1-1,549	-
	unitig_3	CP018819.1	85,161	22,320	26.2	9,162-24,777, 28,718-31,201 and 54,810-59,032	*aac(6')-Ib-cr*, *strA*, *strB*, $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1}, *sul2* and *catB4*
	unnamed2	CP023922.1	129,106	18,090	14.0	20,660-33,556 and 117,275-122,469	*qnrB1*
	pKp_Goe_641-1	CP018737.1	72,952	8,706	11.9	51,998-54,455 and 56,466-62,715	-
	pS-3002cz	KJ958927.1	73,581	4,712	6.4	49,988-54,700	-
	pKPN1482-1	CP020842.1	180,210	10,917	6.1	42,775-42,925 and 93,928-104,695	*rmtF*, *aac(6')-Ib-cr* and *arr-2*
	unnamed1	CP023937.1	279,104	12,070	4.3	155,289-166,538 and 189,241-190,062	*sul1, dfrA1* and *tet*(A)
	unnamed2	CP029142.1	340,462	3,501	1.0	43,459-44,498, 55,803-55,985 and 76,282-78,562	-
	p18-43_02	CP023555.1	169,145	793	0.5	146,599-147,392	-

Table 17 continued

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST437 (London)	pKp28	CP011999.1	83,399	21,232	25.5	1-4,909, 5,698-12,851, 18,397-18,613, 44,935-50,707, 51,075-51,813, 56,640-56,928 and 81,243-83,399	-
	pS-3002cz	KJ958927.1	73,581	16,868	22.9	41,884-54,768 and 55,191-59,175	-
	pGMI16-006_2	CP028178.1	100,222	12,809	12.8	13,280-16,978, 17,110-25,894 and 36,728-37,055	*aadA1*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}9}$ and $bla_{TEM\text{-}1}$
	unnamed 3	CP015503.1	84,941	10,558	12.4	46,966-57,524	*rmtF* and *aac(6')-Ib-cr*
	pEc20/2xEcTOP	MH514861.1	60,204	4,418	7.3	124-4,542	-
	pKp11-42	KF295829.1	146,695	5,484	3.7	113,239-115,652 and 116,631-119,702	*arr-2*
	unnamed 2	CP015502.1	140,874	1,577	1.1	92,635-93,353 and 95,042-95,901	*dfrA14*
	pM206-NDM1 DNA	AP018830.1	209,679	1,562	0.4	174,371-175,933	-
	unnamed3	CP033628.1	110,020	740	0.7	50,536-50,716 and 59,625-60,185	-

Table 10: Plasmid DNA sequences identified in a *rmtF* plasmid that had <90.0% identity to published plasmids on GenBank.

Isolate (geographical distribution)	Plasmid	Accession number	Published plasmid size (bp)	Amount of published plasmid identified (bp)	% of published plasmid	Range matched (bp)	Antibiotic resistance genes
K. pneumoniae ST231 (London)	pIncFIBpQil	CP036321.1	127,300	112,932	88.7	1-14,353, 14,819-20,467, 20,986-21,132, 22,846-23,594, 30,901-32,644, 33,319-43,255, 44,088-50,984, 52,241-52,455 and 54,052-127,300	*rmtF*, *aac(6')-Ib-cr*, *catA1* and *arr-2*
	pKPN1482-1	CP029740.1	103,250	4,585	4.4	65,108-69,693	bla_{SHV-12}

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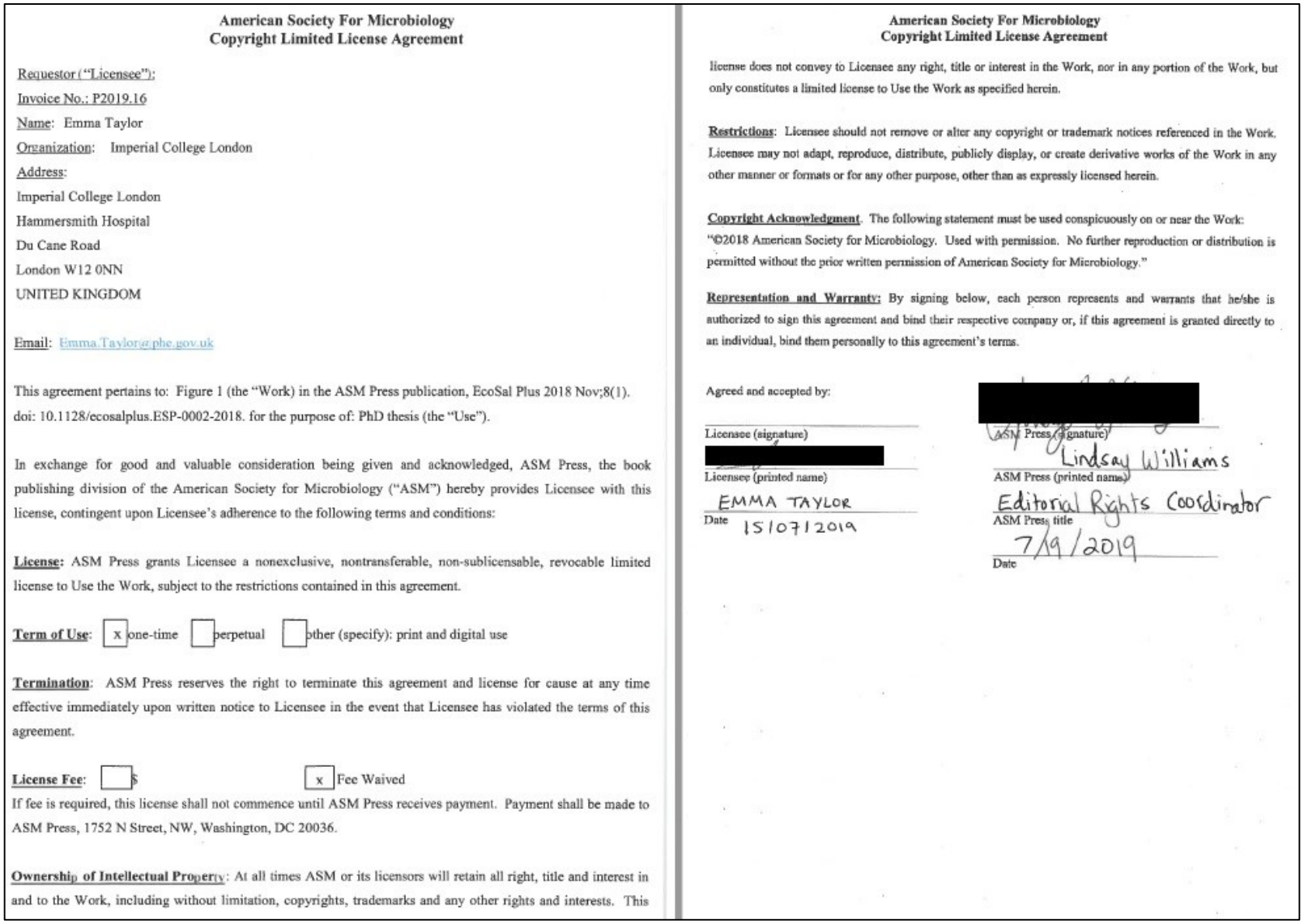
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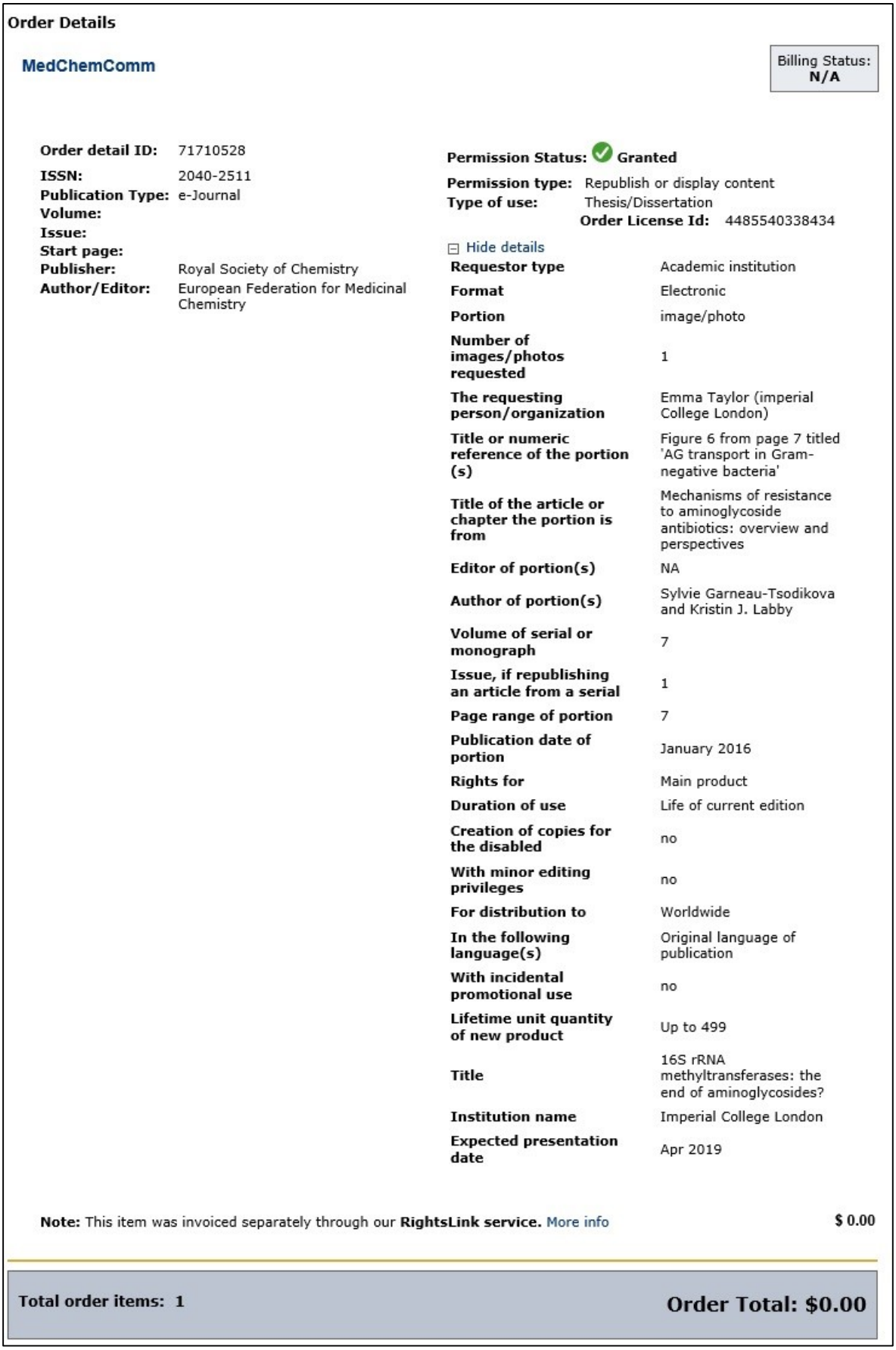
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Short Communication

High prevalence of 16S rRNA methyltransferases among carbapenemase-producing Enterobacteriaceae in the UK and Ireland

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ARTICLE INFO

Article history:
Received 23 November 2017
Accepted 21 March 2018

Keywords:
16S RMTase
Aminoglycoside resistance
bla_{NDM}
Multiplex PCR

ABSTRACT

The emergence of 16S rRNA methyltransferases (16S RMTases) worldwide is a growing concern due to their ability to confer high-level resistance (minimum inhibitory concentrations (MICs) >256 mg/L) to all clinically relevant aminoglycosides. As the occurrence of 16S RMTases in the United Kingdom has not been investigated to date, we screened 806 Enterobacteriaceae isolates displaying high-level aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥64, ≥32 and ≥32 mg/L, respectively) for 16S RMTases either by analysing whole-genome sequence (WGS) data (which were available for 449 isolates) or by polymerase chain reaction. A total of 94.5% (762/806) pan-aminoglycoside-resistant Enterobacteriaceae were positive for one or more 16S RMTase genes; *armA* was the most common (340, 44.6%) followed by *rmtC* (146, 19.2%), *rmtF* (137, 18.0%), *rmtB* (87, 11.4%) and various two-gene combinations (52, 6.8%). Most (93.4%; 712/762) 16S RMTase producers also carried acquired carbapenemase genes, with bla_{NDM} the most common (592/712; 83.1%). Additionally, high-risk bacterial clones associated with bla_{NDM} were identified in the subset of isolates with WGS data. These included *Escherichia coli* sequence types (STs) 405 (21.8%, 19/87), 167 (20.7%, 18/87) 410 (12.6%, 11/87) and *K. pneumoniae* STs 14 (35.6%, 112/315), 231 (15.6%, 49/315) and 147 (10.5%, 33/315). These accounted for 4.2% (15/358), 5.0% (18/358), 3.1% (11/358), 28.2% (101/358), 3.1% (11/358) and 7.0% (25/358) bla_{NDM}-producing isolates, respectively. This study shows that 16S RMTases occur in the UK and Ireland and carbapenemases are particularly prevalent in 16S RMTase-producing Enterobacteriaceae. This association poses a risk to the treatment of multidrug-resistant Gram-negative infections in the clinical setting.

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1. Introduction

16S rRNA methyltransferases (16S RMTases) are emerging aminoglycoside resistance mechanisms, first discovered in various Enterobacteriaceae and *Pseudomonas aeruginosa* in the late 1990s, but which have since been identified globally in Enterobacteriaceae, *Acinetobacter baumannii* and *P. aeruginosa*. Ten 16S RMTase-encoding genes are known to date (*armA*, *rmtA*-rmt*H* and *npmA*) [1]. These enzymes confer high-level resistance to all clinically relevant aminoglycosides (minimum inhibitory concentration (MIC) >256 mg/L) [2] and the genes encoding them are typically located on plasmids along with those for extended-spectrum beta lactamases (ESBLs), carbapenemases and fluoroquinolone resistance determinants. This potentially renders ineffective multiple classes of antimicrobials used to treat multidrug-resistant Gram-negative infections [1].

Rates of 16S RMTases around the world appear to differ between the East and the West, with rates of 0.12% (19/15,386) in Belgium [3] and 3.9% (38/985) in China [4] in Enterobacteriaceae. However, the occurrence of 16S RMTases in the UK and Ireland is currently unknown. We sought the presence of 16S RMTase genes in a collection of Enterobacteriaceae isolates displaying high-level pan-aminoglycoside resistance that had been submitted to Public Health England's Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit for the investigation of unusual antibiotic resistance (primarily carbapenem resistance).

2. Materials and Methods

2.1. Bacterial Isolates

A panel of 806 Enterobacteriaceae referred between June 2004 and December 2015 was recovered from the AMRHAI Reference

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https://doi.org/10.1016/j.ijantimicag.2018.03.016
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Table 1
Primer sequences for the identification of 16S RMTase genes.

Multiplex	16S RMTase gene	Primer	Primer sequence (5′ to 3′)	Temperature (°C)	Amplicon size	Reference
Multiplex 1	*armA*	Forward	AAAGTACAATCAGGGGCAGTT	61.4	269	This study
		Reverse	TCGTCGTCTTTAACTTCCCAA	63.4		
	rmtA	Forward	CTAGCGTCCATCCTTTCCTC	57.48	634	[4]
		Reverse	TTGCTTCCATGCCCTTGCC	61.29		
	rmtB	Forward	GCTTTCTGCGGGCGATGTAA	61.37	173	[4]
		Reverse	ATGCAATGCCGCGCTCGTAT	63.60		
	rmtC	Forward	CGAAGAAGTAACAGCCAAAG	54.66	711	[4]
		Reverse	ATCCCAACATCTCTCCCACT	57.72		
	rmtD	Forward	CGGCACGCGATTGGGAAGC	64.87	401	[4]
		Reverse	CGGAAACGATGCGACGAT	58.31		
Multiplex 2	*rmtE*	Forward	TGGTTGCAGAGGTTCTGTCGAGC	71.8	518	This study
		Reverse	CGGCGTAACAGACACGGCATCA	74.1		
	rmtF	Forward	ATTCATCTGGGCTGCGTGCGAC	74.4	338	This study
		Reverse	ACCAGCTCGTCCGACACCGTAA	71.9		
	rmtG	Forward	CGTGTATGCGCGTCTGTTGGGT	73.1	420	This study
		Reverse	ACGGTGCGTTCGATTCGCCATT	75.0		
	rmtH	Forward	ACAAAAAGCCCAAGCAGGCGGT	73.2	259	This study
		Reverse	CGGTGCAGCATCAGCGGGTTTA	74.8		
	npmA	Forward	GGTCAGTTTGATCGTGTGCA	58.77	195	This study
		Reverse	AGCTGCAATAACAAACACCACA	59.31		

Unit's culture collection. Isolates had been submitted for reference service investigation of unusual (primarily carbapenem) resistance. Approval for this study was sought from Public Health England's R&D Office, who deemed the project enhanced surveillance, therefore ethical review was not required. All demonstrated high-level pan-aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥64, ≥32 and ≥32 mg/L, respectively) as determined by agar dilution and 729 (90.4%) had been confirmed to be carbapenemase-producing Enterobacteriaceae by in-house polymerase chain reaction (PCR).

2.2. DNA extraction and multiplex PCR assay

Crude DNA extracts were subjected to two multiplex PCRs where Multiplex 1, designed by Doi and Arakawa [5], detected *armA*, *rmtA-rmtD* and Multiplex 2, designed in this study, detected *rmtE-rmtH* and *npmA*, including subvariants *rmtD2*, *rmtE2* and *rmtF2* (see Table 1 for primer sequences). The cycling conditions were based on those proposed by Doi and Arakawa [5] and were: initial denaturation at 96°C for 5 min; followed by 30 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by a final elongation step at 72°C for 5 min. Following PCR, the DNA fragments were analysed using gel electrophoresis on 2% agarose gels.

2.3. Bacterial typing

Escherichia coli and *Klebsiella pneumoniae* isolates sent to the AMRHAI Reference Unit for bacterial typing were tested by pulsed-field gel-electrophoresis (PFGE) [6] or variable-number tandem-repeat (VNTR) analysis [7], respectively.

2.4. Whole-genome sequencing

Whole-genome sequencing (WGS) data were available for 449 isolates, and had been derived from a Hiseq sequencing system (Illumina, San Diego, CA, USA) and analysed using AMRHAI's in-house 'GeneFinder' bioinformatics pipeline to infer multilocus sequence typing (MLST) profiles and determine total antibiotic resistance gene contents [8].

3. Results

Seven hundred and sixty-two (94.5%) of 806 isolates displaying high-level pan-aminoglycoside resistance were positive for one or more 16S RMTase genes (Table 2). The 16S RMTase genes identified included *armA* (340/762, 44.6%), *rmtC* (146, 19.2%), *rmtF* (137, 18.0%), *rmtB* (87, 11.4%), *armA* + *rmtC* (23, 3.0%), *armA* + *rmtF* (17, 2.2%), *rmtB* + *rmtF* (5, 0.7%), *rmtC* + *rmtF* (3, 0.4%), *armA* + *rmtB* (2, 0.3%) and *rmtB* + *rmtC* (2, 0.3%). No *rmtA*, *rmtE*, *rmtG*, *rmtH* or *npmA* genes were detected in this study.

The 16S RMTase genes were found in the following species: *K. pneumoniae* (n=502), *E. coli* (n=152), *Enterobacter cloacae* complex (n=33), *Klebsiella* spp. (n=25), *Citrobacter freundii* (n=11), *Providencia* spp. (n=14), *Enterobacter* spp. (n=10), *Proteus mirabilis* (n=6), *Morganella morganii* (n=2), *Serratia marcescens* (n=2), *Citrobacter* spp. (n=4) and *Escherichia* sp. (n=1).

Collectively, *K. pneumoniae* was the most common host species for *rmtF*, *armA* and *rmtC* genes, accounting for 95.7% (155/162), 74.3% (284/382) and 51.7% (90/174) of *rmtF*, *armA* and *rmtC* isolates, respectively. Conversely, *E. coli* was the most common host species for *rmtB*, accounting for 82.3% (79/96) of the *rmtB*-positive isolates.

16S RMTase-producing isolates were submitted by 109 hospital laboratories across 13 regions in the UK and Ireland and were obtained from 627 patients and seven environmental swabs. Six hundred and six patients were from England where the national distribution of the patients was as follows: London (n=328), West Midlands (n=79), North West (n=48), Yorkshire and the Humber (n=40), East Midlands (n=31), East (n=26), South East (n=25), South West (n=17), and North East (n=12). The 21 remaining patients were from Scotland (n=11), Northern Ireland (n=3), Isle of Man (n=2) and Republic of Ireland (n=5). This includes one patient that was treated in both London and the South East as well as another patient treated in both the East of England and East Midlands. Five of the environmental swabs were from London, one was from the North West and one was from the West Midlands.

Five hundred and forty-one (70.0%) isolates were from patients in the hospital setting, 53 (7.0%) were from general practice clinics and 168 (22.0%) came from an unknown setting. The most common specimen type was 'screening swab' (32.8%, 250/762), followed by urine (31.1%, 237), tissue and fluid (16.8%, 128), blood and line tip (8.1%, 62), respiratory (6.4%, 49), faecal (1.6%, 12), environmental (0.9%, 7), and 17 (2.2%) were from an unknown source.

Information on travel history was unavailable for 475/627 (75.8%) patients. However, 98 (15.6%) patients had reported recent travel abroad, with travel to India (n=61), Pakistan (n=13), Egypt (n=4), Bangladesh (n=3), Greece (n=2), Middle East

Table 2
Total number of pan-aminoglycoside resistant Enterobacteriaceae with 16S RMTases between 2004 and 2015 from Antimicrobial Resistance and Healthcare Associated Infections, Public Health England.

	Negative	*armA*	*rmtB*	*rmtC*	*rmtD*	*rmtE*	*rmtF*	*armA* + *rmtB*	*armA* + *rmtC*	*armA* + *rmtF*	*rmtB* + *rmtC*	*rmtB* + *rmtF*	*rmtC* + *rmtF*	Total
Citrobacter freundii	1	5	0	5	0	0	0	0	0	0	1	0	0	12
Citrobacter sp.	1	3	0	1	0	0	0	0	0	0	0	0	0	5
Enterobacter cloacae	5	11	3	17	0	0	0	0	2	0	0	0	0	38
Enterobacter sp.	2	6	0	4	0	0	0	0	0	0	0	0	0	12
Escherichia coli	10	33	74	38	0	0	0	1	1	1	1	3	0	162
Escherichia sp.	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Klebsiella pneumoniae	19	251	8	70	0	0	135	1	17	15	0	2	3	521
Klebsiella sp.	3	16	0	5	0	0	2	0	1	1	0	0	0	28
Morganella morganii	0	1	1	0	0	0	0	0	0	0	0	0	0	2
Proteus mirabilis	0	5	0	1	0	0	0	0	0	0	0	0	0	6
Proteus sp.	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Providencia sp.	0	6	1	5	0	0	0	0	2	0	0	0	0	14
Serratia marcescens	2	2	0	0	0	0	0	0	0	0	0	0	0	4
Total	44	340	87	146	0	0	137	2	23	17	2	5	3	806

Table 3
Total number of carbapenemase genes in Enterobacteriaceae with pan-aminoglycoside resistance between 2004 and 2015 from Antimicrobial Resistance and Healthcare Associated Infections, Public Health England.

16S RMTase	Carbapenemase									
	Negative	NDM	OXA-48-like	NDM + OXA-48-like	KPC	VIM	GES	NDM + VIM	VIM + OXA-48-like	Total
Negative	26	2	6	0	8	1	0	0	1	44
ArmA	28	249	31	26	4	0	1	1	0	340
RmtB	5	66	3	10	1	2	0	0	0	87
RmtC	3	130	1	12	0	0	0	0	0	146
RmtF	12	54	65	6	0	0	0	0	0	137
ArmA + RmtB	0	2	0	0	0	0	0	0	0	2
ArmA + RmtC	2	21	0	0	0	0	0	0	0	23
ArmA + RmtF	0	4	11	2	0	0	0	0	0	17
RmtB + RmtC	0	2	0	0	0	0	0	0	0	2
RmtB + RmtF	0	3	1	1	0	0	0	0	0	5
RmtC + RmtF	0	3	0	0	0	0	0	0	0	3
Total	77 (9.6%)	535 (66.4%)	118 (14.6%)	57 (7.1%)	13 (1.6%)	3 (0.4%)	1 (0.1%)	1 (0.1%)	1 (0.1%)	806

(n=2), and single patients had travelled to the Asian continent, Democratic Republic of the Congo, Equatorial Guinea, India and Pakistan, Kuwait, Nigeria, Oman, Saudi Arabia, Singapore, Sri Lanka, Syrian Arab Republic, Thailand and Vietnam.

Most (93.4%; 712/762) of the 16S RMTase-positive isolates produced an acquired carbapenemase (Table 3), with clear associations found with NDM (83.1%, 592/712) and OXA-48-like (23.7%, 169/712) carbapenemase families, either alone or in combination with other carbapenemases. bla_{KPC}, bla_{VIM} and bla_{GES-5} were also identified amongst 16S RMTase-positive isolates, but in very low numbers (Table 3).

MLST data were derived from WGS data of 87 *E. coli* and 315 *K. pneumoniae* isolates, where 25 *E. coli* and 30 *K. pneumoniae* STs were identified. The top three *E. coli* STs (accounting for 55.2%, 48/87 isolates) were ST405 (n=19), ST167 (n=18) and ST410 (n=11), which were found in 14 hospitals spanning 5 regions, 14 hospitals spanning 7 regions and 8 hospitals spanning 4 regions, respectively. PFGE was performed on 52.6% (10/19), 55.6% (10/18) and 72.7% (8/11) isolates, respectively, where all isolates had different PFGE profiles indicating that these STs encompass different strains. The top three *K. pneumoniae* STs (accounting for 61.6%, 194/315 isolates) were ST14 (n=112), ST231 (n=49) and ST147 (n=33), which were found in 25 hospitals spanning 7 regions, 24 hospitals spanning 7 regions and 21 hospitals spanning 8 regions, respectively. VNTR was performed on 73.2% (82/112), 69.4% (34/49) and 84.8% (28/33) isolates, respectively. Different strains were identified in STs 14 and 167 with isolates belonging to three and two different VNTR profiles, respectively (data not shown). However, all ST231 isolates shared an identical VNTR profile, which was found in 20 hospitals spanning seven regions.

Analysis of co-resident antibiotic resistance genes in the 449 16S RMTase-positive isolates with WGS data confirmed that bla_{NDM} (71.7%, 358/499) was the most common carbapenemase (Table 3) with bla_{NDM-1} (82.1%, 294/358) the most common variant identified. This carbapenemase allele was found associated with all 16S RMTases (*armA* (85.7%, 180/210), *rmtC* (91.5%, 65/71), *rmtF* (40.2%, 35/87), *rmtB* (10.9%, 7/64), *armA* + *rmtC* (100%, 2/2), *armA* + *rmtF* (25.0%, (2/8), *armA* + *rmtB* (100%, 1/1), *rmtB* + *rmtC* (100%, 1/1), *rmtC* + *rmtF* (100%, 1/1)) but as not found in isolates with *rmtB* + *rmtF* (n=4). bla_{NDM-5} had a clear association with *rmtB*-positive isolates as it was found in 62.5% (40/64) of *rmtB*-positive isolates compared to bla_{NDM-4} (14.1%, 9/64) and $bla_{NDM-1,}$ (10.9%, 7/64). *rmtF* had a slightly higher association with $bla_{OXA-232}$ (51.7%, 45/87) compared with bla_{NDM-1} (40.2%, 35/87), which may be due to 31/45 (68.9%) $bla_{OXA-232}$ genes being found in *K. pneumoniae* isolates belonging to ST231. Other antibiotic genes that were found to be commonly associated with 16S RMTase producers were the quinolone resistance gene *aac(6′)-Ib-cr* and the extended-spectrum beta-lactamase $bla_{CTX-M-15,}$ which were found in 341/499 (75.9%) and 302/499 (67.3%) isolates, respectively. Both were associated with all 16S RMTase genes but were found to be most common in *rmtF*-positive isolates as they were identified in 86/87 (98.9%) and 79/87 (90.8%) isolates, respectively.

4. Discussion

This study has shown that the vast majority (94.5%; 762/806) of Enterobacteriaceae isolates displaying high-level pan-aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥64, ≥32 and ≥32 mg/L, respectively) from AMRHAI's isolate collection were positive for 16S RMTase genes.

Other studies investigating the presence of 16S RMTase genes in pan-aminoglycoside-resistant Enterobacteriaceae have also found high rates of 100% (20/20), 93.6% (204/218) and 86.4% (19/22) in Poland [9], South Korea [10] and Belgium [3], respectively. Absence of a 16S RMTase gene in isolates expressing pan-aminoglycoside resistance may be due to combinations of other aminoglycoside resistance mechanisms such as aminoglycoside-modifying enzymes and upregulation of efflux pumps. Alternatively, it could be due to novel 16S RMTase genes or variants of existing genes that could not be detected with existing PCR assays. These isolates will be investigated further using WGS to attempt to identify underlying resistance mechanisms.

armA was the most frequently identified 16S RMTase gene in this study and was mainly found in *K. pneumoniae*. It is the one of the most widely distributed 16S RMTase genes [1]. In contrast to reports from other countries, *rmtC* and *rmtF* were more frequent in our isolate collection than *rmtB*, which is one of the most predominant global 16S RMTase genes alongside *armA* [1]. This may be due to their higher association with bla_{NDM-1} when compared to *rmtB* or perhaps plasmids or bacterial clones with *rmtC* and *rmtF* are more prominent in the UK and Ireland than those with *rmtB*.

Most (93.4%; 712/762) of the 16S RMTase-producing bacteria in our collection were carbapenemase producers, indicating a strong association between these resistance mechanisms and providing a possible explanation for the emergence of 16S RMTases in the UK and Ireland. Carbapenemases belonging to the NDM family were the most common, which may be due to co-location of 16S RMTase genes and bla_{NDM} on the same plasmids [11,12]. Other antibiotic resistance genes were also highly associated with 16S RMTases, e.g. *aac(6′)-Ib-cr*, *fosA3* and $bla_{CTX-M-15}$ were particularly associated with *rmtF*, as has been reported in Egypt and the USA [13,14]. This suggests that these genes may be linked on the same plasmids, which has implications for the treatment of bacterial infections as co-selection of genes causing resistance to aminoglycosides, beta-lactams and fluoroquinolones removes the use of the three key antibiotics used to treat Gram-negative infections.

High-risk clones may also play a role in 16S RMTase emergence in the UK and Ireland as the most common STs identified in this study (*E. coli* ST405, ST167 and ST410 as well as *K. pneumoniae* ST14, ST231 and ST147) have all been reported to carry bla_{NDM} [15–18]. However, the reason for the predominance of these STs is unknown as there is insufficient evidence to determine whether this is due to carriage of particular virulence genes or from the presence of 16S RMTase genes and/or carbapenemase genes. Additionally, the lack of clonality in Enterobacteriaceae isolates that had undergone PFGE and VNTR, apart from *K. pneumoniae* ST231 isolates, suggests that different bacterial strains across the UK harbour 16S RMTase genes.

India and Pakistan are known reservoirs of bla_{NDM} carbapenemases [19] and it appears that they may also act as reservoirs of 16S RMTase genes as 62.2% (61/98) and 13.3% (13/98) of patients with a known travel history had visited these countries, respectively. This is also supported by London and the West Midlands having the highest prevalence of 16S RMTase genes as these regions are known to have multicultural populations with links to the Indian Subcontinent.

Public Health England's AMRHAI Reference Unit is the UK and Ireland's national reference laboratory for the investigation of unusual antibiotic resistance in healthcare-associated infections. It receives multidrug-resistant bacteria from across the country (particularly those demonstrating carbapenem resistance) and so the occurrence of antibiotic resistance genes in AMRHAI's isolate collection will be much higher than they actually are in the clinical setting. As there are no denominator data, the national prevalence of 16S RMTases cannot be calculated. Further studies need to be carried out in order to estimate the true prevalence of these resistance genes in the UK and Ireland using unbiased isolate collections.

In conclusion, this is the first report to describe the occurrence of 16S RMTases in pan-aminoglycoside resistant Enterobacteriaceae in the UK and Ireland, albeit in otherwise highly multidrug-resistant isolates. There was a clear association with acquired carbapenemase genes, which is very concerning as these 16S RMTase-positive isolates display resistance to many antibiotic classes used to treat Gram-negative infections, including the new aminoglycoside plazomicin, which is in phase III clinical trials. This highlights that novel agents currently in development with Gram-negative activity will not offer solutions for all multidrug-resistant organisms encountered in the clinical setting. Diagnostic laboratories should be aware of 16S RMTases and consider their presence in isolates displaying high-level pan-aminoglycoside resistance.

Acknowledgements

We would like to thank Professor Bruno González-Zorn, Dr Laurent Poirel and Dr Yohei Doi for providing us with bacterial positive controls for *rmtF*, *rmtG* and *rmtH*, respectively. The research was funded by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Healthcare Associated Infections and Antimicrobial Resistance at Imperial College London in partnership with Public Health England. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health or Public Health England.

Funding: This research was funded by the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Healthcare Associated Infections and Antimicrobial Resistance at Imperial College London in partnership with Public Health England [HPRU-2012-10047].

Competing interests: None.

Ethical approval: Not required.

References

[1] Doi Y, Wachino JI, Arakawa Y. Aminoglycoside resistance: The emergence of acquired 16S ribosomal RNA methyltransferases. Infect Dis Clin North Am 2016;30(2):523–37.

[2] Lee H, Yong D, Yum JH, Roh KH, Lee K, Yamane K, et al. Dissemination of 16s rRNA methylase-mediated highly amikacin-resistant isolates of Klebsiella pneumoniae and Acinetobacter baumannii in Korea. Diagn Microbiol Infect Dis 2006;56(3):305–12.

[3] Bogaerts P, Galimand M, Bauraing C, Deplano A, Vanhoof R, De Mendonca R, et al. Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. J Antimicrob Chemother 2007;59:459–64.

[4] Wu Q, Zhang Y, Han L, Sun J, Ni Y. Plasmid-mediated 16S rRNA methylases in aminoglycoside-resistant Enterobacteriaceae isolates in Shanghai, China. Antimicrob Agents Chemother 2009;53:271–2.

[5] Doi Y, Arakawa Y. 16S ribosomal RNA methylation: Emerging resistance mechanism against aminoglycosides. Clin Infect Dis 2007;45(1):88–94.

[6] Turton JF, Perry C, Elgohari S, Hampton CV. PCR characterization and typing of *Klebsiella pneumoniae* using capsular type-specific, variable number tandem repeat and virulence gene targets. J Med Microbiol 2010:541–7.

[7] Woodford N, Ward ME, Kaufmann ME, Turton J, Fagan EJ, James D, et al. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum β-lactamases in the UK. J Antimicrob Chemother 2004:735–43.

[8] Doumith M, Day M, Ciesielczuk H, Hope R, Underwood A, Reynolds R, et al. Rapid identification of major *E. coli* sequence types causing urinary tract and bloodstream infections. J Clin Microbiol 2015;53:160–6.

[9] Piekarska K, Zacharczuk K, Wołkowicz T, Rzeczkowska M, Bareja E, Olak M, et al. Distribution of 16S rRNA methylases among different species of aminoglycoside-resistant Enterobacteriaceae in a tertiary care hospital in Poland. Adv Clin Exp Med 2016;25:539–44.

[10] Kang HY, Kim KY, Kim J, Lee JC, Lee YC, Cho DT, et al. Distribution of conjugative-plasmid-mediated 16S rRNA methylase genes among amikacin-resistant Enterobacteriaceae isolates collected in 1995 to 1998 and 2001 to 2006 at a university hospital in South Korea and identification of conjugative plasmids mediating dissemination of 16S rRNA methylase. J Clin Microbiol 2008;46:700–6.

[11] Ho PL, Lo WU, Yeung MK, Lin CH, Chow KH, Ang I, et al. Complete sequencing of pNDM-HK encoding NDM-1 carbapenemase from a multidrug-resistant *Escherichia coli* strain isolated in Hong Kong. PLoS One 2011;6:e17989.

[12] Pitart C, Solé M, Roca I, Román A, Moreno A, Vila J, et al. Molecular characterization of bla_{NDM-5} carried on an IncFII plasmid in an *Escherichia coli* isolate from a nontraveller patient in Spain. Antimicrob Agents Chemother 2015;59:659–62.
[13] Gamal D, Fernández-Martínez M, Salem D, El-Defrawy I, Montes LA, Ocampo-Sosa A, et al. Carbapenem-resistant *Klebsiella pneumoniae* isolates from Egypt containing blaNDM-1 on IncR plasmids and its association with *rmtF*. Int J Infect Dis 2016;43:17–20.
[14] Rojas LJ, Hujer AM, Rudin SD, Wright MS, Domitrovic TN, Marshall SH, et al. NDM-5 and OXA-181 beta-lactamases, a significant threat continues to spread in the Americas. Antimicrob Agents Chemother 2017;61 e00454-17.
[15] D'Andrea MM, Venturelli C, Giani T, Arena F, Conte V, Bresciani P, et al. Persistent carriage and infection by multidrug-resistant *Escherichia coli* ST405 producing NDM-1 carbapenemase: report on the first Italian cases. J Clin Microbiol 2011;49:2755–8.
[16] Qin S, Zhou M, Zhang Q, Tao H, Ye Y, Chen H, et al. First identification of NDM-4-producing *Escherichia coli* ST410 in China. Emerg Microbes Infect 2016;5:e118.
[17] Zhu Y, Zhao J, Xu C, Zhao H, Jia N, Yan-nian L. Identification of an NDM-5-producing *Escherichia coli* sequence type 167 in a neonatal patient in China. Sci Rep 2016;6:29934.
[18] Giske CG, Fröding I, Hasan CM, Turlej-Rogacka A, Toleman M, Livermore D, et al. Diverse sequence types of *Klebsiella pneumoniae* contribute to the dissemination of bla_{NDM-1} in India, Sweden, and the United Kingdom. Antimicrob Agents Chemother 2012;56:2735–8.
[19] Wilson ME, Chen LH. NDM-1 and the Role of Travel in Its Dissemination. Curr Infect Dis Rep 2012;14:213–26.