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The genetic analysis of *Enterobacteriaceae* plasmids to provide insights into the acquisition and spread of the *bla*$_{NDM}$ gene

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Abstract

Treatment options for infections caused by pathogenic Gram-negative bacteria, especially those of the *Enterobacteriaceae* family, are becoming limited with the increase of antimicrobial resistance (AMR). β-lactamases are a major mechanism for AMR within the *Enterobacteriaeae*. The most problematic β-lactamases are carbapenemases, which confer resistance to carbapenems, the major last-line antimicrobial. A recently emerged carbapenemase that has globally disseminated is the NDM carbapenemase, provided by *bla*$_{NDM}$ genes. These *bla*$_{NDM}$ genes (and other AMR genes) are able to transmit between strains when inserted on extrachromosomal self-replicating DNA molecules known as 'plasmids'. AMR genes within *Enterobacteriacae* are frequently associated with specific bacterial species, clonal lineage, plasmid Incompatibility (Inc) types or transposable elements. The *bla*$_{NDM}$ genes however do not have this association when oberserved in the *Enterobacteriaceae* family. To address and characterise this new paradigm presented by *bla*$_{NDM}$ genes, this thesis presents the bioinformatic analysis of plasmids associated with the *Enterobacteriaceae* to provide insights into the acquisition and spread of the *bla*$_{NDM}$ gene and an epidemiological approach to assess its plasmid-mediated dissemination between genetically unrelated species.

Specifically these aims were achieved by; firstly, the establishment of a recent account of the *bla*$_{NDM}$ gene from an epidemiological perspective using a novel genetic/molecular approach. This would identify the spread of individual plasmids carrying *bla*$_{NDM}$ across multiple species and patients, both within a single facility and across multiple national facilities. The approach combined in-depth bioinformatic analysis of *bla*$_{NDM}$ genetic contexts (NGCs) with common molecular epidemiology techniques. IncN2 (n=4) and IncA/C (n=3) were identified as the most common plasmids types carrying *bla*$_{NDM}$ across four patients within a Pakistani military hospital. These patients harboured between two and four NDM-1 producing Gram-negative bacilli of different

species coresident in their stool samples. IncFII-types (n=7) and IncX3 (n=4) were the most common plasmid types carrying *bla*$_{NDM}$ amongst 12 *Enterobacteriaceae* isolates, each from different patients across multiple Australian healthcare facilities. These isolates each carried one plasmid harbouring *bla*$_{NDM}$ but only five different *bla*$_{NDM}$ genetic contexts were identified, indicating five particular plasmids with a specific NGC had disseminated amongst these 12 isolates.

Secondly, to investigate transposable elements involved for insertion of the *bla*$_{NDM}$ gene into different plasmid types, the complete sequence of four plasmids carrying *bla*$_{NDM}$ (two IncA/C$_2$ and two IncFII$_Y$) was bioinformatically analysed. These plasmids were from four different clinical samples of four patients, comprised of *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Escherichia coli*. Each plasmid was observed to acquire *bla*$_{NDM}$ by different mechanisms on very similar plasmid backbones. Transposable elements IS*CR1* and either IS*26* or IS*CR27* were involved with *bla*$_{NDM}$ insertion into different locations of the antibiotic resistance island ARI-A on IncA/C$_2$ plasmids. Tn*3*-derived Inverted-repeat Transposable Elements (TIMEs) and an IS*903*-like element were identified for IncFII$_Y$ plasmids. This thesis collectively identified eight different transposable elements associated with *bla*$_{NDM}$: IS*CR27* and/or IS*26* on type 1 IncA/C$_2$; IS*CR1* on IncN$_2$, IncA/C$_2$ and IncFII$_2$; IS*CR6*-like, IS*903*-like and TIMEs on IncFII$_Y$; IS*26* and/or IS*3000* on IncX3; and an IS*26* composite transposon on IncH1B.

Thirdly, to investigate the relationship between plasmid types and bacterial species, *in silico* plasmid typing (via plasmid typing database, PlasmidFinder) and Principal Component Analysis (PCA) was performed to survey the plasmid content across 1683 *Enterobacteriaceae* isolates. These whole genome sequenced isolates comprised of *K. pneumoniae* (n=494), *Shigella sonnei* (n=223), *Yersinia* spp. (n=214), *Shigella flexneri* (n=171), *E. coli* (n=355), *E. cloacae* (n=133) and *Salmonella enteria* serovar Typhimurium (n=95). Twelve main plasmid types were identified distributed into three levels of occurence: common, IncF (~65% of strains); intermediate, IncHI,

IncI, IncR (8-10%); and rare, IncA/C, B/O/K/Z, L/M N, O, P, Q, X, and Y (0.5-3%). PCA of isolates and their shared plasmid content identified specific plasmid sub-types to represent possible routes of gene exchange between different genera. Furthermore, two primary clusters of species were identified based on their shared plasmid sub-type content, Group 1: *K. pneumoniae* and *E. cloacae*, and Group 2: *E. coli*, *S. sonnei* and *S. flexneri*. Species within each group were seen to be phyogenetically similar.

Collectively the analysis presented in this thesis, proposes an underlying network of interactions between AMR genes, transposable elements, plasmids types and the bacterial host, where each interaction may involve a degree of compatibility depending on the genera of the strain. The bla_{NDM} genes appear to have transmitted through this proposed network, from *Acinetobacter* spp. to disseminate amongst the *Enterobacteriaceae* family, following its interactions, compatibilities and limitations. Further surveillance of the *Enterobacteriaceae* family, including environment and community samples, will be required to define the extent plasmid-mediated AMR genes have spread within the *Enterobacteriaceae* family. The combined molecular/genetic approach and subsequent whole plasmid sequence analysis would be recommended for this survelliance. This PhD thesis provides insights into the acquisition and spread of the bla_{NDM} gene and emphasizes the capability of *Enterobacteriaceae* to transmit plasmid-mediated AMR genes amongst themselves to adapt to their environment, especially where antimicrobial pressure is present.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Review articles

- **Wailan AM**, *Paterson DL*. 2014. The spread and acquisition of NDM-1: a multifactorial problem. Expert Rev. Anti. Infect. Ther. 12:91-115. DOI: 10.1586/14787210.2014.856756

Original articles in peer-review journals

1. **Wailan AM**, *Sartor AL, Zowawi HM, Perry JD, Paterson DL, Sidjabat HE*. The genetic contexts of $bla_{\text{NDM-1}}$ in patients carrying multiple NDM producing strains. Antimicrob. Agents Chemother. 2015 Dec;59(12):7405-10. doi: 10.1128/AAC.01319-15
2. **Wailan AM**, *Paterson DL, Caffery M, Sowden D, Sidjabat HE*. Draft Genome Sequence of NDM-5-Producing *Escherichia coli* Sequence Type 648 and Genetic Context of $bla_{\text{NDM-5}}$ in Australia. Genome Announc. 2015 Apr 9;3(2). pii: e00194-15. doi: 10.1128/genomeA.00194-15
3. **Wailan AM**, *Paterson DL, Kennedy K, Ingram PR, Bursle E, Sidjabat, HE*. Genomic characteristics of NDM-producing *Enterobacteriaceae* in Australia and their bla_{NDM} genetic contexts. Antimicrob. Agents Chemother. 2015 Oct 19;60(1):136-41. doi: 10.1128/AAC.01243-15
4. **Wailan AM**, *Sidjabat HE, Yam WK, Alikhan NF, Petty NK, Sartor AL, Williamson DA, Forde BM, Schembri MA, Beatson SA, Paterson DL, Walsh TR, Partridge SR*. Mechanisms involved in acquisition of bla_{NDM} genes by IncA/C_2 and IncFII$_Y$ plasmids. Accepted with minor review at Antimicrob. Agents Chemother, 2016.
5. **Wailan AM**, *Cain AK, Goffau M, Reuter S, Baker KS, Mentzer A, Chung H, Ellington M, Holt KE, Thomson NR*. Surveying plasmid content of the *Enterobacteriaceae* family: a retrospective study. Submitted to Microbial Genomics, 2016.
6. *Baker KS, Burnett E, McGregor H, Deheer-Graham A, Boinett C, Langridge G,* **Wailan AM**, *Cain A, Thomson NR, Russel J, Parkhill J* . The Murray collection of pre antibiotic era

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7. *Zowawi HM, Ibrahim E, Syrmis MW,* **Wailan AM,** *AbdulWahab A, Paterson DL.* PME-1 Producing *Pseudomonas aeruginosa* in Qatar. Antimicrob. Agents Chemother. 2015 Jun;59(6):3692-3. doi: 10.1128/AAC.00424-15.
8. *Toh B, Paterson DL, Pfluege V, Kvaskoff D,* **Wailan AM**, *Riley T, Harper J, Flohr G, Huber C.* Relationships between whole genome sequencing, PCR ribotyping and MALDI - TOF MS in the subtyping of *Clostridium difficile*. Submitted to Diagn. Microbiol. Infect. Dis, 2015.
9. *Toh B, Paterson D, Witchuda Kamolvit W, Zowawi H, Kvaskoff D, Sidjabat H,* **Wailan AM**, *Peleg A and Huber C.* Species identification within the *Acinetobacter calcoaceticus - baumannii* complex using MALDI - TOF MS. J. Microbiol. Methods. 2015 Nov;118:128-32. doi: 10.1016/j.mimet.2015.09.006.

Conference publications (Appendix E-J)

1. Conjugation Rates of the NDM Plasmid Conferring Carbapenem Resistance in *Enterobacteriaceae* ***Wailan A****, Paterson D, Silvey A, Williamson D, Sidjabat H* Australian Society for Antimicrobials, 13rd Annual Scientific Meeting – Antimicrobials 2012, Brisbane, Australia February 2012 (Appendix J)
2. The mechanisms of plasmid acquisition of *bla*$_{NDM}$ in *Enterobacteriaceae* **Wailan AM,** *Sidjabat HE and Paterson DL* Australasian Society for Infectious Diseases, Gram Negative 'Superbugs' Meeting, Gold Coast, Australia August 2013 (Appendix G)
3. Complete Plasmid Sequence of IncA/C pKP1-NDM-1 from South East Queensland Yam WK, **Wailan AW**, Alikhan N-F, Paterson DL, Petty N, Beatson SA, Schembri MA, Sidjabat HE Australasian Society for Infectious Diseases, Gram Negative 'Superbugs' Meeting, Gold Coast, Australia August 2013 (Appendix H)

4. Sequence comparison of plasmids carrying *bla*$_{NDM}$ from Australian and New Zealand *Enterobacteriaceae*, **Wailan AM**, *Petty NK, Sidjabat HE, Beatson SA, Schembri MA, Williamson DA and Paterson DL,* Australian Infectious Diseases, Lorne Infection & Immunity Conference 2014, Lorne, Australia February 2014 (Appendix I)
5. The prevalence plasmids types carrying *bla*$_{NDM}$ and genetic context of *bla*$_{NDM}$ providing carbapenem resistance to the *Enterobacteraceae* family in Australia. **Wailan AM**, *Paterson DL*, *Nimmo GR*, *Karina K*, *Ingram PR*, *Sidjabat HE*. Australian Society for Antimicrobials, 16th Annual Scientific Meeting – Antimicrobials 2015, Brisbane, Australia February 2015 (Appendix F)
6. The genetic analysis of various NDM-producing Gram-negatives to investigate the inter-species and inter-patient transmission of *bla*$_{NDM}$ within a clinical setting. **Wailan AM**, *Sartor AL*, *Paterson DL*, *Perry JD*, *Sidjabat HE*. 25th European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, Denmark, April 2015 (Appendix E)

Publications included in this thesis

Chapter 1: Introduction and Review of the Literature

Wailan AM, Paterson DL. 2014. The spread and acquisition of NDM-1: a multifactorial problem. Expert Rev. Anti. Infect. Ther. 12:91-115. DOI: 10.1586/14787210.2014.856756.

Contributor	Statement of contribution
Wailan AM (Candidate)	Concept and Design (80%) Wrote the paper (100%)
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Chapter 2: The prevalence of plasmids within the *Enterobacteriaceae* family

Wailan AM, *Sartor AL, Zowawi HM, Perry JD, Paterson DL, Sidjabat HE.* The genetic contexts of bla_{NDM-1} in patients carrying multiple NDM producing strains. Antimicrob. Agents Chemother. 2015 Dec;59(12):7405-10. doi: 10.1128/AAC.01319-15

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Zowawi HM	Concept Design (10%) Edited manuscript (30%)
Perry JD	Edited manuscript (10%)
Paterson DL	Edited manuscript (10%)
Sidjabat HE	Designed experiments (10%) Execution of experiments (10%) Concept Design (20%) Edited manuscript (30%)

Chapter 3: Characterisation of NDM plasmids within *Enterobacteriaceae* in Australia

Wailan AM, *Paterson DL, Kennedy K, Ingram PR, Bursle E, Sidjabat, HE.* Genomic characteristics of NDM-producing *Enterobacteriaceae* in Australia and their bla_{NDM} genetic contexts. Antimicrob. Agents Chemother. 2015 Oct 19;60(1):136-41. doi: 10.1128/AAC.01243-15

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Ingram PR	Edit and revision of manuscript (10%) Clinical isolate information (30%)
Evan Bursle	Experimental work (15%)
Sidjabat HE	Designed experiments (30%) Bioinformatic analysis (20%) Experimental work (35%) Clinical isolate information (40%) Edit and revision of manuscript (30%)

***Wailan AM**, Paterson DL, Caffery M, Sowden D, Sidjabat HE*. Draft Genome Sequence of NDM-5-Producing *Escherichia coli* Sequence Type 648 and Genetic Context of bla_{NDM-5} in Australia. Genome Announc. 2015 Apr 9;3(2). PMID: 25858833 (Appendix A)

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Sowden D	Supplement of clinical data (50%) Edit and revision of manuscript (10%)
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Chapter 4: Complete NDM plasmid backbone analysis and mechanisms of plasmid capture.

Wailan AM, *Sidjabat HE, Yam WK, Alikhan NF, Petty NK, Sartor AL, Williamson DA, Forde BM, Schembri MA, Beatson SA, Paterson DL, Walsh TR, Partridge SR*. Mechanisms involved in acquisition of *bla*$_{NDM}$ genes by IncA/C$_2$ and IncFII$_Y$ plasmids. Accepted with minor review at Antimicrob. Agents Chemother, 2015.

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Alikhan N	Edit and revision of manuscript (5%)
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Paterson DL	Edit and revision of manuscript (5%)

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Contributions by others to the thesis

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Keywords

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List of Abbreviations

Abbreviation	Term
Δ	partial sequence
AmpC	ampC β-Lactamase
AMR	Antimicrobial resistance
BLAST	Basic Local Alignment Search Tool
bla	β-lactamase gene
ble_{MBL}	bleomycin resistance
BSI	blood stream infection
C. freundii	*Citrobacter freundii*
CMY	cephamycinase
CTX-M	Cefotaximase-Munich
dct	divalent cation tolerance protein
ECCMID	European Congress of Clinical Microbiology and Infectious Diseases
EBI	European Bioinformatics Institute
ESBL	Extended-Spectrum β-Lactamase
ETEC	*Enterotoxigenic Escherichia coli*
groEL	heat resistant chaperonin
groES:	cofactor of *groEL*
HGT	Horizontal Gene Transfer
IMP	Imipenemase
Inc	Incompatibility
IR_L	Inverted Repeat Left
IR_R	Inverted Repeat Right
IS	Insertion Sequence
IS*CR*	IS Common Region
KPC	*Klebsiella pneumoniae* carbapenemase
LB	Luria Bertani
MBL	Metallo-β-lactamases
MDR	Multi-Drug Resistant
MH	Mueller Hinton
MIC	Minimum Inhibitory Concentration
MITE	Miniature Inverted-Repeat Transposable Element

MLST	Multi-Locus Sequence Typing
MSM	Men who have sex with men
NCBI	National Center for Biotechnology Information
NDM-1	New Delhi Metallo-β-lactamase-1
NGC	*bla*$_{NDM}$ Genetic Context
OXA	Oxacillinase
Δ*pac*	truncated phospholipid acetyletransferase
PacBio	Pacific Biosciences
PBRT	PCR-Based Replicon Typing
PCR	Polymerase Chain Reeaction
PCA	Principal Component Analysis
SNP	Single Nucleotide Polymorphism
ST	Sequence type
tat	twin-arginine translocation pathway signal sequence protein
TIME	Tn*3*-derived Inverted-repeat Transposable Elements
trpF	phosphoribosylanthranilate isomerise
Tn	Transposon
UTI	uniary tract infections
U.K.	United Kingdom
WGS	Whole Genome Sequence

Chapter 1: A new paradigm presented by the *bla*$_{NDM}$ gene

This chapter presents a background on genes conferring antimicrobial resistance, defining the factors involved in their spread within the *Enterobacteriaceae* family. Those factors will be described for the *bla*$_{NDM}$ gene according to literature at the commencement of PhD candidature (2013), summarised in manuscript form. A thesis outline and overall aim, supplemented with specific chapter aims will also be presented.

1.1 Introduction

Gram-negative bacteria are pathogens that can cause a range of serious human infections including pneumonia, urinary tract, intra-abdominal, and bloodstream infections, but also asymptomatic colonisation in the gastro-intestinal tract. These infections are usually treated with antimicrobials such as β-lactams. Unfortunately, treatment options are diminishing with multi-drug resistant (MDR) strains frequently reported as resistant to the carbapenemes the major "last line" antimicrobial group (1-4). The genes responsible for an antimicrobial resistance (AMR) phenotype comprise of an array of genes, including different β-lactamase genes (*bla*), which produce enzymes able to hydrolyse specific β-lactam compounds. These β-lactamase enzymes can be divided into four different groups based on the Ambler molecular classification system (5, 6). This system categorises the enzyme according to their amino acid sequence, primarily on their active site. Classes A (Extended-spectrum β-lactamases; ESBLs), C (AmpC) and D (OXA type β-lactamases) are serine β-lactamases and Class B are metallo-β-lactamases (MBL) which require a bivalent metal ion for activity, usually Zn^{+2} (7). ESBLs or AmpC β-lactamases genes such as *bla*$_{CTX-M-15}$ and *bla*$_{CMY-2}$, are able confer resistance to extended-spectrum cephalosporins, a frequently used sub-group of β-lactams (8, 9). Pathogens producing ESBLs or AmpC are often treated with carbapenems (10-12). There are β-lactamase genes however, able to produce carbapenemase enzymes that

hydrolyse carbapenems, for example the *bla*$_{NDM}$ and *bla*$_{KPC}$ genes (1). The emergence of carbapenemases and their high prevalence within species of *Enterobacteriaceae* family e.g. *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter cloacae*, has brought carbapenemase-producing-*Enterobacteriaceae* to the attention of those within the health-care and infectious disease profession (4, 13).

A recently identified carbapenemase to disseminate globally is New-Delhi Metallo-β-lactamase (NDM-1), which was first described in 2009, produced by a *K. pneumoniae* strain isolated from a Swedish patient who received medical treatment in New-Delhi, India (2, 14). This gene (*bla*$_{NDM}$) provides resistance to all β-lactams (except aztreonam), and unlike other classes of β-lactamases has no commercially available inhibitors. The *bla*$_{NDM}$ gene has also rapidly spread to every inhabitable continent (in over 40 countries (15-17)) by 2013, with the Indian sub-continent described as the major reservoir for human acquisition of NDM producing Gram-negative bacteria (18, 19). NDM producers also frequently carry other antimicrobial resistance determinants, including ESBLs, AmpC cephalosporinases, other carbapenemases (OXA type), and those that provide resistance to aminoglycosides (16S RNA methylases), quinolones (*qnr*), rifampicin, sulfamethoxazole and chloramphenicol (20-23). As a consequence of strains co-harbouring these resistance determinants with *bla*$_{NDM}$, antimicrobial treatment for the majority of NDM producing strains is limited to only three available drugs, colistin, tigecycline and fosfomycin (24). Unfortunately colistin is unfavourable because of its nephrotoxicity and neurotoxicity adverse effects. Tigecycline has a large volume of distribution resulting in a limited efficacy. For blood stream infection (BSI), uninary tract infections (UTI) and pneumonia treatment (25-30) this is not optimal, as presence of tigecycline in blood, the urinary tract, and the lung would be in low concentrations. Alternatively fosfomycin, which is an older antimicrobial, is being revisited for treatment because of its excellent clinical efficacy for UTI. However, is not as widely

available compared to colisin and tigecycline, and the lack of randomised control trials leaves fosfomycin restricted to only UTI treatment. Resistance to these antimicrobials has also been reported in NDM producers (31-34).

The rapid spread of the *bla*$_{NDM}$ gene is central to this thesis and has been attributed to several factors including epidemiological aspects such as medical tourism, asymptomatic carriage, and personal travel (18, 35). Additonal factors include those linked to the Asian continent, such as the poor sanitation problems, polluted water sources which was highlighted during the first report of NDM as well as the lack of control and monitoring of over the counter prescriptions. Molecular epidemiology and bioinformatic investigations have made numerous associations with previously identified AMR genes. These include frequently observed genetic contexts (the structure of the DNA/sequence surrounding the AMR gene) (36, 37), types of plasmids (circular extrachromosomal pieces of DNA carried by strains) where the gene has inserted (38-40), clonal lineages (41, 42), or bacterial species (43, 44) frequently carrying the gene, or a combination of these. The following sections of this chapter will introduce and summarise these epidemiological aspects and associations with previously identified AMR genes. These associations will be described for the *bla*$_{NDM}$ gene, with further details listed in a literature review manuscript.

1.2 Addressing the spread of MDR strains

There are three major interventions implemented by health-care professionals in response to the numerous strains carrying AMR genes, primarily identified by epidemiological studies. These interventions were implemented to control and address major factors involved in AMR development and the spread MDR strains. The first is infection control, which contains the spread of MDR organisms from its source (e.g. infected patients) in order to prevent additional infections during an outbreak, especially within health-care facilities where

hospital-acquired (nosocomial) infection is common (45). The second is surveillance via screening populations for MDR organisms to inform professionals in infection control and rapid diagnostics of their underlying spread. This includes asymptomatic carriage, as natural human flora are capable of carrying AMR genes e.g. gut colonisers (46). Asymptomatic carriers have been recently identified as 'vehicles' for the spread of resistance by commuting internationally, for purposes of medical tourism or personal travel (47-49). The third intervention is antimicrobial stewardship, which aims to limit the selection and development of antibiotic resistance within health-care facilities and the community, as the over- or misuse of antimicrobials has been theorised to have led to the emergence and dissemination of antimicrobial resistance (50). This legislation involves monitoring and promotion of appropriate use of antimicrobial prescriptions. These concepts essentially are interventions for human-to-human transmission, and antibiotic resistance selection and development in MDR bacteria, which has been associated with controlling the transmission of AMR.

1.3 Molecular epidemiology investigations of plasmid-mediated AMR genes

Multi-drug resistant organisms have become frequently reported. In response, there have been studies conducted to understand the mechanisms involved in the spread of AMR amongst Gram-negative bacteria. Previously, public health and epidemiological investigations have used molecular typing to determine the basis of carbapenem resistance spread. This would involve a comparison of bacterial strains isolated from a cohort of patients to identify clonal strains and possible outbreaks sources. A standard procedure to identify clonal strains is determining their sequence type (ST) by analysing the DNA sequence of multiple conserved house keeping genes within each strain. Those with the same ST are considered 'clonal'. The Multi-Locus Sequence Typing (MLST) scheme is a widely used typing method for these purposes (available at: http://pubmlst.org (51)). These

molecular epidemiology investigations would frequently associate the AMR gene with, either certain bacterial species where they were intrinsic such as *Acinetobacter baumannii* (*bla*$_{\text{OXA-51-like}}$; carbapenemase (52)) and, *K. pneumoniae* and *E. coli* (the chromosomal *ampC* gene (10, 43)), or commonly spread by specific clones, for example *E. coli* ST131 (*bla*$_{\text{CTX-M-15}}$ (41)), *K. pneumoniae* ST258 (*bla*$_{\text{KPC}}$ (53)) and *A. baumannii* clonal complex 92 (*bla*$_{\text{OXA-23}}$; carbapenemase (54)). The *bla*$_{\text{NDM}}$ gene however is not solely associated with a specific species or clone (although there are some clones that are frequently observed to carry *bla*$_{\text{NDM}}$ e.g. *E. coli* ST101), reported in different species within the *Enterobacteriaceae* family, non-*Enterobacteriaceae* genera (*Acinetobacter* spp. and *Pseudomonas* spp.), and various sequence types (20). A likely part of the explanation that the *bla*$_{\text{NDM}}$ gene does not follow this paradigm is because the gene is frequently located on plasmids. Plasmids carry genes able to produce enzymes for its horizontal gene transfer between two bacterial cells, in a process known as conjugation. Plasmid-mediated AMR genes such as the *bla*$_{\text{NDM}}$ gene are unfortunately unable to be properly and entirely assessed by this type of molecular epidemiology alone, and may need additional techniques to assess the entire situation, such as conjugation and/or transformation experiments.

1.4 Investigations of plasmid-mediated AMR genes

Plasmids are circular elements of DNA that replicate independently from the chromosome and do not encode genes essential for bacterial cell growth (55). Each gene on a plasmid can be categorised in groups/modules according to their respective function such as plasmid replication e.g. replicon genes, stability, transfer (for conjugation), establishment, partitioning and accessory/adaptive genes e.g AMR genes. Plasmids exist in a range of sizes from small sized plasmids e.g 1 Kbp, while others can be large for example up to 100 Kb in size. Large sized plasmids usually are present in low copy number while smaller plasmids have higher

copy numbers. Those that are associated with AMR are typically plasmids with low-copy number and often carry genes for conjugational transfer to other strains. Plasmids associated with the *Enterobacteriaceae* family are categorised into different Incompatibility (Inc) types or groups. This incompatibility was originally based on the observation that plasmid backbones with the same replication system cannot be co-retained within the same cell line (40, 56). Those plasmid backbones with the same replication system were then categorised within the same Inc group. Plasmids belonging to different Inc groups are able to be co-harboured within the same strain. Typing for plasmids into their Inc groups was initially developed in the late 1980s, utilising DNA-DNA hybridisation between strain DNA immobilised on filters and purified DNA fragments/probes which are specific for each Inc group and labelled via nick translation to allow for colourmetric detection (57). Typing of plasmids was then updated in 2005 as a typing scheme based on primers after the identification of their major role in the spread of AMR through conjugation, reducing time and labor in molecular epidemiology. The plasmid-typing scheme was named 'PCR-based replicon typing' (PBRT) and used a multiplex PCR for 18 major plasmid types to identify the Inc type of the plasmid carrying AMR genes (58). Similar to sequence typing, PBRT targets conserved sequence regions representive for each plasmid type, such as the plasmid replicon region responsible for replication. Through this typing certain AMR genes were frequently identified on specific plasmid types and became highly associated, such as the IncA/C plasmids carrying $bla_{\text{CMY-2-like}}$ genes, and IncF types plasmids carrying $bla_{\text{CTX-M-15}}$ within the *E. coli* ST131 clone, or carrying bla_{KPC} in *K. pneumoniae* (40). The bla_{NDM} gene however is not associated with a particular plasmid type unlike these prominent AMR genes. The bla_{NDM} gene has been reported on numerous *Enterobacteriaceae* plasmid backbones/ Inc types, IncA/C, IncF types, IncHI types, IncL/M, IncX types, and IncN types (40, 59). Acquisition of a "NDM plasmid" by a strain could be considered responsible for their carbapenem resistant

phenotype; in a similar fashion previous AMR genes such as *bla*$_{\text{CMY-2-like}}$ genes were associated with acquisition of an IncA/C plasmid type. The spread of the *bla*$_{\text{NDM}}$ gene however, is far more complex as some plasmids carrying the *bla*$_{\text{NDM}}$ gene are genetically different despite being characterised as the same plasmid type. This was indicated by IncA/C plasmids available during commencement of PhD candidature, pNDM10469 (Accession no JN861072.1), pNDM102337 (Accession no. JF14412.2), and pNDM10505 (Accession no. JF503991.1). Typing of plasmids (Inc) and the bacterial host (ST) therefore can only partially explain the mechanisms of acquisition and spread of this carbapenemase gene.

1.5 Investigations for transposition of AMR genes

In parallel to the mentioned molecular epidemiology investigations, bioinformatic studies began to analyse these plasmids in high detail with the increased availability to commercial whole genome sequencing. These analyses would characterise another mechanism that contributes to the movement of AMR genes called “transposition”. Transposition events involve the movement of DNA from one DNA site to another within a bacterial host. These events are facilitated by numerous transposable element enzymes such as insertion sequence (IS) elements (60-62). This gene movement can occur between two sites on the chromosome, between chromosome and a plasmid or between different plasmids. Each transposable element has a specific mechanism involving recognition sites on the DNA for insertion and the amount of adjacent sequence upstream or downstream of the transposable element that is mobilised (62). Frequently encountered mechanisms will be briefly reviewed in the following paragraphs.

Homologous recombination is a natural and major process in which DNA can be exchanged within a bacterial cell. The recombination process requires the breaking and joining of DNA strands at regions that have high similarity, which is dependent on the degree and length of

homology (60). This process can occur on the same DNA molecule or between different DNA molecules in the same bacterial cell. Recombination between two highly similar sequences on two different DNA molecules such as two plasmids can fuse the two molecules together. Further, a 'double crossover' event can occur where recombination involving two separate pairs of highly similar sequences that are in close proximity (60). A recombination event can result in the exchange of DNA segments between the two pairs. This homologous recombination process has the potential to transpose regions carrying antimicrobial resistance determinants to different sites on the chromosome and plasmids. Numerous elements such as Insertion Sequences (IS) or transposons (Tn) are also able to act as the high similar sequences during the mentioned recombination when present in multiple copies.

There are multiple transposable elements involved in the transposition of antimicrobial resistance determinants. The major types are IS elements, composite and unit transposons, and integrons. 'Classical' insertion sequences elements are sequences bound by two short identical or imperfect inverted repeats (IR) that define their boundary. Between both IR are one or two genes that encode the transposase enzyme which facilitates movement of the IS element. The direction of transcription for the transposase gene(s) designates the IR, 'IR_L' upstream of the gene or left end of the IS element and 'IR_R' downstream or right end of the IS element. The IR are recognised by the transposase enzyme to facilitate the movement of the DNA sequence between the IR to a new location either using a 'cut and paste' and/or 'copy and paste' process depending on the specific IS element (63). The movement for the majority of IS elements creates 2-14 bp direct repeats (DR) flanking the IS element's new location. The DR indicates and provides evidence of the occurrence of a transposition event when bioinformatic analysis of bacterial genomes is performed. IS elements do not carry any additional genes, however when two copies of the same (or similar) IS element are located in

close proximity to each other the transposase is able to recognize and mobilise the sequence between both IS elements as a 'composite transposon'. Transposition however is not always simple. There are instances which IS elements such as IS*Ecp1*, which are unlike classical IS elements fail to recognised their IR_R and instead recognise a sequence similar to the IR_R further downstream of the element. Tranposition would then proceed to mobilise the sequence between the IR_L and the similar IR downstream, which can carry antimicrobial resistance determinants (60).

Unit transposons were originally defined as larger than an IS element and in addition to their transposition function carried other genes such as antimicrobial resistance determinants (60). The Tn*3* transposon family is a frequently observed unit transposon consisting of two sub-groups, Tn*3*-like and Tn*21*-like. Both sub-groups consist of a transposase (*tnpA*), resolvase (*tnpR*) and a resolution site (*res)* and are flanked by a 38bp IR. Mobilisation involves IR recognition by the TnpA enzyme, generating a co-integrate intermediate consisting of the donor and recipient DNA molecules. The co-integrate intermediate is resolved by tnpR-mediated site-specific recombination and generates a 5 bp DR upon transposition. The differences between the two sub-groups are the sequence and the organisation of the *tnpA*, *tnpR* and *res* genes. Elements of the Tn*3*-like subgroup have the *res* located between the *tnpA* and *tnpR* which are transcriptionally orientated in opposite directions. In contrast, Tn*21*-like sub-group members have *tnpA* and *tnpR* orientated in the same direction and the *res* is not located between both genes but downstream of *tnpR* i.e. 5'-*tnpA*|*tnpR*|*res*-3'. In addition to the mentioned transposable elements, there are other elements that utilise different mechanisms for the transposition of DNA sequences, including IS*CR*s, ICE and integrons.

Insertion sequence common regions (IS*CR*s) are IS*91*-like sequences e.g IS*91*, IS*801* and IS*1294* and are not flanked by terminal IRs like classical IS elements (64). IS*CR*s use a 'rolling-circle' replication mechanism to mobilise the DNA upstream of the transposase gene, essentially mobilising in the reverse direction when compared to classical IS elements. In order to mobilise, the transposase named 'rcr' recognises a flanking origin (*ori*IS) downstream, and a terminus (*ter*IS) upstream. The rcr can fail to recognise the upstream *ter*IS and continue to replicate and transpose into the adjacent genetic structures until a surrogate *ter*IS is recognised (65). Through this mechanism, these elements can mobilise at least 28 Kb of DNA in a single movement (65). Thus, they are able to transpose genes conferring antibiotic resistance without the usual flanking invert repeats (66).

Integrative and Conjugative Elements (ICE) are self-transmissible mobile genetic elements which encode proteins for their own excision from a DNA molecule, conjugation to another bacterial cell and reintegration/insertion into the chromosome or plasmid. Prior to its first classification in 2002, ICE were known previously as different groups i.e. IncJ plasmids, conjugative tranpsosons (CTn), and constins, but they all described the same element. The nomenclature has been revised and such elements types are currently known as CTn or ICE (67). These mobile elements (similar to plasmids) are able to transmit antimicrobial resistance determinants between locations on the chromosome, between chromosome and plasmids as well as between bacterial cells.

Integrons were discovered through their association with antimicrobial resistance determinants. The identification of other integron types such as chromosomal integrons and 'mobile resistance integrons' led to the classification of the original integrons as class 1 integrons, according to their integron-integrase (*intI*) sequences. Class 1 integrons are genetic

structures composed of a recombinase called integrase and the recombination site *attI*. The IntI1 protein facilitates the insertion or excision of circularised gene cassettes into the integron by catalyzing recombiniation between the *attC* site on the gene cassette and the recombination site *attI* of the integron (68, 69). These gene capturing integrons can also be mobilised due their placement into transposon structures such as the Tn*402*/Tn*5090*-like transposon. The most frequently encountered in terms of antimicrobials resistant determinants are those derived from Tn*402*-like transposons, sometimes referred to as the 'clinical' or 'sul1-type' transposons. These transposons have two conserved segments (CS). The 5'-CS contains the IRi of the Tn*402*-like transposon and *intI1*, while the 3'-CS carries two ORFs (unknown function) and a truncated *qacEΔ1* gene overlapped with a *sul1*, which are remnants of an integration event of *sul1* into a previous Tn*402*-like transposon carrying *qacEΔ1* as the final cassette. These conserved segments are frequently observed flanking cassette arrays that provides the opportunity for entire arrays to be exchanged between two 'clinical' transposons if present within the same bacterial cell via double crossover events. Gene cassettes of integrons are also able to mobilise into other integrons in the same cell via homologous recombination between similar gene cassettes of different arrays.

Transposition events in addition to mobilising antimicrobial resistance genes also have the opportunity to generate antimicrobial resistance genes via fusion of genes. The *bla*$_{NDM}$ gene has been described as a chimeric gene. In 2012, Toleman and colleagues, theorised that *bla*$_{NDM-1}$ may be a chimeric gene, fused with a previous aminoglycoside resistant gene, *aphA6* by alignment of several sequences containing the genetic environment around *bla*$_{NDM-1}$ (70). The authors first aligned genetic contexts of the *bla*$_{NDM-1}$ gene available at the time. The alignment showed that the intergenetic region between IS*Aba125* and *bla*$_{NDM-1}$ were identical. Furthermore the GC% content was found to change dramatically from below 50% to above 50% after the first 19 nucleotides (encoding the first 6 amino acids) into the *bla*$_{NDM-1}$

gene. A second alignment was conducted between several sequences containing either 5'-IS*Aba125*|*bla*$_{\text{NDM-1}}$-3' or 5'-IS*Aba125*|*aphA6*-3' (both with IS*Aba125* upstream). This revealed that IS*Aba125* was located at the same distance upstream from both *bla*$_{\text{NDM-1}}$ and *aphA6*, and the intergenetic regions between IS*Aba125* and their respective gene had a 100% identical sequence. The alignment of the genetic contexts of *bla*$_{\text{NDM}}$ and *aphA6* interestingly breaks at 19bp within *bla*$_{\text{NDM-1}}$, the same point at where the GC% content changes, thus suggesting that *bla*$_{\text{NDM-1}}$ is a chimeric gene produced by in-frame fusion of *aphA6* with a pre-existing MBL gene.

The authors proposed two theories for the events generating the chimeric *bla*$_{\text{NDM-1}}$. The first theory is an in-frame deletion event between an *aphA6* gene within an IS*Aba125* composite transposon upstream of a pre-existing *bla*$_{\text{MBL}}$ gene. The second theory involves the insertion of a previous *bla*$_{\text{MBL}}$ via an IS*CR* element and its rolling circle mechanism, to insert into *aphA6* with IS*Aba125* upstream. The latter is more favourable as Tn*125*$_{\text{NDM}}$ also contains IS*CR27* downstream to *groEL* and gro*ES*.

With the above knowledge, bioinformatic analysis would frequently describe genetic contexts for particular AMR genes, detailing adjacent transposable elements such as IS elements, transposons, capture systems e.g. class 1 integrons (a common location for the insertion of AMR genes cassettes), and other genes in close proximity (60). These analyses would determine the sequences of events that genetic contexts would arise via sequence comparison with other strains and plasmids. Consequently certain transposable elements were associated with AMR genes by frequent co-observation. These associated transposable elements would infer the transposition mechanisms involved for insertion of the AMR gene onto the chromosome and plasmid. Mobilisation between two locations would be inferred by their comparison with other similar genetic contexts. One example of the association between

AMR genes and transposable elements is the *bla*$_{\text{CMY-2-like}}$ and *bla*$_{\text{CTX-M-15}}$ genes with the IS*Ecp1* transpositional unit (60, 71). The *bla*$_{\text{NDM}}$ gene has been associated with transposon Tn*125* (bound by two IS*Aba125*) within *Acinetobacter* spp. (72, 73), however when observed on *Enterobacteriaceae* plasmids and compared to the genetic contexts such as those of *bla*$_{\text{CMY-2-like}}$ and *bla*$_{\text{CTX-M-15}}$ genes (frequently observed presence in the IS*Ecp1* transpositional unit), the *bla*$_{\text{NDM}}$ gene does not have an associated and frequent genetic context apart from a fragmented Tn*125* (ΔTn*125*) of different lengths. ΔTn*125* would frequently encompass a single ΔIS*Aba125* upstream of the *bla*$_{\text{NDM}}$ and a *ble*$_{\text{MBL}}$ gene downstream, with fluctuations in the presence of other Tn*125*-associated genes (2, 74, 75). Although not widely known by health-care professionals, the transposition mechanism is an important factor to understand the acquisition and spread of the *bla*$_{\text{NDM}}$ gene amongst various plasmid types and bacterial species.

The above and literature during candidature commencement is summarised, presented and discussed further in manuscript format published in *Expert Reviews of Anti-infective Therapy*. **Wailan AM**, *Paterson DL.* 2014. The spread and acquisition of NDM-1: a multifactorial problem. Expert Rev. Anti. Infect. Ther.12:91-115. DOI: 10.1586/14787210.2014.856756

The University of Queensland requires the presentation of the submitted or accepted article. The numbers of the figures, tables and references of the presented article have been amended to suit the structure of this thesis. The article remains in American English spelling enforced by journal guidelines.

1.6 Chapter 1 - Review: The spread and acquisition of NDM-1: a multifactorial problem.

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Abstract: *bla*$_{NDM}$ is a major mechanism of resistance of gram-negative bacteria to β-lactam antibiotics including the carbapenems. *bla*$_{NDM}$ has been acquired by a large range of gram-negative bacilli, especially by the *Enterobacteriaceae* and *Acinetobacter* spp. The combination of human factors (suboptimal antibiotic stewardship and infection control, movement of people between countries) plus bacterial factors (hospital adapted clones, environmental persistence and prolific horizontal gene transfer) has led to global spread of *bla*$_{NDM}$ at a rapid pace. Treatment options for NDM producers are very limited. For serious infections, combination therapy including a polymyxin is preferred. However, resistance to polymyxins is emerging. Clearly, substantial international efforts must be made to control the spread of NDM-producers or else many of the advances of modern medicine may be undermined by untreatable infections.

Keywords: NDM-1, nosocomial, community, transmission routes, medical tourism, plasmid, transposition, Tn*125*, genetic mobilization, polymyxins

Gram-negative bacteria have developed resistance to many antibiotics including the last line β-lactams, carbapenems. This is achieved by developing or acquiring genes conferring antibiotic resistance. A number of mechanisms of antibiotic resistance are possible. These mechanisms include antibiotic modification, modification of the target site of antibiotic activity and membrane alteration & efflux systems (76). For example, carbapenem resistance may occur due to membrane impermeability to the carbapenems (typically linked to use or misuse of this class of antibiotics).

The most important mechanism of resistance to β-lactam antibiotics is the production of enzymes called β-lactamases. These enzymes are encoded by the *bla* genes which hydrolyze the β-lactam ring, rendering the antibiotic inactive (77).

Carbapenem resistance is most frequently due to carbapenemases, β-lactamases capable of hydrolyzing the carbapenem antibiotics. A variety of carbapenemases have emerged such as KPC (particularly prominent in the United States, Greece, Italy and Israel) and OXA-48 (particularly prominent in Turkey, North Africa and the Middle East). Another such carbapenemase is the New Delhi metallo-β-lactamase (NDM), which is defined as a Class B metallo-β-lactamase using the Ambler classification of β-lactamases. The NDM-type β-lactamase was first isolated in 2009 from a Swedish patient returning from India, who was infected with *Klebsiella pneumoniae* resistant to multiple antibiotics including all carbapenems (2). bla_{NDM} has now spread to all inhabited continents and is carried by multiple gram negative species (78-84). This review will provide a detailed overview focusing on the different aspects allowing for the successful global spread of NDM including epidemiology and genetics. The article will conclude with a brief review of infection control and current treatment options available to treat infections due to NDM producers.

Epidemiology

"Reservoirs" of NDM producers

Since the first report of NDM produced by *K. pneumoniae* from Sweden, *bla*$_{\text{NDM-1}}$ has been reported throughout the world (Fig. 1) (2). In the first few years following this initial case, NDM producing organisms have been strongly linked with the Indian subcontinent (India, Pakistan, Bangladesh and Nepal), through epidemiologic studies in this area and travel history in individuals returning to other countries with infections (16). This suggests the Indian sub-continent is a major/ primary reservoir for *bla*$_{\text{NDM}}$ acquisition. China is also known to be a reservoir country, emerging as a reservoir shortly after the Indian sub-continent's association with *bla*$_{\text{NDM}}$. At this stage it does not appear that NDM producers are as widely prevalent in China as in the Indian subcontinent. The Balkan states (for example, Serbia, Montenegro, and Bosnia-Herzegovina) may be considered as a "secondary" reservoir area for *bla*$_{\text{NDM}}$ acquisition since a number of cases have been reported with no travel history to Asia.

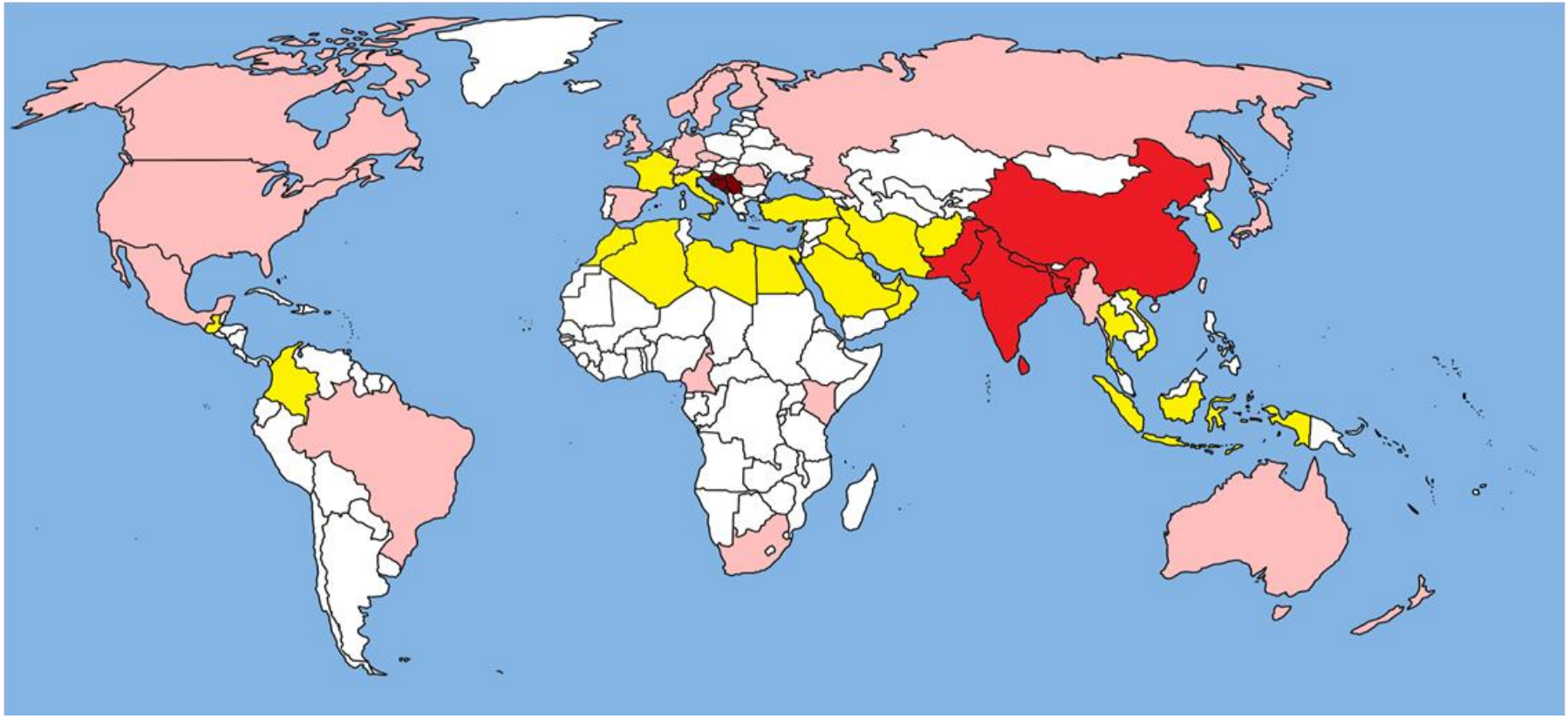

Figure 1. International dissemination of *bla*$_{NDM}$ as reported in the literature till July 2013

Red: Initial reservoirs (Indian sub-continent and China); Dark Red: Secondary reservoirs (Balkan States); Yellow: "Expanded" Reservoirs; Light Red: Countries reported cases linked with international travel.

Expansion of Reservoirs

The situation is, of course, quite fluid and secondary cases are occurring in many parts of the world. In particular, the greater Middle East (Morocco (45, 85, 86), Algeria (87), Libya, Egypt (88, 89), Iraq (90, 91), Kuwait (32), Oman (92), Lebanon (93), Afghanistan (94)), South East Asia (South Korea (95), Indonesia (96), Vietnam (81) and Thailand (85)) and parts of Europe (France (97), Italy (98)) may be additional reservoir areas since they have recently been linked with nosocomial acquisition, community acquisition or have been the area of origin for *bla*$_{NDM}$ in cases involving personal travel.

Additionally, community acquired NDM producing bacteria have been reported in Russia (99), Guatemala (100) and Colombia (79) indicating the possibility that the number of NDM reservoir countries is still increasing and expanding.

Travel: medical tourism and personal travel

Travel appears to be the major means by which NDM producing bacteria have spread throughout the world. Europe provided the first case in 2009 in Sweden and shortly after other European countries began reporting travel related NDM acquisition from the Indian Sub-continent or the Balkan states. These countries include Sweden, United Kingdom, Spain, Belgium, Norway, Switzerland, Denmark, Netherlands, Germany, Belgium, and Croatia. The only European country to not have been linked via travel from reservoir areas (Balkan States and/or the Indian sub-continent), is Czech Republic which involved travel from Egypt.

The countries outside of Europe that have reported NDM, initially only through travel to the Indian sub-continent, are the United States, Canada, Japan, Australia, New Zealand, Oman and Kuwait (15, 45, 101, 102). Other countries with NDM reports involving travel, are countries linking NDM acquisition from other potential reservoir countries previously

mentioned. These countries include Singapore reporting links to Vietnam and Indonesia, and Turkey and Lebanon reporting epidemiological links with Iraq.

It must be noted that the reports mentioned above have included clinical information concerning travel history. Many reports do not state (due to its unavailability), the patient travel history or any possible indication of where the NDM producing organism was acquired. Therefore the expansion of NDM reservoir areas may be certainly underestimated.

Three major routes of NDM acquisition

In terms of international spread and countries reporting NDM, there are three major routes of NDM acquisition. These are nosocomial acquisition (Table1), personal travel (Table 2) and community acquisition (Table 3).

The nosocomial acquisition route we define as hospital acquisition in the patient's home country or abroad. The presumed most likely modes of nosocomial acquisition are inadequate hand hygiene and spread from a contaminated hospital environment.

Community acquired bla_{NDM} provides the second main route for human route of acquisition. We define this as acquisition in the person's country of residence without recent international travel. These reports indicate reservoirs and common means by which NDM producers can be acquired. Community acquired bla_{NDM} also involves the presence of NDM within the environment and thus this category of acquisition also includes reports of NDM producers retrieved from the environment, which in the most common forms include water seepage and tap waste (78, 103).

Table 1. Nosocomial acquired NDM including medical tourism

Travel Acquired	Introduced Country	Species	Sample	Reference
Indian sub continent origin				
India	Sweden	*Klebsiella pneumoniae*	Urine	(2)
	France	*Salmonella enterica* subsp. *enterica* serotype Westhampton	Urine	(104)
	France	*Citrobacter freundii*	Catheter, Urine	(84)
	France	*Klebsiella pneumoniae*	Faecal sample	(104)
	France	*Escherichia coli*	Unknown (2)	(105)
	New Zealand	*Klebsiella pneumoniae*	Rectal swab	(101)
	New Zealand	*Escherichia coli*	Rectal swab (2), Urine	(101)
	New Zealand	*Proteus mirabilis*	Rectal swab	(101)
	Oman	*Klebsiella pneumoniae*	Wound, Intra-adominal, sputum	(92)
	Oman	*Klebsiella pneumoniae*	Urinary catheter	(106)
	United Kingdom	*Klebsiella pneumoniae*	Blood, Urine, wound swab, sputum	(16)
	United Kingdom	*Escherichia coli*	Blood, Urine, wound swab, sputum	(16)
	United Kingdom	*Enterobacter* spp.	Blood, Urine, wound swab, sputum	(16)
	United Kingdom	*Citrobacter freundii*	Blood, Urine, wound swab, sputum	(16)
	United Kingdom	*Morganella morgannii*	Blood, Urine, wound swab, sputum	(16)
	United Kingdom	*Providencia* spp.	Blood, Urine, wound swab, sputum	(16)
	United Kingdom	*Klebsiella pneumoniae*	Wound	(107)
	United Kingdom	*Escherichia coli*	Wound	(107)
	United Kingdom	*Vibro cholerae*	Blood culture, wound	(107)
	Italy	*Escherichia coli*	Wound	(108)
	Italy	*Escherichia coli*	Urine	(98)
	Norway	*Klebsiella pneumoniae*	Catheter	(109)
	Norway	*Escherichia coli*	Urine, blood cultures	(109)
	Spain	*Escherichia coli*	Stool sample	(82)
	Spain	*Klebsiella pneumoniae*	Peritoneal fluid	(110)
	Canada	*Escherichia coli*	Urine	(111)
	Canada	*Escherichia coli*	Thigh tissue	(112)
	Canada	*Klebsiella pneumoniae*	Thigh tissue	(112)
	Germany	*Escherichia coli*	Tracheal secretions	(113)
	Hong Kong	*Escherichia coli*	Rectal swab/ stool sample	(114)
	Japan	*A. baumannii*	Sputum	(115)
	United States	*Escherichia coli*	Catheter Sample	(116)

	Switzerland	*Klebsiella pneumoniae*	Urine	(117)
	Kuwait	*Klebsiella pneumoniae*	Wound swab	(32)
Bangladesh	Australia	*Escherichia coli*	Urine	(75)
	Singapore	*Escherichia coli*	Blood culture	(118)
Pakistan	Belgium	*Escherichia coli*	Pus	(83)
	Denmark	*Escherichia coli*	Faecal sample	(119)
	United States	*Klebsiella pneumoniae*	Urine, sputum, nasal wash specimen	(102)
Sri Lanka	Czech Republic	*Enterobacter cloacae*	Rectal swab	(120)
Balkan states origin				
Serbia	Switzerland	*Klebsiella pneumoniae*	Urine	(117)
	Netherlands	*Klebsiella pneumoniae*	Throat, rectal and urine	(121)
	France	*Pseudomonas aeruginosa*	Urine, rectal swabs	(122)
Montenegro	Belgium	*Enterobacter cloacae*	Pus	(83)
	Belgium	*Morganella morgannii*	Wound swab	(83)
Bosnia-Herzegovina	Croatia	*Klebsiella pneumoniae*	Blood culture	(123)
Middle East and North Africa origin				
Iraq	France	*Klebsiella pneumoniae*	Rectal swabs	(90)
	Turkey	*Klebsiella pneumoniae*	Blood culture	(124)
	Lebanon	*Klebsiella pneumoniae*	Blood culture, Urine	(91)
Egypt	France	*Klebsiella pneumoniae*	Stool sample	(88)
	Czech Republic	*Acinetobacter baumannii*	Oral cavity swab	(89)
Morocco	Norway	*Klebsiella pneumoniae*	Rectal screening	(85)
Algeria	Belgium	*Acinetobacter baumannii*	Rectal swab	(87)
South East Asia origin				
Thailand	Norway	*Escherichia coli*	Urine	(85)
Indonesia	Singapore	*Klebsiella pneumoniae*	Stool sample	(96)
Vietnam	Singapore	*Klebsiella pneumoniae*	Urine	(125)

ST: Sequence type, Nosocomial cases without travel history have been reported from India (126-128), Bangladesh (129), Morocco (86), Oman (92), Kuwait (32), France (97), Italy (98), United Kingdom (128), China (81, 130), South Korea (95), Guatemala (100), Canada (112) and Colombia (79).

Table 2. NDM-1 acquired through personal travel involving colonization and long-term carriage

Country Acquired	Introduced Country	Species	Sample	Reference
India	France	*Escherichia coli*	Rectal swabs	(131)
	France	*Escherichia coli*	Fecal sample	(88)
	Italy	*Escherichia coli*	Rectal swab	(108)
	Japan	*Escherichia coli*	Blood culture	(132)
	Australia	*Klebsiella pneumoniae*	Urine	(133)
Pakistan	Switzerland	*Proteus mirabilis*	Rectal	(117)
Serbia (Balkan states)	Switzerland	*Escherichia coli*	Rectal	(117)
Montenegro (Balkan states)	Belgium	*Klebsiella pneumoniae*	Sputum	(83)
Montenegro (Balkan states)	Belgium	*Escherichia coli*	Fecal swab	(83)
Romania	Norway	*Klebsiella pneumoniae*	Rectal screening	(85)
Libya	Denmark	*Acinetobacter baumannii*	Nostrils, tonsils or perineum	(134)
Ireland	India	*Klebsiella pneumoniae*	Urine	(135)

Table 3. Community acquired NDM-1 and NDM producing environmental isolates

Country	Species	Sample	Reference
Community acquired			
France	*Klebsiella pneumoniae*	Urine	(33)
	Klebsiella pneumoniae	Urine	(136)
India	*Acinetobacter baumannii*	Donor corneal rim	(137)
Russia	*Klebsiella pneumoniae*	Urine	(99)
Afghanistan	*Providencia stuartii*	Blood	(94)
Environmental samples			
India	*Escherichia coli*	Waste seepage	(78)
	Pseudomonas putida	Waste seepage	(78)
	Pseudomonas pseudoalcaligenes	Waste seepage	(78)
	Pseudomonas oryzihabitans	Waste seepage	(78)
	Pseudomonas aeruginosa	Tap water	(78)
	Vibrio cholerae	Waste seepage	(78)
	Aeromonas caviae	Waste seepage	(78)
	Stenotrophomonas maltophilia	Waste seepage	(78)
	Klebsiella pneumoniae	Waste seepage	(78)
	Achromobacter spp.	Tap water	(78)
	Achromobacter spp.	Tap water	(78)
	Kingella dentrificans	Tap water	(78)
	Shigella boydii	Waste seepage	(78)
	Suttonella indologenes	Waste seepage	(78)
	Citrobacter freundii	Waste seepage	(78)
China	*Acinetobacter johnsonii*	Hospital sewage	(138)
	Acinetobacter lwoffii	Chicken	(103)
Vietnam	*Klebsiella pneumoniae*	Seepage water	(139)

Following nosocomial acquisition in the incidence of NDM cases is community acquisition during personal travel. We define this as international travel for purposes other than seeking medical care. Reports of this route have usually involved colonization of the gut by NDM producing organisms usually identified by rectal screening, the most common being *E. coli*, a natural component of the flora within the gut. However *K. pneumoniae*, *Acinetobacter baumannii* and *Proteus mirabilis* have also been detected in this way.

These three routes of NDM acquisition clearly have some overlap, and exceptions to how these three major routes occur. For example, community acquisition may have occurred prior to presentation to a health care facility leading to the international transfer. Furthermore there are reported cases in which travel history is not stated in the published report (Table 4). Nevertheless, it is clear that both nosocomial and community acquisition of NDM producers can occur. Individuals who travel can then transfer NDM producers to new geographic locations. The origin and sources of NDM producers in the environment needs greater investigation.

Table 4. NDM cases with no travel history and unknown travel history

Country	Species	Sample	Reference
No travel history (possible nosocomial or community acquisition)			
India	*Klebsiella pneumoniae*	Endotracheal aspirate, blood culture	(140)
	Klebsiella pneumoniae	Sputum, tracheal aspirate (2), blood culture	(127)
	Klebsiella pneumoniae	Unknown	(105)
	Klebsiella pneumoniae	Urine	(141)
	Klebsiella pneumoniae	Umbilical cord tip, sputum, pus, urine, blood, umbilical cord, endotracheal tube	(142)
	Klebsiella pneumoniae	Skin, blood, respiratory tract	(143)
	Klebsiella pneumoniae	Intra-abdominal infection	(144)
	Klebsiella pneumoniae	Blood, urine	(16)
	Escherichia coli	Abscess (pus), tissue, blood culture	(127)
	Escherichia coli	Intra-abdominal infection	(144)
	Escherichia coli	Skin, blood	(143)
	Escherichia coli	Unknown	(145)
	Escherichia coli	Urine, catheter, pus, blood, endotracheal tube	(142)
	Escherichia coli	Blood, urine	(16)
	Enterobacter cloacae	Blood culture, Tracheal aspirate	(127)
	Enterobacter cloacae	Intra-abdominal infection	(144)
	Enterobacter cloacae	Blood	(143)
	Enterobacter cloacae	Blood, urine	(16)
	Citrobacter freundii	Wound/drainage	(127)
	Citrobacter freundii	Urine, tissue, pus, catheter tip, endotracheal tube	(142)
	Citrobacter freundii	Blood, urine	(16)
	Providencia rettgeri	Intra-abdominal infection	(144)
	Providencia rettgeri	Blood, urine	(16)
	Morganella morganii	Intra-abdominal infection	(144)
	Acinetobacter spp.	Pus, CSF, sputum, fluid	(146)
	Proteus spp.	Blood, urine	(16)
	Klebsiella oxytoca	Blood, urine	(16)
	Providencia stuartii	Unknown	(147)
	Stenotrophomonas maltophilia	Umbilicus	(126)
	Pseudomonas spp.	Pus, CSF, Sputum, Fluid	(146)

Pakistan	*Acinetobacter baumannii*	Stool samples	(148)
	Enterobacter cloacae	Stool samples	(148)
	Klebsiella pneumoniae	Stool samples	(148)
	Citrobacter freundii	Stool samples	(148)
	Citrobacter braakii	Stool samples	(148)
	Escherichia coli	Stool samples	(148)
	Providencia rettgeri	Stool samples	(148)
	Aeromonas caviae	Stool samples	(148)
Bangladesh	*Klebsiella pneumoniae*	Urine, endotrachael tube, wound swab, urine	(129)
	Klebsiella pneumoniae	Urine, Tracheal aspirate	(149)
	Escherichia coli	Tracheal aspirate	(129)
	Citrobacter freundii	Urine	(129)
	Providencia rettgeri	Urine	(129)
China	*Escherichia coli*	Stool sample	(150)
	E. aerogenes	Stool sample	(150)
	Klebsiella pneumoniae	Stool sample	(150)
	Acinetobacter junii	Blood culture	(151)
	Acinetobacter lwoffii	Urine	(73)
Serbia (Balkan states)	*Pseudomonas aeruginosa*	Urine, wound	(152)
	Klebsiella pneumoniae	Urine	(153)
Oman	*Klebsiella pneumoniae*	Perineal swab, cannula site	(92)
	Klebsiella pneumoniae	Urine	(106)
Thailand	*Escherichia coli*	Urine	(154)
	Klebsiella pneumoniae	Urine	(154)
	Citrobacter freundii	Urine	(154)
Algeria	*Acinetobacter baumannii*	Blood cathered, rectal swabs	(155)
Brazil	*Providencia rettgeri*	Soft tissue of the toe	(156)
Cameroon	*Escherichia coli*	Urine	(157)
Japan	*Klebsiella pneumoniae*	Urine	(158)
Kenya	*Klebsiella pneumoniae*	Urine, uretheral pus	(80)
Lebanon	*Klebsiella pneumoniae*	Unknown	(93)
Mauritius	*Klebsiella pneumoniae*	Urine	(159)
Singapore	*Klebsiella pneumoniae*	Urine	(125)
South Africa	*Klebsiella pneumoniae*	Urine	(160)
USA	*Klebsiella pneumoniae*	Urine	(161)

Bacterial Hosts

A variety of bacterial hosts are able to harbor *bla*$_{NDM.}$ The most predominant are those in the *Enterobacteriaceae* family. However, non-fermentative Gram negative bacteria such as *Acinetobacter* spp. are also potentially important and may even be the originators of *bla*$_{NDM.}$ Reports of *bla*$_{NDM}$ in Gram positive organisms are highly likely to be spurious.

Klebsiella pneumoniae

Klebsiella pneumoniae was the first bacterial host to be isolated with *bla*$_{NDM}$ and continues to be the most predominant pathogen producing NDM (Table 5). Multilocus sequence typing (MLST) has been the molecular epidemiologic tool most frequently used to evaluate the global epidemiology of NDM producers. Various *K. pneumoniae* sequence types (STs) have been reported to harbor *bla*$_{NDM}$. The predominant ST types are 14 (2, 105, 106, 117, 127, 144), 147 (90, 92, 117, 144), 11 (85, 92, 101, 127, 144), 340 (95, 99, 106, 144, 162), 15 (33, 83, 86, 92, 121), 1043 (79), and 231 (144, 159, 162), with a small number of cases presenting with ST types 17 (100, 127), 37 (162), 38 (124, 127, 162), 42 (158, 162), 101 (92, 144), 149 (162), and 625 (162). In addition to these frequently reported ST types, there are also novel ST types including 1 (136), 16 (112), 20 (144), 25 (123), 29 (127), 43 (162), 273 (162), 283 (139), 307 (162), 372 (92), 391 (144), 405 (85), 483 (150), 525 (85), 571 (144), 572 (144), 610 (162), 623 (162), 624 (162), and 924 (112). The diversity of *K. pneumoniae* STs described highlights its ability to spread via various clones. Globally, ST 14, 15 and 147 are the most predominant amongst *K. pneumoniae*. Interestingly, ST258 has been the *K. pneumoniae* most frequently associated with spread of *bla*$_{KPC}$, which is the most common carbapenemase found in North America. ST258 has not been found to harbor *bla*$_{NDM}$ but single locus variants (ST11, 340 and 572) have been found to harbor this enzyme.

The ability to be acquired and spread amongst a large range of ST types, not only novel single ST types, but those capable of high clonal spread begins to indicate in detail how *bla*$_{NDM}$ was able to spread at a rapid rate. However this ST diversity of *bla*$_{NDM}$ acquisition is not restricted in *K. pnuemoniae* but continues to be mirrored within another *Enterobacteriaceae* species, *E. coli*.

Table 5. Reported cases of NDM producing *Klebsiella pneumoniae*

ST type	Country Acquired	Introduced Country	Sample	Reference
14 (n=20)	India	Sweden	Urine	(2)
	India	N/A	Endotracheal tube	(127)
	India	None	Unknown	(105)
	India	Oman	Urinary catheter	(106)
	India	N/A	Intra-abdominal infection	(144)
	India	Unknown	Urine, lower respiratory tract samples	(162)
	United Kingdom	Unknown	Urine, lower respiratory tract samples	(162)
	Sweden	Unknown	Urine, lower respiratory tract samples	(162)
147 (n=18)	India	N/A	Intra-abdominal infection	(144)
	India	Switzerland	Urine	(117)
	India	Australia	Urine	(15)
	Oman	None	Wound, rectal swab, urine, supra-pubic catheter	(92)
	Iraq	France	Rectal swabs	(90)
	United Kingdom	Unknown	Urine, lower respiratory tract samples	(162)
11 (n=12)	India	Norway	Catheter	(109)
	India	N/A	Intra-abdominal infection	(144)
	India	N/A	Sputum, tracheal aspirate	(127)
	India	New Zealand	Rectal swab	(101)
	None	Oman	Urine, Blood, Perineal swab	(92)
	India	Unknown	Urine, lower respiratory tract samples	(162)
	United Kingdom	Unknown	Urine, lower respiratory tract samples	(162)
	Sweden	Unknown	Urine, lower respiratory tract samples	(162)
	India	Spain	Peritoneal fluid	(110)
340 (n=9)	Russia	No travel	Urine	(99)
	South Korea	N/A	Stool, Urine	(95)
	India	N/A	Intra-abdominal infection	(144)
	Oman	Unknown	Urine	(106)
	Sweden	Unknown	Urine, lower respiratory tract samples	(162)
15 (n=8)	Morocco	None	Urine, Blood culture, Pancreatic abscess	(86)
	Serbia (Balkan States)	Netherlands	Throat, rectal, urine	(121)
	India	Oman	Intra-adominal sputum	(92)

	France	None	Urine	(33)
	Montenegro (Balkan states)	Belgium	Sputum	(83)
1043 (n=6)	Colombia	None	Neonatal	(79)
231 (n=5)	India	N/A	Intra-abdominal infection	(144)
	Mauritius	Unknown	Urine	(159)
	United Kingdom	Unknown	Urine, lower respiratory tract samples	(162)
17 (n=3)	Guatemala	Unknown	Catheter, Tracheal secretions	(100)
	India	N/A	Blood culture	(127)
38 (n=3)	India	N/A	Tracheal aspirate	(127)
	Iraq	Turkey	Blood culture	(124)
	India	Unknown	Urine, lower respiratory tract samples	(162)
149 (n=3)	India	Unknown	Urine, lower respiratory tract samples	(162)
625 (n=3)	India	Unknown	Urine, lower respiratory tract samples	(162)
37 (n=2)	India	Unknown	Urine, lower respiratory tract samples	(162)
	United Kingdom	Unknown	Urine, lower respiratory tract samples	(162)
42 (n=2)	India	Japan	Urine	(158)
	United Kingdom	Unknown	Urine, lower respiratory tract samples	(162)
101 (n=2)	Oman	Unknown	Perineal swab, cannula site	(92)
	India	N/A	Intra-abdominal infection	(144)
340 (n=2)	India	Unknown	Urine, lower respiratory tract samples	(162)
	Sweden	Unknown	Urine, lower respiratory tract samples	(162)
1 (n=1)	Europe	None	Urine	(136)
16 (n=1)	India	Canada	Rectal swab	(112)
20 (n=1)	India	N/A	Intra-abdominal infection	(144)
25 (n=1)	Bosnia-Herzegovina (Balkan states)	Croatia (Balkan states)	Blood culture	(123)
29 (n=1)	India	N/A	Blood culture	(127)
43 (n=1)	United Kingdom	Unknown	Urine, lower respiratory tract samples	(162)
273 (n=1)	United Kingdom	Unknown	Urine, lower respiratory tract samples	(162)
283 (n=1)	Vietnam	N/A	Seepage water	(139)
307 (n=1)	India	Unknown	Urine, lower respiratory tract samples	(162)
372 (n=1)	India	Oman	Wound	(92)
391 (n=1)	India	N/A	Intra-abdominal infection	(144)
405 (n=1)	Romania (Balkan States)	Norway	Rectal screening	(85)
483 (n=1)	China	None	Stool sample	(150)

525 (n=1)	Romania	Norway	Rectal screening	(85)
571 (n=1)	India	N/A	Intra-abdominal infection	(144)
572 (n=1)	India	N/A	Intra-abdominal infection	(144)
610 (n=1)	Sweden	Unknown	Urine, lower respiratory tract samples	(162)
623 (n=1)	India	Unknown	Urine, lower respiratory tract samples	(162)
624 (n=1)	India	Unknown	Urine, lower respiratory tract samples	(162)
972 (n=1)	India	Canada	Thigh tissue	(112)

ST: Sequence type, N/A: Not applicable. In the following reference, sequence type of NDM producers was not recorded (16, 32, 78, 80, 88, 91, 93, 96, 98, 102, 104, 107, 125, 126, 129, 135, 140-143, 148, 149, 153, 154, 160, 161).

Escherichia coli

Escherichia coli is another predominant carrier of *bla*$_{NDM}$ and is commonly observed during rectal screening as it is naturally found as part of gut flora. It is also the most common cause of urinary tract infection (UTI). As is the case with *K. pneumoniae*, a broad range of *E. coli* STs may be NDM producers but a group of predominant clonal strains also exists (Table 6). The predominant *E. coli* ST types producing NDM include STs 101 (75, 83, 101, 113, 119, 127, 143), 405 (85, 157, 163), 88 (144), 410 (109, 112, 117, 163), 648 (105, 163), 156 (82, 163), 744 (150) and the already predominant strain responsible for the spread of *bla*$_{CTX-M-15}$, ST131 (116, 131). Single ST type reports include ST2 (144), 10 (105), 38 (132), 44 (144), 88 (144), 156 (82), 167 (143), 361 (101), 471 (144), 501 (144), 648 (105), 744 (150), 782 (83), 2488 (101) and 2527 (92).

It can be seen that *bla*$_{NDM}$ is found in a variety of *E. coli* STs, including those that are known for clonal pandemic spread such as *E. coli* ST131 (164). This particular ST has been found worldwide and is of major clinical importance since it confers multidrug resistance as well as possessing multiple virulence factors. The diversity of *bla*$_{NDM}$ amongst STs also demonstrates that *bla*$_{NDM}$ is not restricted to a single ST type. Furthermore, to add more diversity, *bla*$_{NDM}$ can also be found in various Gram-negatives including other *Enterobacteriaceae* species and Acinetobacter species.

Other *Enterobacteriaceae* species

After *Klebsiella* and *Escherichia*, the most common genera to be reported harboring *bla*$_{NDM}$ from the *Enterobacteriaceae* (Table 7) are *Citrobacter* and *Enterobacter*. *Citrobacter freundii* and *Enterobacter cloacae* are the most common species, however there have been single reports of *Citrobacter braakii* (148) and *Enterobacter aerogenes*. Other less predominant *Enterobacteriaceae* that have been reported include *Klebsiella oxytoca* (16,

165), *Providencia rettgeri* (129, 144, 153), *Providencia stuartii* (94, 105), *Proteus mirabilis* (103, 117), *Morganella morganii* (83, 144), *Salmonella enterica* (104) and *Shigella boydii* (78).

Table 6. Reports of NDM producing *Escherichia coli*

ST type	Country Acquired	Introduced Country	Sample	Reference
101 (n=16)	India	N/A	Abscess (pus), tissue	(127)
	India	New Zealand	Rectal swab	(101)
	India	None	Skin, blood	(143)
	India	Germany	Tracheal secretions	(113)
	India	Canada	Urine	(111)
	Pakistan	Denmark	Faecal sample	(119)
	Pakistan	Belgium	Pus	(83)
	Pakistan	ND	Unknown	(163)
	Bangladesh	Australia	Urine	(75)
	Unknown	United Kingdom	Blood, Urine, Feces	(163)
405 (n=11)	India	Italy	Fectal swab, wound	(108)
	Unknown	United Kingdom	Blood, Urine, Feces	(163)
	Cameroon	France	Rectal swab	(157)
88 (n=8)	India	N/A	Intra-abdominal infection	(144)
410 (n=4)	Serbia (Balkan states)	Switzerland	Rectal	(117)
	India	Norway	Urine, blood cultures	(109)
	India	Canada	Thigh tissue	(112)
	Unknown	United Kingdom	Blood, Urine, Feces	(163)
648 (n=4)	India	France	Unknown	(105)
	Pakistan	ND	Unknown (2)	(163)
	Unknown	United Kingdom	Blood, Urine, Feces	(163)
131(n=2)	India	United States	Catheter sample	(116)
	India	France	Rectal swabs	(131)
156 (n=2)	India	Spain	Stool sample	(82)
	Unknown	United Kingdom	Blood, Urine, Feces	(163)
744 (n=2)	China	N/A	Stool sample	(150)
2 (n=1)	India	N/A	Intra-abdominal infection	(144)
10 (n=1)	India	France	Unknown	(105)
38 (n=1)	India	Japan	Blood culture	(132)
44 (n=1)	India	N/A	Intra-abdominal infection	(144)
90 (n=1)	India	ND	Unknown	(163)
167 (n=1)	India	ND	Skin	(143)
361 (n=1)	India	New Zealand	Rectal swab	(101)

471 (n=1)	India	N/A	Intra-abdominal infection	(144)
501 (n=1)	India	N/A	Intra-abdominal infection	(144)
782 (n=1)	Montenegro (Balkan states)	Belgium	Fecal swab	(83)
2488 (n=1)	India	New Zealand	Urine	(101)
2527 (n=1)	Oman	None	Perineal swab	(92)

ST: Sequence type, ND: Not Defined, N/A: Not applicable. In the following references, sequence type of NDM producing was not recorded (16, 78, 88, 97, 98, 107, 114, 118, 126, 127, 129, 142, 145, 148, 154)

Table 7. Reports of other New Delhi metallo-β-lactamase-producing *Enterobacteriaceae* species

Species	Country Acquired	Introduced Country	Sample	Reference
Enterobacter species				
E. cloacae	India	N/A	Intra-abdominal infection	(144)
	India	N/A	Blood culture, tracheal aspirate	(127)
	India	ND	Blood, urine	(16)
	Pakistan	ND	Stool samples	(148)
	India	None	Blood	(143)
	Montenegro (Balkan states)	Belgium	Pus	(83)
	Sri Lanka	Czech Republic	Rectal swab	(120)
E. aerogenes	China	None	Stool sample	(150)
Enterobacter spp.	UK	India	Blood, urine	(16)
Citrobacter species				
C. freundii	India	France	Catheter	(84)
	India	N/A	Wound/drainage	(127)
	India	UK	Wound	(107)
	India	N/A	Waste seepage	(78)
	India	None	Urine, tissue, pus, catheter tip, endotracheal tube	(142)
	India	ND	Blood, urine	(16)
	India	France	Urine	(84)
	UK	India	Blood, urine	(16)
	Thailand	ND	Urine	(154)
	Bangladesh	None	Urine	(129)
	Pakistan	ND	Stool samples	(148)
C. braakii	Pakistan	ND	Stool samples	(148)
Providencia species				
P. rettgeri	Pakistan	ND	Stool samples	(148)
	Bangladesh	ND	Urine	(129)
	N/A	India	Intra-abdominal infection	(144)
	Unknown	Brazil	Soft tissue of the toe	(156)
P. stuartii	Afghanistan	N/A	Blood	(94)

	India	None	Unknown	(105)
Providencia spp.	UK	India	Blood, urine	(16)
Other *Enterobacteriaceae* species				
Klebsiella oxytoca	India	ND	Blood, Urine	(16)
Morganella morganii	Montenegro (Balkan states)	Belgium	Wound swab	(83)
	N/A	India	Intra-abdominal infection	(144)
	UK	India	Blood, Urine, wound swab, sputum	(16)
Proteus mirabilis	India	New Zealand	Rectal swab	(101)
	Pakistan	Switzerland	Rectal	(117)
Proteus spp.	India	Unknown	Blood, urine	(16)
Salmonella enterica	India	France	Urine	(104)
Shigella boydii	N/A	India	Waste seepage	(78)

ST: Sequence type, ND: Not Defined, N/A: Not applicable

NDM producing Non-*Enterobacteriaceae*

Acinetobacter species

The most common genus reported carrying *bla*$_{NDM}$ outside of the *Enterobacteriaceae* family is *Acinetobacter* (Table 8). *Acinetobacter baumannii* is the most predominant bacterial species to harbor *bla*$_{NDM}$, alongside *K. pneumoniae* and *E. coli*. *A. baumannii* has also been theorized to be the organism involved in the genesis of the *bla*$_{NDM}$ gene (70, 166), which will be discussed in the following genetic sections. The STs that have been identified for *A. baumannii* thus far are ST1 (89), ST92 (87) and ST222 (115), however many remain undefined by MLST. Other species of *Acinetobacter* which may produce NDM include *A. johnsonii* (138), *A. junii* (151), *A. lwoffii* (73), and *A. pittii* (130) which have predominantly been reported from China within the clinical setting as well as the environment i.e. hospital sewage or meat producing animals (103, 138).

Pseudomonas species and other unique bacterial hosts

Pseudomonas species follows the *Enterobaceriaceae* family and the *Acinetobacter* genus in predominant reports of species producing NDM, being first reported in 2011 (Table 9). *Pseudomonas aeruginosa* is the most commonly reported *Pseudomonas* spp. to produce NDM and in most cases are commonly linked to Serbia within the Balkan states with one case reporting a NDM producing *P. aeruginosa* ST235 (147), the primary founder of the epidemic clonal complex 235 (167). There are other species of this genus known to produce NDM including *P. putida*, *P. pseudoalcaligenes* and *P. ozyzihabitans*, most of which were found in the environment (waste seepage and tap water) within India by Walsh and colleagues (78). It was also noted that the NDM gene was not stable within these hosts and was lost within 48 hours. Other bacterial hosts harboring *bla*$_{NDM}$ were usually found within

the environment (78). These isolates include *Vibrio cholerae*, *Aeromonas caviae*, *Stenotrophomonas maltophilia*, *Achromobacter* spp., *Kingella denitrificans* and *Suttonella indologenes*.

Table 8. Reports of NDM producing Acinetobacter species

Species	ST type	Country Acquired	Introduced Country	Sample	Reference
A. baumannii	222	India	Japan	Sputum	(115)
	92	Algeria	Belgium	Rectal swab	(87)
	1	Egypt	Czech Republic	Oral cavity swab	(89)
	ND	China	None	Sputum (2), Blood, Secretions	(81)
	ND	Bangladesh	None	Tracheal aspirate (2), Sputum	(129)
	ND	India	None	Donor corneal rim	(137)
	ND	India	None	Mouth	(126)
	ND	Algeria	Unknown	Blood cathered, rectal swabs	(155)
	ND	Libya	Denmark	Nostrils, tonsils or perineum	(134)
	ND	Pakistan	Unknown	Stool samples	(148)
A. johnsonii	-	China	N/A	Hospital sewage	(138)
A. junii	-	China	Unknown	Blood culture	(151)
A. pittii	63	China	N/A	ICU draw handle, groin	(130)
A. lwoffii	ND	China	Unknown	Urine	(73)
Acinetobacter spp.	ND	India	Unknown	Pus, CSF, sputum, fluid	(146)

ST: Sequence type, ND: Not Defined, - : No current MLST scheme

Table 9. Reports of other NDM producing gram negative bacteria

Species	Country Acquired	Introduced Country	Sample	Reference
Pseudomonas species				
P. aeruginosa	Serbia (Balkan states)	France	Urine, rectal swabs	(122, 147)
	Serbia (Balkan states)	None	Urine	(152)
	Serbia (Balkan states)	None	Wound	(152)
	India	N/A	Tap water	(78)
P. putida	India	N/A	Waste seepage	(78)
P. pseudoalcaligenes	India	N/A	Waste seepage	(78)
P. ozyzihabitans	India	N/A	Waste seepage	(78)
Pseudomonas spp.	India	Unknown	Pus, CSF, sputum, fluid	(146)
Other gram negative species				
Vibrio chloerae	India	UK	Blood culture, wound	(107)
	India	N/A	Waste seepage	(78)
Aeromonas caviae	India	N/A	Waste seepage	(78)
	Pakistan	Unknown	Stool samples	(148)
Stenotrophomonas maltophilia	India	N/A	Waste seepage	(78)
	India	None	Umbilicus	(126)
Achromobacter spp.	India	N/A	Tap water	(78)
Kingella dentrificans	India	N/A	Tap water	(78)
Sutonella indologenes	India	N/A	Waste seepage	(78)

N/A: Not applicable

The role of bacterial hosts in terms of spread

The four most predominant species within the *Enterobacteriaceae* family harboring bla_{NDM}, *K. pneumoniae*, *E. coli*, *C. freundii* and *E. cloacae*, are faecal coliforms able to colonize the gut. This has allowed the gut to act as a reservoir for bla_{NDM} which has facilitated colonization and asymptomatic long-term carriage leading to the spread of bla_{NDM}. These hosts are also pathogens which are able to produce clinical infections such as bloodstream infections (BSI) and urinary tract infections (UTI). Water-borne transmission of these organisms has allowed for environment reservoirs to be observed in not only waste seepage and tap water (generally documented within the Indian sub-continent), but also hospital

sewage samples (168). There are also reports of NDM producers on surfaces within the hospital environment (130) - mostly commonly this has been linked with the *Acinetobacter* species.

Due to the nature of *Acinetobacter* species being a difficult bacterium to remove in the clinical setting, they are a perfect reservoir host from which other species can acquire bla_{NDM}. Furthermore, as with the *Enterobacteriaceae* hosts, *Acinetobacter* species (in particular *A. baumannii*) are also opportunistic pathogens. This may raise the point that a restricting characteristic of bla_{NDM} in terms of pathogenicity, is that the most common bacterial hosts carrying bla_{NDM} are opportunistic and are often only clinically significant when a patient has presented with an altered health condition e.g. immunocompromised or prolonged hospitalization. However it is only a matter of time before bla_{NDM} is combined with a highly virulent pathogen or gene, as is the case with documented virulent strains of *E. coli* harboring bla_{NDM} i.e. ST131.

The number of STs in which NDM has been identified in *K. pneumoniae*, *E. coli* and *A. baumannii* highlights the ability of this gene to be acquired by different bacterial strains which are all capable of clonal spread and causing outbreaks. The diversity of NDM producing *K. pneumoniae*, *E. coli* and *A. baumannii* ST types may be underestimated because many have not been defined by MLST. Further to clonal diversity of NDM, is the ability to be acquired by a vast number of Gram negative bacterial hosts. This is because the NDM gene is commonly encoded genetically on circular mobile elements known as plasmids, capable of transmitting antibiotic resistance between bacterial hosts. Interestingly, as mentioned previously, *V. cholerae* has been reported to harbor bla_{NDM}. *V. cholerae* is not usually treated with carbapenems and thus it may seem strange for this pathogen to harbor this gene. However, the genetic vehicle on which bla_{NDM} resides upon is compatible with *V. cholerae* as a recipient and may also carry other genes providing resistance to tetracycline

and ciprofloxacin (commonly used to treat *V. cholerae). V. cholerae* is also able to reside in the gut and in this case would be able to transfer bla_{NDM} to other available gut flora, providing carbapenem resistance. This extra evasive factor of the bacterial host acting as a bacterial host reservoir makes carbapenem resistant pathogens difficult to control. Furthermore, this demonstrates that the case of bla_{NDM} is unlike the well known paradigms of resistance genes being intrinsic to certain bacterial species e.g. *K. pneumoniae* (bla_{SHV-1}) *A. baumannii* (bla_{OXA-51}) (169) or commonly spread by specific clones e.g. *E. coli* ST131 ($bla_{CTX-M-15}$), *K. pneumonia*e ST258 (bla_{KPC}) and *A. baumannii* clonal complex 92 (bla_{OXA-23}) (170).

Genetics

Plasmids harboring *bla*$_{NDM-1}$

Different plasmid backbones

bla$_{NDM}$ is known to reside on mobile DNA elements known as plasmids, which are capable of transferring resistance genes from one bacterial cell to another through a mechanism known as conjugation, a type of horizontal gene transfer (HGT). Each plasmid has a backbone known as an Inc (Incompatibility)/replicon type. *bla*$_{NDM}$ has been known to be encoded on at least six different backbones with many remaining undefined by standard plasmid replicon typing (58). Plasmid backbones harboring *bla*$_{NDM}$ include common Inc types such as IncH types, IncL/M, IncF type and also the rare Inc type X3. However, the most common plasmid Inc type to harbor *bla*$_{NDM}$, is IncA/C. IncA/C plasmids are vehicles that have an extremely broad bacterial host range which include *A. baumannii*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. stuartii*, *S. enterica*, *V. cholerae* and *Yersinia pestis*. A combined range with the other plasmid backbones allows additional bacterial hosts to harbor *bla*$_{NDM-1}$, such as *Citrobacter freundii* (IncHI1) (171), *Enterobacter cloacae* (IncHI2 type) (172) as well as undefined plasmids that can spread *bla*$_{NDM}$ to *A. pittii* and *A. lwoffii*. Further to the acquisition of the carbapenemase, these plasmids harboring *bla*$_{NDM-1}$ also co-harbor other beta-lactamases but also quinolone and aminoglycoside resistance genes.

Co-harboring of other resistance genes with *bla*$_{NDM}$

The resistance genes which can be co-harbored on the same plasmid backbone encoding *bla*$_{NDM}$ range from ESBLs, AmpC, quinolone and aminoglycoside resistance genes (Table 10). Common plasmids harboring *bla*$_{NDM}$ such as IncF type, IncL/M, IncA/C, can also carry

other beta-lactamases such as *bla*$_{\text{TEM-1}}$, ESBL genes such as *bla*$_{\text{CTX-M-15,}}$ AmpC genes such as *bla*$_{\text{CMY-6}}$ and *bla*$_{\text{CMY-16}}$, Oxacillinase genes such as *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-9}}$, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{OXA-21}}$, aminoglycoside resistance genes such as *armA*, *aacA4*, *aac(6')*, *aac6-1b*, *aadA1*, *aadA2*, *aac2*, *aacC2*, *rmtA*, *rmtC* and quinolone resistance genes such as *aac(6')-lb-cr*, *qnrA*, *qnrA6*, *qnrA6*-like, *qnrB1*. Combine this plasmid co-harborization with chromosomally encoded resistance genes and treatment options become seriously limited. An *E. coli* producing NDM has been reported to have acquired tigecycline resistance within 4 months (34), however it is unknown whether the mechanism of resistance was chromosomally or plasmid encoded. There are also cases of NDM producing *K. pneumoniae* resistant to colistin and tigecycline (32, 33).

bla$_{\text{NDM}}$ as part of an accessory module

As previously mentioned plasmids are composed of a backbone (Inc) which contains several genes which can be categorized into modules or groups according to their function. These groups include plasmid replication/partitioning, stability, transfer, establishment and accessory/adaptive genes. In short, plasmid replication/partitioning is required as its name suggests for determining plasmid replication; stability is necessary to ensure plasmids are retained within its bacterial host and their subsequence daughter cells; transfer is the major feature of plasmids providing its ability to transfer to other bacterial hosts; and establishment to compliment transfer genes by ensuring the plasmid is stable within its new host and is capable of increasing the recipient host range (170, 173, 174). The accessory/ adaptive genes are highly variable and usually contribute to the variation amongst plasmid backbones. These genes include the secretion of products which enhance growth, bacteriocins, virulence and antibiotic/chemical resistance genes. The jargon term "NDM plasmid" is actually a plasmid backbone, for example IncA/C, acquiring the *bla*$_{\text{NDM-1}}$ gene as an accessory module.

Furthermore, the other co-harbored resistance genes residing on the plasmid are also genes of other accessory modules captured prior to *bla*$_{NDM}$ acquisition. The common mechanism by which accessory modules e.g. novel antibiotic resistance, are able to traverse amongst different plasmid backbones and become acquired by plasmids is via transposition.

Table 10. Reported plasmid backbones and sequences carrying *bla*$_{NDM}$

Plasmid backbone (Inc type)	Co-harboring resistance genes			Isolate species (ST type)	Country of Isolation	Reference
	β-Lactamases Genes (*bla*)	Quinolone Resistance Genes	Aminoglycoside Resistance Genes			
A/C	-	-	-	*P. stuartii*	Afghanistan	(94)
	-	-	-	*K. pneumoniae* (ST14)	Kenya	(80, 175)
	-	-	-	*E. coli*	Canada	Unpublished
	-	-	-	*E. coli* (ST38)	Japan	(176, 177)
	-	-	-	*E. coli*	Canada	Unpublished
	bla$_{CMY-16}$ *bla*$_{OXA-10}$	-	-	*E. coli* (ST10)	France	(105)
	-	-	*rmtA*	*K. pneumoniae* (ST147)	Switzerland	(117)
	bla$_{OXA-10}$ *bla*$_{CMY-16}$	*qnrA6*	-	*K. pneumoniae* (ST25)	Switzerland	(117)
	bla$_{CMY-6}$	-	*rmtC*	*K. pneumoniae* (ST14)	Kenya	(80)
	bla$_{OXA-10}$, *bla*$_{CMY-16}$	-	*armA*	*P. mirabilis*	Switzerland	(117)
	-	-	-	*E. coli*	India	(78)
	-	-	-	*V. cholerae*	India	(78)
	-	-	-	*C. freundii*	India	(78)
	-	-	-	*A. baumannii*	Switzerland	(178)
	bla$_{CTX-M-15}$ *bla*$_{SHV-12}$	*aac(6')-Ib-cr qnrS*	-	*K. pneumoniae* (ST147)	Canada	(179, 180)
N	Unknown	Unknown	Unknown	*K. pneumoniae*	UK	(107)
N2	-	-	-	*E. coli*	Australia	(75, 181)
	-	-	-	*K. pneumoniae*	Singapore	(125)
	-	-	-	*K. pneumoniae*	Singapore	(125)
HI1	-	-	-	*C. freundii*	India	(171)
	-	-	-	*E. coli* (ST156)	Spain	(82)
HI1B	*bla*$_{TEM-1}$ *bla*$_{CTX-M-15}$ *bla*$_{SHV-12}$ *bla*$_{OXA-1}$	-	*armA*	*K. pneumoniae*	Oman	(92)
	bla$_{CTX-M-15}$	-	*armA*	*K. pneumoniae*	Oman	(92)
	bla$_{SHV-12}$	-	*armA*	*K. pneumoniae*	Oman	(92)
	bla$_{CTX-M-15}$ *bla*$_{OXA-1}$	-	*armA*	*K. pneumoniae*	Oman	(92)
L/M	-	-	-	*E. coli*	Hong-Kong	(74)
	bla$_{TEM-1}$	-	-	*K. pneumoniae* (ST14)	Oman	(106)
	bla$_{TEM-1}$	-		*K. pneumoniae*	Oman	(92)

F	*bla*$_{TEM-1}$		*armA*	*E. coli*	Switzerland	(117)
	-	-	*armA*	*E. coli* (ST648)	India	(105)
	bla$_{TEM-1}$ *bla*$_{CTX-M-14}$	-	-	*E. coli*	Oman	(92)
FII	*bla*$_{OXA-1}$	-	*aacA4* *aadA2* *aac2*	*E. coli* (ST131)	India	(182)
FIIA	*bla*$_{CTX-M-15}$ *bla*$_{SHV-12}$	*aac(6')-Ib-cr*	-	*K. pneumoniae* (ST340)	Canada	(180)
FIIs	*bla*$_{TEM-1}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{OXA-9}$	-	-	*K. pneumoniae*	Oman	(92)
H	-	-	-	*K. pneumoniae* (ST15)	Morocco	(86, 183)
X3	-		-	*K. pneumoniae*	China	Unpublished
P	-	-	-	*E. cloacae*	India	(127)

ST: Sequence type, Inc type: Plasmid backbone/incompatibility type (Inc) via Carattoli replicon typing; ND: Not defined; -: Not present

Transposition

Transposition is the mobilization of a DNA sequence from one DNA site to another. This mechanism is facilitated by transposons (Tn), insertion sequence (IS) elements and the most recent, insertion sequence common region (IS*CR*). IS elements are genes encoding a transposase enzyme capable of copying the DNA sequence of the IS element into new DNA locations. Transposons on the other hand can be divided into two sub groups, composite and unit transposons. Composite transposons incorporate two IS elements flanking a sequence and the two IS element sequences work in tandem to mobilize the DNA sequence between the two flanking IS elements. Unit transposons work in a similar mechanism but utilize other enzymes on top of the transposase such as recombinases, to mobilize the sequence within the unit transposon. IS*CR* elements are transposases thought to utilize a rolling-circle mechanism to mobilize DNA. Amongst these elements IS*CR1* are only associated with class 1 integron capture systems (64).

Tn*125* carrying *bla*$_{\text{NDM-1}}$

Through sequence analysis of the genetic surroundings of *bla*$_{\text{NDM}}$, it has been theorized that NDM-1 was originally mobilized in transposon 125 (Tn*125*). Originally found in *A. baumannii*, Tn*125* is flanked by two IS elements known as IS*Aba125*. Within Tn*125*, there are several genes apart from *bla*$_{\text{NDM}}$ which are found downstream. Downstream from the *bla*$_{\text{NDM-1}}$ are the following genes, *ble*$_{\text{MBL}}$ – conferring bleomycin resistance, *trpF* - phosphoribosylanthranilate isomerize, *tat* - twin-arginine translocation pathway signal sequence protein, *dct* - divalent cation tolerance protein, *groES* – cofactor of groEL, *groEL* – heat resistant chaperonin, IS*CR27* – transposase, Δ*pac* – truncated phospholipid acetyltransferase (Δ i.e. partial sequence) before the other flanking IS*Aba125*. Tn*125*, 5'-IS*Aba125*|*bla*$_{\text{NDM-1}}$|*ble*$_{\text{MBL}}$|*trpF*|*tat*|*dct*|*groES*|*groEL*|IS*CR27*|Δ*pac*|IS*Aba125*-3' (Figure 2), is

the platform on which *bla*$_{NDM}$ has been theorized to be originally mobilized from *Acinetobacter* species into plasmids and then facilitating mobilization into other species such as those found in the *Enterobacteriaceae* family (113).

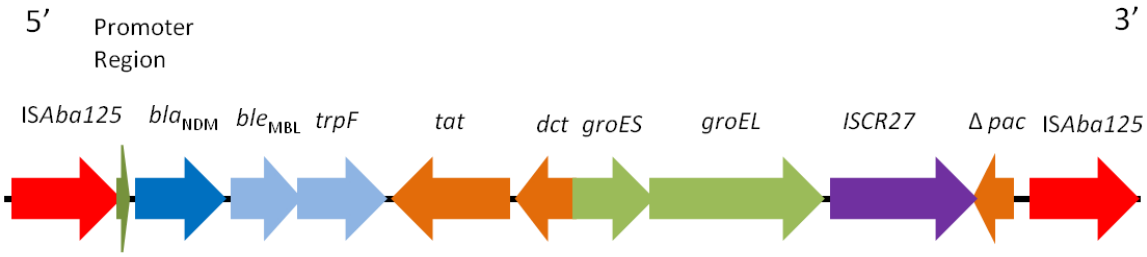

Figure 2. Schematic representation of Tn*125* carrying *bla*$_{NDM}$ from *Acinetobacter baumannii* (Accession no. HQ857107)

IS*Aba125*: IS element native in *A. baumannii*; Promoter Region: promoter sequences driving expression; *ble*$_{MBL}$: bleomycin resistance; *trpF*: phosphoribosylanthranilate isomerise; *tat*: twin-arginine translocation pathway signal sequence protein; *dct*: divalent cation tolerance protein; *groES*: cofactor of *groEL*; *groEL* : heat resistant chaperonin; IS*CR27*: transposase; Δ*pac*: truncated phospholipid acetyletransferase; Red arrows: IS elements; Green (Dark) arrow : Promoter; Blue arrow : β-lactamase; Blue (light) arrows: highly conserved gene with *bla*$_{NDM}$; Orange arrows: Environmental genes; Green arrows : Heat:resistant chaperonin proteins; Purple arrow: IS Common Region (IS*CR*) element; *Δ*: partial sequence.

Analyzing however, the NDM sequences outside of *Acinetobacter* species and within *Enterobacteriaceae* species, reveals that Tn*125* becomes truncated both at the beginning, truncating the 5' end IS*Aba125* (184), and at the end (3' end) losing certain genes (Δ*pac*, IS*CR27*, *groEL* etc.), leaving a range of truncated Tn*125* structures of different lengths. However, even with the different lengths of Tn*125* carrying *bla*$_{NDM}$, certain Tn*125* structures are still repeatedly observed in different plasmid backbones. Interestingly, various IS elements have been identified directly upstream of *bla*$_{NDM-1}$ when the 5'end IS*Aba125* is truncated. These IS elements, may have been inserted close to *bla*$_{NDM}$ to provide a one-ended transposition mechanism to mobilize *bla*$_{NDM}$ into new plasmid backbones, however this remains as speculation. *bla*$_{NDM}$ residing within close proximity to a transposition mechanism may explain why *bla*$_{NDM}$ has been reported on various plasmid backbones therefore allowing

bla$_{NDM}$ to spread to various bacterial hosts. Essentially, transposition allows an antibiotic resistance gene an additional mechanism of acquisition and spread below the horizontal gene transfer level, a gene mobilization level. Gene mobilization allows a gene to spread between different plasmid backbones, which in turn increases the bacterial host range in which that gene can be acquired.

Class one integrons

Class one integrons are able to capture gene cassettes (a section of DNA containing multiple genes) and insert them into a DNA site on which the integron sequence resides. This mechanism is commonly associated with antibiotic resistance - however in terms of spread it is strictly unable to provide a mechanism to mobilize the resistance further to other sites (chromosome or plasmid). However mobilization of the gene cassettes to other locations in the chromosome and/or plasmids is still possible. If there are two integrons present within the cell, exchange of gene cassettes can occur between the two integrons via homologous recombination. Additionally, if the resistance gene or the integron itself is incorporated within a transposon or is in close proximity to an IS element or IS*CR* e.g. IS*CR1*, the gene or gene cassettes will also be able to mobilize into various plasmid backbones or the chromosome. Incorporating such mobilization elements increases the potential to spread to various species via the different host ranges of each plasmid.

bla*$_{NDM-1}$ in *Pseudomonas aeruginosa

Most of the *bla*$_{NDM}$ and Tn*125* genetic investigations reported are either from the *Acinetobacter* spp. or from the *Enterobacteriaceae* family, and are likely mobilized by transposition and plasmid mobilization i.e. conjugation. Recently, a unique genetic sequence (Accession no. HF546976) has been published involving the integration of two copies of

bla$_{NDM-1}$ into the chromosome of *P. aeruginosa* (152) via a Class 1 integron mechanism. The two copies of *bla*$_{NDM-1}$ are found in two short truncated Tn*125* sequence structures 5'-IS*Aba125*|*bla*$_{NDM}$|Δ*ble*$_{MBL}$-3' with *aphA6* upstream. Integrated after the first two cassette arrays, were two aminoglycoside resistance genes *aacA7* and *aadA6.* This as well as the chromosomally encoded *bla*$_{NDM}$ within *P. aeruginosa* ST235 (147) demonstrates the ability for *bla*$_{NDM}$ to be also acquired onto the chromosome and complexity of where *bla*$_{NDM}$ is able to reside and mobilize from. Interestingly, *aphA6* is found upstream of both copies of ΔTn*125*. Thus the source of the Tn*125* and therefore *bla*$_{NDM}$ may have been from a conjugation event from *A. baumannii* to *P. aeruginosa,* as *aphA6* has been theorized to be involved in the genesis of *bla*$_{NDM-1}$ in *A.baumannii* (70).

Characteristics of the NDM gene

Genesis of *bla*$_{NDM-1}$

In 2012, Toleman and colleagues, by alignment of several genetic sequences containing the genetic environment around *bla*$_{NDM-1}$, proposed that *bla*$_{NDM-1}$ may be a chimeric gene within its origin, involving the aminoglycoside resistant gene, *aphA6* (70). There are reasons to believe that the origin/genesis of *bla*$_{NDM-1}$ occurred by fusion between *aphA6* and a previous *bla*$_{MBL}$ in *A. baumannii*. Reasons include (1) The IS*Aba125* associated with *bla*$_{NDM-1}$ is commonly found in *A. baumannii* (2) *aphA6* is highly associated with *A. baumannii* (3) the first NDM-producing *A. baumannii* in Europe (isolated in Germany, 2007 (72)), contained two full IS*Aba125* composite transposons, one with *bla*$_{NDM-1}$ and the other with *aphA6*. Thus it could be suggested that *A. baumannii* has played a large role in the origin of *bla*$_{NDM-1}$.

Expression of NDM-1 provided by IS*Aba125*

It is known that IS elements commonly encode a promoter sequence to drive expression of downstream genes. It has also been shown that the promoters provided by IS*Aba125* in conjunction with *bla*$_{NDM}$ itself provide a high level of carbapenem resistance (imipenem MIC >32 μg/mL with promoters versus only 0.5 μg/mL without the promoters) (185). However, it is not as simple as the presence or absence of a promoter, there are other factors that influence the level of carabapenem resistance which have yet to be charactersised. In 2006, 15 clinical NDM producing *Enterobacteriaceae* isolates were identified, most of which has an MIC of 8 μg/mL or less. Only three had an imipenem MIC greater than 16 μg/mL i.e. 16, 16, 64 μg/mL (143).

***bla*$_{NDM-1}$ variants**

Hitherto, there are up to seven published different variants of *bla*$_{NDM}$ due to different point mutations from the original *bla*$_{NDM-1}$ (Table 11). There are also two more variants of NDM-1 (NDM-9 and NDM-10) assigned however remain unpublished at this time [201]. Amongst the seven published variants, there are several common features. Firstly, is that they were all isolated in *E. coli* with the exception of *bla*$_{NDM-2}$ from *A. baumannii*. Secondly, all were found to have originally acquired from India, excluding *bla*$_{NDM-2}$ (Egypt) and *bla*$_{NDM-7}$ (Yemen). The *E. coli* ST type ST648 is the only type thus far to harbor two novel *bla*$_{NDM-1}$ variants, *bla*$_{NDM-4}$ and *bla*$_{NDM-5}$.

All of NDM-1 variants excluding NDM-4 and NDM-8, provide high levels of resistance to carbapenems with an MIC of ≥32 ug/mL against imipenem and meropenem. NDM-4 and NDM-8 provided lower carbapenemase resistance compared to the other NDM variant

counterparts with MIC levels for imipenem and meropenem of 8 ug/mL and 16 ug/mL for NDM-4 (186), and 0.5 μg/mL and 0.25 μg/mL for NDM-8 (187), respectively.

Table 11. Variants of NDM-1

NDM-1 Variant	Country of Isolation	Country origin	Sample	Species	ST type	Point mutation position from NDM-1 (Amino acid change)	Reference
NDM-1	Sweden	India	Urine	*K. pneumoniae*	14	-	(2)
NDM-2	Germany	Egypt	Catheter	*A. baumannii*	103	82 (Pro➔Ala)	(188)
NDM-3	Australia	India	Urine	*E. coli*	ND	95 (Asp➔Asn)	(189)
NDM-4	India	India	Urine	*E. coli*	648	154 (Met➔Leu)	(186)
NDM-5	United Kingdom	India	Perineum and throat	*E. coli*	648	88 (Val➔Leu) 154 (Met➔Leu)	(190)
NDM-6	New Zealand	India	Rectal swab	*E. coli*	101	698 (Cys➔Tyr)	(101)
NDM-7	German	Yemen	Wounds, throat, rectum	*E. coli*	599	388 (Gly➔Ala) 460 (Ala➔Cys)	(191)
NDM-8	Nepal	Unknown	Pus	*E. coli*	ND	130 (Asp➔Gly) 154 (Met➔Leu)	(187)
NDM-9	China	Unknown	Urine	*K. pneumoniae*	ND	152 (Glu➔Lys)	Unpublished

ST: Sequence type, ND: Not defined, Amino Acids- Pro: Proline; Ala: Alanine; Asp: Aspartic Acid; Asn: Asparagine; Met: Methionine; Leu: Leucine; Lys: Lysine; Val: Valine; Tyr: Tyrosine; Gly: Glycine; Glu: Glutamic acid; Cys: Cystein

A tiered model for the spread and acquisition of antibiotic resistance

bla$_{\text{NDM-1}}$ has been able to spread rapidly because of the combination of travel, its bacterial host (predominately gut colonizers), plasmid encoding and transposition. Thus this situation can be thought in the perspective of the acquisition and the ability of spread of a resistance gene. In terms of *bla*$_{\text{NDM}}$, it could be defined on various tiers deeper than the three routes of transmission mentioned previously. These tiers include the bacterial host, horizontal gene transfer (HGT) and genetic mobilization and are determined upon where the gene is genetically encoded (Fig. 3). The bacterial host determines the three routes of acquisition as well as international spread, and also potential reservoirs of the resistance gene.

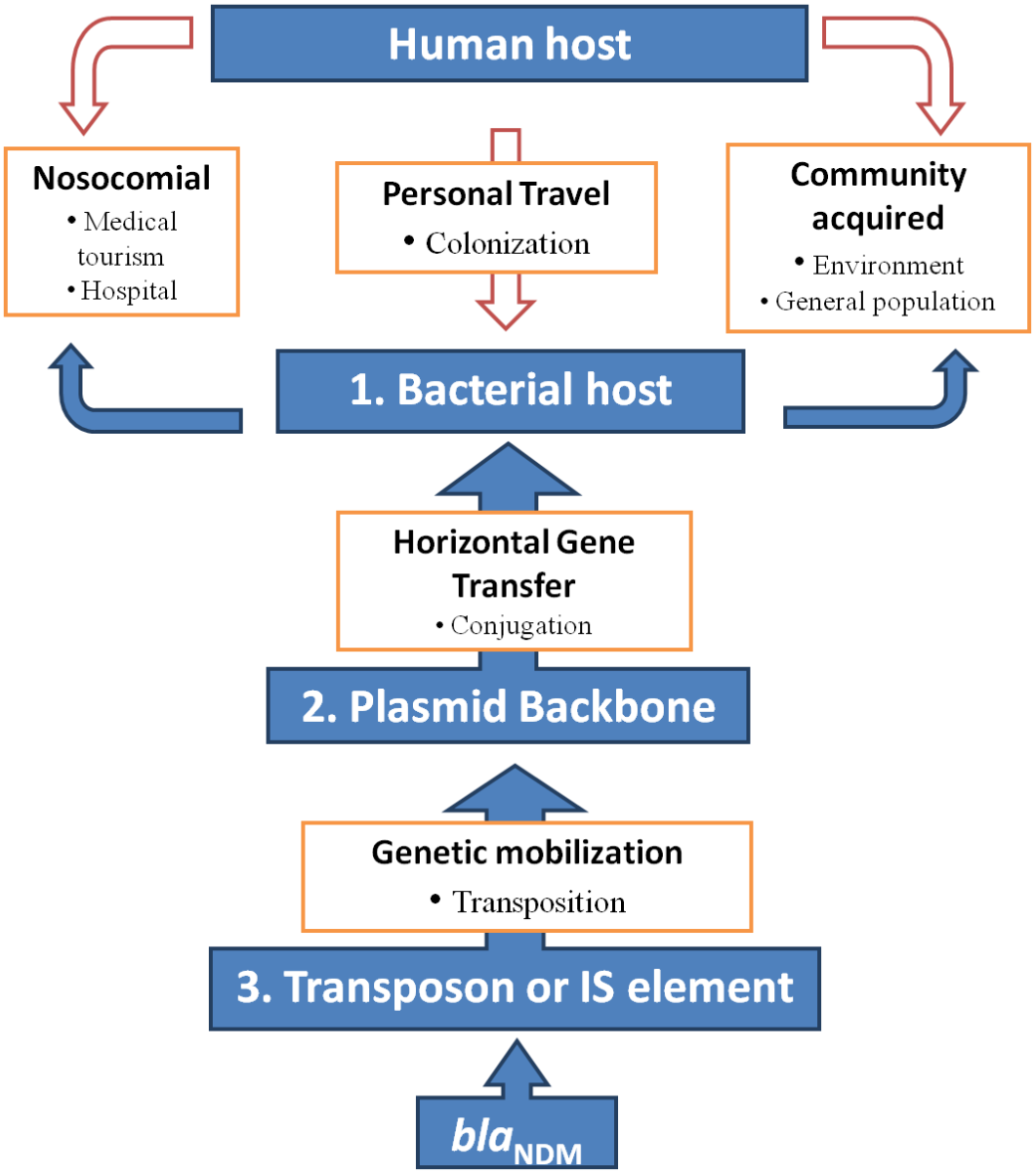

Figure 3. Model of *bla*$_{NDM}$ spread and acquisition

bla$_{NDM}$ is encoded on the 3 genetic tiers by mechanisms of 1. Clonal spread (bacterial host), 2. Conjugation (horizontal gene transfer) and 3. Transposition (genetic mobilization). Each tier flows into and broadens the range of the tier above until the bacterial host, which determines how *bla*$_{NDM}$ can be acquired by a person. The bacterial host determines how the gene can spread to a human host and will remain in certain reservoirs until acquired, in the case of *bla*$_{NDM}$ is either nosocomial or within the community. In terms of human hosts, there are three major routes to acquire an NDM producing organism: nosocomial, personal travel and community acquisition. Blue box: Host/platform harboring *bla*$_{NDM}$; Orange box: Mode of transmission; Bullet point: mechanism of acquisition/spread; Blue arrows: *bla*$_{NDM}$ spread; Red arrows: *bla*$_{NDM}$ acquisition.

The bacterial host essentially determines how the resistance gene can be humanly acquired and the reservoirs in which the gene will remain until acquired. For example, *bla*$_{\text{NDM-1}}$ harbored by *K. pneumoniae* and *E. coli* is essentially acquired via faecal-oral transmission and in terms of a reservoir will reside in the environment e.g. contaminated water, hospital surfaces and the GI tract. Colonization rate of species from the *Enterobacteriaceae* family in humans is variable. While *E. coli* is the most commensal aerobic bacterium colonized in the human gut (192), colonization of *K. pneumoniae* varies according to many factors including geographical region, health care facility, ward and patient group with some studies reporting colonization prevalence of KPC producing *Enterobacteriaceae* at 45.8% within metropolitan Chicago, USA, (193), while others report a prevalence of ESBL producing *Enterobacteriaceae* in inpatients, intensive care patients, and adults as 7.4%, 9.3% and 12% within Hungarian university wards and clinics (194). The GI tract as a reservoir generally allows for colonization and long-term asymptomatic carriage leading to the route of international spread. In the example of the *bla*$_{\text{NDM}}$ gene, the ability of this gene to spread would be amplified if the bacterial hosts (*K. pneumoniae* and *E. coli*) harboring *bla*$_{\text{NDM}}$ were capable of high clonal spread, i.e. highly predominant ST types. Bacterial hosts provide the basis for routes of transmission, reservoirs and thus international spread of an antibiotic resistance gene. The bacterial host range can broaden depending on where and how the resistance gene is encoded.

The genetic platform on which the resistance gene is encoded (chromosome or plasmid) determines the bacterial host range. A resistance gene encoded on the chromosome is restricted to spread by only the bacterial host level, meaning only via clonal spread and patient acquisition is limited to that of one clone. However if the gene is encoded within an ICE or on a plasmid, it is capable of HGT to other bacterial hosts and thus more ST types for clonal spread and increasing the potential for human acquisition. The resistance gene's

bacterial host range however is determined and restricted by the plasmid backbone it is encoded upon because each plasmid backbone (Inc types) has a different bacterial host range (broad and narrow). This bacterial host range can be further broadened by the presence of genetic mobilization mechanisms.

Genetic mobilization provides a mechanism in which genes can traverse to different DNA locations including the chromosome and plasmids. A gene captured or localized in close proximity to a genetic element capable of genetic mobilization is able to traverse the gene from the chromosome to various different plasmid backbones or vice-versa, therefore further broadening the range of bacterial hosts able to acquire *bla*$_{\text{NDM}}$.

The spread of an antibiotic resistance gene is dependent on the genetic platform which encodes the gene. These genetic platforms can be arranged into tiers describing the genetic mechanism of spread and acquisition i.e. 1. Bacterial host (chromosome), 2. Horizontal gene transfer (plasmid or ICE) and 3. Genetic mobilization (transposons/IS elements). The further genetic tiers the gene is encoded and associated with, the range of the tiers above broadens e.g. Genetic mobilization increases the number of plasmid backbones which in turn increases the bacterial host range, therefore determining the routes and reservoirs for human acquisition. *bla*$_{\text{NDM}}$ traverses all three genetic mechanism tiers and having the correct combination of genetic tiers, gut colonizers (Bacterial host), broad and narrow bacterial host sprectrum plasmids (HGT) and Tn*125*/various IS elements (Genetic mobilization) has made an optimal platform for rapid spread. Combining these three genetic mechanism tiers of spread with the human-bacterial host interaction determining the three routes of human acquisition, begins to explain how *bla*$_{\text{NDM}}$ was able to successfully spread on a global scale. This leads to the issue that even with the current protocols to prevent bacterial host spread amongst the human population, there are further issues to address concerning reservoirs and more mechanisms of spread other than clonal. In addition there are also environmental issues

such as the poor sanitation of regions in the Asian continent and the lack prescription regulation that have and continue to promote the spread of the *bla*$_{NDM}$ gene.

Infection Control

Approaches to the prevention of spread of NDM producing organisms will vary depending on whether the setting is a reservoir country (high prevalence country) or a low prevalence country.

Infection control in a high prevalence setting

Given that a substantial proportion of the general population in the Indian subcontinent are colonized with NDM producers (195) infection control in high prevalence settings is challenging. Compounding this is a lack of resources for infection control in many publicly funded healthcare facilities. In this setting, attention to hand hygiene with inexpensive alcohol based products must be paramount. High levels of hand hygiene before and after patient care should be maintained in accordance with the World Health Organization's Five Moments of Hand Hygiene. All reusable patient equipment should be cleaned before and after each use. Patient rooms should be cleaned, and preferably disinfected, on a daily basis. Basic attention to antibiotic management such as reduction in duration of antibiotic therapy should be reinforced.

Additional measures to reduce cross-transmission of NDM producers should be used in private healthcare settings which are likely to have additional resources. Here, contact isolation precautions should be used with the patient accommodated in a single room with its own toilet facilities. The use of contact isolation precautions implies use of a gown and gloves for those entering the patient's room. Unnecessary transfer of patients within the healthcare facility should be avoided. This applies both to patients colonized with NDM

producers and compromised patients (such as neutropenic patients). Antibiotic stewardship activities should be undertaken in order to reduce inappropriate use of cephalosporins, fluoroquinolones, beta-lactam/beta-lactamase inhibitor combinations, aminoglycosides, fosfomycin, tigecycline and polymyxins. Measurement of use of these antibiotics should be performed. Additionally, monitoring of antimicrobial resistance at a facility level for key Gram-negative organisms at significant sites of infection (for example, blood cultures) is essential.

Infection control in a low prevalence setting

The key measures of infection control used in high prevalence settings should still be undertaken in low prevalence settings. This includes high compliance with hand hygiene, use of contact isolation precautions, environmental cleaning and antibiotic stewardship. Additionally, active screening for colonization with NDM producing organisms should be performed on patients at high risk for this organism. This includes patients who have been admitted to a hospital or a residential aged care facility in a high prevalence country within the last 12 months. In practice, it is important to remember that there are other carbapenemases which cause the same antibiotic resistance phenotype as NDM. Therefore, a more general approach to detection of carbapenemase production should be undertaken – this may involve screening rectal swabs on selective media and using rapid tests (such as the carbaNP test) to detect carbapenemase production (196).

In addition to screening, there may be justification to use pre-emptive contact isolation in patients directly transferred from an overseas hospital, patients who have been admitted to an overseas hospital within the last 12 months or who have previous demonstrated colonization or infection with NDM producers.

Treatment Options

The spectrum of NDM producers ranges from asymptomatic colonization to overwhelming and potentially fatal sepsis. There is insufficient data to support decolonization of patients colonized with NDM-producing organisms. Clinical studies of simple cystitis have observed natural resolution in most women, albeit delayed when compared with susceptible antimicrobial therapy. In this setting of a highly resistant pathogen, however, withholding therapy must be weighed against a potential risk of upper tract UTI and subsequent sepsis with a difficult to treat organism. Many patients with urinary tract infection (UTI) are best treated with orally administered antibiotics. Most NDM producers are resistant to the orally administered antibiotics commonly used in the treatment of UTI (such as trimethoprim/sulfamethoxazole or fluoroquinolones). Fosfomycin has been used successfully in the therapy of cystitis of multiply resistant Gram-negative bacilli. Many NDM producers remain susceptible to fosfomycin (75, 111, 113, 180). Nitrofurantoin represents another orally administrable option for NDM producers and successful use in this setting has been reported (189). Chloramphenicol and tetracyclines may represent other orally administered options which may be active against NDM producers, but this would need to be confirmed with susceptibility testing.

Upper tract UTIs and more serious infections with NDM producers (such as pneumonia and bloodstream infection) will require therapy with intravenously administered antibiotics. Options include the polymyxins and tigecycline. Both antibiotics have pharmacokinetic profiles that make them imperfect as antibiotic choices for serious infections. Recent investigations have led to greater understanding of the need for a loading dose and appropriate dosage modification of colistin for serious infections (197). Studies of the treatment of other carbapenemase producers (e.g. KPC producers) suggest that combination antibiotic therapy may lead to superior outcomes compared to use of colistin or tigecycline

alone (27-29). Combinations may include a carbapenem (such as meropenem) even though the treated organism is a carbapenemase producer (27-29). No randomized controlled trials have yet been performed on optimal treatment of NDM producers, yet such studies are urgently needed. This is particularly important given reports of polymyxin and tigecycline resistance.

Expert commentary

Carbapenem resistance in Gram-negative bacilli is of major clinical significance since it denotes resistance to a major class of antibiotics used in critically ill patients. Carbapenem resistance is usually mediated by carbapenemases, which are beta-lactamases which can hydrolyze the carbapenem antibiotics. NDM is an example of just such a carbapenemase. Significantly, the genes encoding NDM are in close proximity to genes encoding resistance to other antibiotic classes such as the aminoglycosides. Additionally, NDM inactivates all penicillins and cephalosporins (except aztreonam), and is not inhibited by currently used beta-lactamase inhibitors. The end result is that NDM producers are typically resistant to all commercially available first line antibiotics. "Reserve" antibiotics such as colistin are used to treat serious infections with NDM producers. Unfortunately colistin resistance has now been reported (198), resulting in organisms resistant to all available antibiotics. NDM producers therefore can pave the way to the "post-antibiotic era".

Five-year view

Over the next five years, it is highly likely that NDM producers will become endemic in many countries which have only seen sporadic isolates thus far. Major mechanisms for this will likely include the ongoing globalization of medicine and the carriage of bla_{NDM} by highly successful clones such as ST131 *E. coli* and ST258 *K. pneumoniae*. It is quite possible that

these strains will also be associated with colistin resistance, meaning that this major antibiotic used to treat NDM producers will be ineffective against many NDM producing organisms. It is possible that aztreonam-based regiments will be trialed in the near future. If successful, this may represent a treatment option for NDM producing organisms.

Key Issues

- Reservoir countries of *bla*$_{NDM}$ have expanded from the Indian sub-continent, China and the Balkan states to South-western Europe, the Greater Middle East and southeast Asia.
- Medical tourism and personal travel are major factors that have allowed the globalization of the carbapenem resistance provided by NDM-1.
- The three routes of human NDM acquisition are Nosocomial, Personal travel and Community acquired.
- Predominant bacterial hosts producing NDM are *K. pneumoniae*, *E. coli*, *A. baumannii* and *Pseudomonas* spp.
- *bla*$_{NDM}$ is encoded on various plasmid backbones capable of horizontal gene transfer, most predominant being IncA/C.
- Genetic mobilization through transposons and IS elements, initially via Tn*125* and IS*Aba125*, has allowed *bla*$_{NDM}$ to traverse amongst various plasmid backbones.
- The more genetic tiers a resistance gene is incorporated within, the broader the range of potential bacterial hosts, which in turn determines the routes of acquisition by a human host.
- Infection control intervention to prevent spread of NDM producers include compliance with hand hygiene, use of contact isolation precautions, vigorous hospital cleaning, good antibiotic stewardship.

- The key antibiotic for serious infections with NDM producers is colistin.

1.7 Thesis Aim

The *bla*$_{NDM}$ gene is not solely associated to a clonal lineage, plasmid type or a transposable element mechanism responsible for its mobilisation when observed within the *Enterobacteriaceae* family. To address the new paradigm of AMR acquisition and spread presented by the *bla*$_{NDM}$ gene, this thesis endeavours to provide, an insight and basic understanding of the mechanisms involved in *bla*$_{NDM}$ acquisition and spread, and an approach to assess its plasmid-mediated dissemination amongst the *Enterobacteriaceae* family. This will be achieved through bioinformatics analysis and characterisation of *Enterobacteriaceae* plasmids, primarily those that harbour the *bla*$_{NDM}$ gene. Investigations will begin by establishing a recent account of the *bla*$_{NDM}$ gene from an epidemiological perspective. A novel molecular/ genetic approach will be used, incorporating characterisation of the *bla*$_{NDM}$ genetic context (NGC) carried by strains, firstly within the context of a single medical institute, followed by a national level investigation of strains isolated from multiple health-care facilities. Secondly, NGCs will be analysed in the context of the plasmid itself by construction and analysis of complete plasmid sequences, to explain the transposable element mechanisms involved for insertion of *bla*$_{NDM}$ genes onto plasmids, leading to the NGCs observed. Lastly, the plasmid types harbouring *bla*$_{NDM}$ described here and within the literature will be placed into a greater context of overall *Enterobacteriaceae* plasmid types. *In silico* plasmid typing will be performed to retrospectively survey the plasmid content within a large data set of *Enterobacteriaceae* strains. Principal component analysis (PCA) of the shared plasmid context between genera will provide insights into the interactions between plasmid types and bacterial species, necessary for the spread of AMR genes. Characterisation of this paradigm would hopefully lead to the development of potential approaches to assess plasmid-mediated AMR spread.

1.8 Thesis outline and chapter aims:

This thesis is composed of six chapters. Chapter 1 is an introductory chapter, Chapters 2 to 5 each address a specific aim (listed below) comprised of a chapter introduction, the study investigating this aim presented in manuscript form, and a chapter summary/ conclusion. Chapter 6 provides a discussion of Chapters 1 to 5 as a collective whole for insights of *bla*$_{NDM}$ acquisition and spread, as well as suggestions for future investigations and a final conclusion of the entire thesis.

Chapter 2: Aim 1 – Establishing a recent account of the *bla*$_{NDM}$ gene from an epidemiological perspective using a novel genetic/molecular approach. The approach to assess *bla*$_{NDM}$ plasmid-mediated dissemination within a single facility, involves characterisation of sequence type, plasmid types and NGCs.

Chapter 3: Aim 2 – The combined approach to assess *bla*$_{NDM}$ plasmid-mediated dissemination will be applied to multiple healthcare facilities to verify if this approach is applicable at a national level, as well as to survey and establish an update on the mechanisms of acquisition and spread for NDM producing *Enterobacteriaceae* within Australia.

Chapter 4: Aim 3 – To investigate the acquisition of *bla*$_{NDM}$ genes in the context of the plasmid backbone and understand the process which NGCs arise; through characterisation of different transposable element mechanisms involved in its insertion upon the same plasmid backbone/ type.

Chapter 5: Aim 4 – To investigate the occurrence of *Enterobacteriaceae* plasmid types regardless of AMR genes through a retrospective plasmid content survey, and analyse the shared plasmid content between genera to provide insights into plasmid-mediated gene transmission between bacterial strains, possibly used in dissemination of the *bla*$_{NDM}$ gene.

Chapter 2: Characterisation of plasmids carrying *bla*$_{NDM}$ within Pakistan and an approach to assess NDM plasmid spread within a facility

2.1 Chapter Introduction

The Indian sub-continent is a geographical reservoir for acquisition of NDM producing Gram-negatives (Chapter 1; (20)). International travel to the Indian sub-continent for Australians is a common route of acquiring an NDM producer, where hospitalisation can lead to acquisition of an NDM producing *Enterobacteriaceae*, *Acinetobacter* spp. or *Pseudomonas* spp. In the perspective of clinical microbiology, epidemiology and infection control, the bacterial species ST type (via MLST) of carbapenem producers is frequently investigated for the purposes of tracing a predominant bacterial clone responsible for infection within a clinical setting. This typing alone has previously been sufficient to describe and loosely associate an antimicrobial resistance phenotype exhibited by a bacterial clone. Further detail however is required for plasmid-mediated AMR, especially those harbouring the *bla*$_{NDM}$ gene. It might also be suggested that stating the plasmid Inc type would provide sufficient information for the spread of antimicrobial resistance. However, as stated previously (Chapter 1), regions carrying genes conferring carbapenem resistance can vary between each plasmid and even upon the same plasmid backbone type, indicating a different capture event. This means that each plasmid type does not have a steady antimicrobial resistance gene profile. Therefore detailing ST type and plasmid type alone may be misleading for investigating plasmid-mediated antimicrobial resistance. Combining genomic characteristics of ST type and plasmid type with the in-depth characterisation of the genetic context for a target resistance gene, can elucidate insights and clarification upon the transmission of AMR i.e. the spread of a specific plasmid providing carbapenem resistance.

The study presented in this chapter, investigates a clinical setting within Pakistan where patients co-harboured multiple NDM producers of different species within their stool. Characterisation of the antimicrobial gene profile, ST type, plasmid Inc type and *bla*$_{NDM}$ genetic context of each strain was utilised as an approach to clarify *bla*$_{NDM}$ inter-species and inter-patient transmission.

This investigation was summarised and presented in a manuscript format published in *Antimicrobial Agents and Chemotherapy*:

Wailan AM, *Sartor AL, Zowawi HM, Perry JD, Paterson DL, Sidjabat HE.* The genetic contexts of $bla_{\text{NDM-1}}$ in patients carrying multiple NDM producing strains. Antimicrob. Agents Chemother. 2015 Dec;59(12):7405-10. doi: 10.1128/AAC.01319-15

The University of Queensland requires the presentation of the submitted or accepted article. The numbers of the figures, tables and references of the presented article have been amended to suit the structure of this thesis. The article remains in American English spelling enforced by journal guidelines.

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2.2. Chapter 2 – Original Article: The genetic contexts of *bla*$_{NDM-1}$ in patients carrying multiple NDM producing strains.

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Running title:

Plasmids carrying *bla*$_{NDM-1}$ from Pakistan

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Abstract

The carbapenem resistance determinant *bla*$_{NDM-1}$, has been found in various Gram-negative bacteria and upon different plasmid replicon types (Inc). Here, we present four patients within two hospitals in Pakistan harboring between two and four NDM-1-producing Gram-negative bacilli of different species co-resident in their stool samples. We characterize the *bla*$_{NDM-1}$ genetic context of these 11 NDM-1-producing Gram-negative bacilli in addition to other antimicrobial resistance mechanisms, plasmid replicon profile and sequence type (ST), in order to understand the underlying acquisition mechanisms of carbapenem resistance within these bacteria. Two common plasmid types (IncN2 and IncA/C) were identified to carry *bla*$_{NDM-1}$, among the six different bacterial species isolated from the four patients. Two of these strains were novel *Citrobacter freundii* ST 20 and ST 21. The same IncN2 type *bla*$_{NDM-1}$ genetic context was found in all four patients and within four different species. The IncA/C type *bla*$_{NDM-1}$ genetic context was found in two different species and in two of the four patients. Combining genetic context characterization with other molecular epidemiology methods we were able to establish the molecular epidemiological links between genetically unrelated bacterial species by linking their acquisition of an IncN2 or IncA/C plasmid carrying *bla*$_{NDM-1}$ for carbapenem resistance. By combining plasmid characterization and in-depth genetic context assessment, this analysis highlights the importance of plasmids in antimicrobial resistance. It also provides a novel approach for investigating the underlying mechanisms of *bla*$_{NDM-1}$ related spread between bacterial species and genera via plasmids.

Introduction

Infectious pathogens have the ability to transmit from one person to another. Antimicrobial resistance in certain instances has been observed to follow this paradigm of transmission; specifically, bacterial species have been described transmitting antimicrobial resistance determinants. A well-known example of a successful international clone is the *Escherichia coli* Sequence Type (ST) 131 transmitting $bla_{\text{CTX-M-15}}$ (41, 42). However, numerous reports in the last few years provide evidence that plasmids are a major factor in the transmission of antimicrobial resistance (40, 199).

Since the first report (2), bla_{NDM} has been reported to be harbored by a diverse range of bacterial species, the most frequent are within the *Acinetobacter* genus and *Enterobacteriaceae* family (20, 200) Furthermore, bla_{NDM} has also been identified to reside within different plasmid replicon types (Inc) amongst the *Enterobacteriaceae* family, including IncA/C (201), IncF types (202), IncL/M (74), IncN (203), IncX (204) and IncH (183). In addition, the genetic structure or context in which bla_{NDM} resides varies between different plasmid types, and even with the same plasmid type (205). In contrast, bla_{NDM} genetic contexts within *Acinetobacter* spp. have been reported with less variation; since bla_{NDM} can be commonly found within the 10,099 bp transposon known as Tn*125*, composed of two flanking IS*Aba125* (72, 138, 184, 200). These $bla_{\text{NDM-1}}$ genetic contexts observed with *Enterobacteriaceae* plasmids appear to frequently involve parts of Tn*125* carrying blaNDM as the common substrate and various mechanisms of gene acquisition used to acquire this substrate, including different IS*CR* elements (177), class one integrons (94), flanking IS elements (177) and singleton IS elements present in close proximity (74, 201).

The Indian sub-continent is recognized as a major reservoir for bla_{NDM} acquisition and has been hypothesized as the geographical origin of bla_{NDM} (20, 206). In a previous study from our group, 66 NDM-1 producing Gram-negatives from stool samples of patients in two

Pakistani hospitals were reported (206). Amongst patients harboring these isolates, four patients were found to carry multiple NDM-1 producers of different species. Sartor and colleagues also identified that the species within each patient harbored different plasmid replicon type profiles (206). This warranted further investigation in order to elucidate the underlying nature of $bla_{\text{NDM-1}}$ acquisition by these different species, when the bacterial hosts have different plasmid replicon profiles. The aim of this study was to further characterize these different NDM producing bacterial species co-existing in multiple patients through whole genome sequencing, noting their resistance mechanisms and Sequence Type (ST) with further focus on characterizing the $bla_{\text{NDM-1}}$ genetic contexts.

Methods

Bacterial isolates. Eleven clinical isolates were acquired in 2010 from stool samples from four different patients in two hospitals in Rawalpindi, Pakistan (148). In the study conducted by Perry and colleagues (148), one patient carried 4 species, 3 patients had 3 species and 5 patients had 2 species of NDM-1-producers (data not shown). Isolates were selected based on the number of isolates present in the patients and the diversity of their plasmid type profile which was determined by PCR Based Replicon Typing (PBRT) of *Enterobacteriaceae* isolates as previously described (58, 206). The strains from each patient are as follows (Table 1): Patient 1 - *Enterobacter cloacae* Pn2, *Acinetobacter baumannii* Pn3, *Klebsiella pneumoniae* Pn4, *Citrobacter freundii* Pn5; Patient 2 – *Pseudocitrobacter faecalis* Pn13, *Escherichia coli* Pn14; Patient 3 – *P. faecalis* Pn27, *E. coli* Pn28; Patient 4 – *E. coli* Pn66, *C. freundii* Pn67 and *E. cloacae* Pn68.

Plasmid transfer experiments through conjugation. Plasmids carrying $bla_{\text{NDM-1}}$ from clinical strains were transferred by conjugation to confirm their transferability and plasmid replicon type. For conjugation experiments, the eleven clinical isolates (donors) were verified

to be susceptible to sodium azide via agar dilution. Donor strains and sodium azide resistant *E. coli* J53 recipients were grown in LB broth at 200 rpm to logarithmic phase (207). The conjugation mixture consisted of donor and recipient strains in a 1:1 ratio plated onto MacConkey agar, and incubated at 37°C overnight (78). The conjugation mixture of approximately 20 µL of confluent growth on the plate was then harvested into 1 mL of saline and serially diluted in saline to 10^{-8}. This serial dilution method was performed to obtain single colonies of both donors and transconjugants between the serial dilutions of 10^{-4} and 10^{-6}. One hundred microliters of each dilution was then inoculated onto MacConkey agar supplemented with meropenem (0.1 µg/mL) and sodium azide (100 µg/mL), sodium azide only (100 µg/mL) and a control plate without additives, incubated at 37°C for 24 h. Successful transconjugants were confirmed phenotypically and through PCR for $bla_{\text{NDM-1}}$. PBRT was performed on transconjugants to identify the plasmid replicon type carrying $bla_{\text{NDM-1}}$ (58).

Sequencing and Bioinformatics. Paired-end libraries of whole genomic DNA of all 11 clinical isolates were prepared and sequenced by the Illumina MiSeq platform (Illumina, San Diego, USA). All sequences were *de novo* assembled using CLC Genomic Workbench v7.5 (CLC Bio, Aarhus, Denmark) with at least 50-fold coverage. pNDM-BJ01 (Genbank accession no. JQ001791) (208) was manually annotated and used as a reference for Tn*125* structure annotation. Sequences from the Genbank database and IS finder (https://www-is.biotoul.fr/) were used to identify and characterize genes flanking the Tn*125* region. CLC Genomic Workbench was used to BLAST (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*), analyze and manually annotate the genetic context of $bla_{\text{NDM-1}}$ according to the aforementioned reference sequences. Contigs containing $bla_{\text{NDM-1}}$ were named as follows: Patient 1 - pPN2-ECL-NDM-1 (*E. cloacae* Pn2), PN3-AB-NDM-1 (*A. baumannii* Pn3), pPN4-KP-NDM-1 (*K.*

pneumoniae Pn4), PN5-CF-NDM-1 (*C. freundii* Pn5); Patient 2 - pPN13-PCF-NDM-1 (*P. faecalis* Pn13), pPN14-EC-NDM-1 (*E. coli* Pn14); Patient 3 – pPN27-PCF-NDM-1 (*P. faecalis* Pn27), PN28-EC-NDM-1 (*E. coli* Pn28); Patient 4 – pPN66-EC-NDM-1 (*E. coli* Pn66), pPN67-CF-NDM-1 (*C. freundii* Pn67) and pPN68-ECL-NDM-1 (*E. cloacae* Pn68). Contigs of each clinical strain were submitted to Resfinder 2.1 (209) (http://cge.cbs.dtu.dk/services/ResFinder/) and Plasmidfinder 1.1 (210) databases (available at the Center of Genomic Epidemiology website (http://www.genomicepidemiology.org/)) to characterize their resistance mechanism genes and plasmid Inc types.

Sequence Typing via MLST scheme. Each clinical isolate was submitted to the MLST 1.7 database (211) (https://cge.cbs.dtu.dk/services/MLST/) for Sequence Typing via respective MLST schemes, except *P. faecalis* strains Pn13 and Pn27 as no MLST was available (212). Both *C. freundii* strains Pn5 and Pn65 were identified as novel sequence types and subsequently submitted to the *C. freundii* MLST website (http://pubmlst.org/ cfreundii/) (213) for assigning of new sequence type.

Nucleotide sequence accession number. Each nucleotide sequence was deposited in the GenBank database with the following accession numbers. pPN2-ECL-NDM-1, KP770024; PN3-AB-NDM-1, KP770025; pPN4-KP-NDM-1, KP770033; PN5-CF-NDM-1, KP770032; pPN13-PCF-NDM-1, KP770031; pPN14-EC-NDM-1, KP770030; pPN27-PCF-NDM-1, KP770029; PN28-EC-NDM-1, KP770023; pPN66-EC-NDM-1, KP770028; pPN67-CF-NDM-1, KP770027 and pPN68-ECL-NDM-1, KP770026.

Results and Discussion

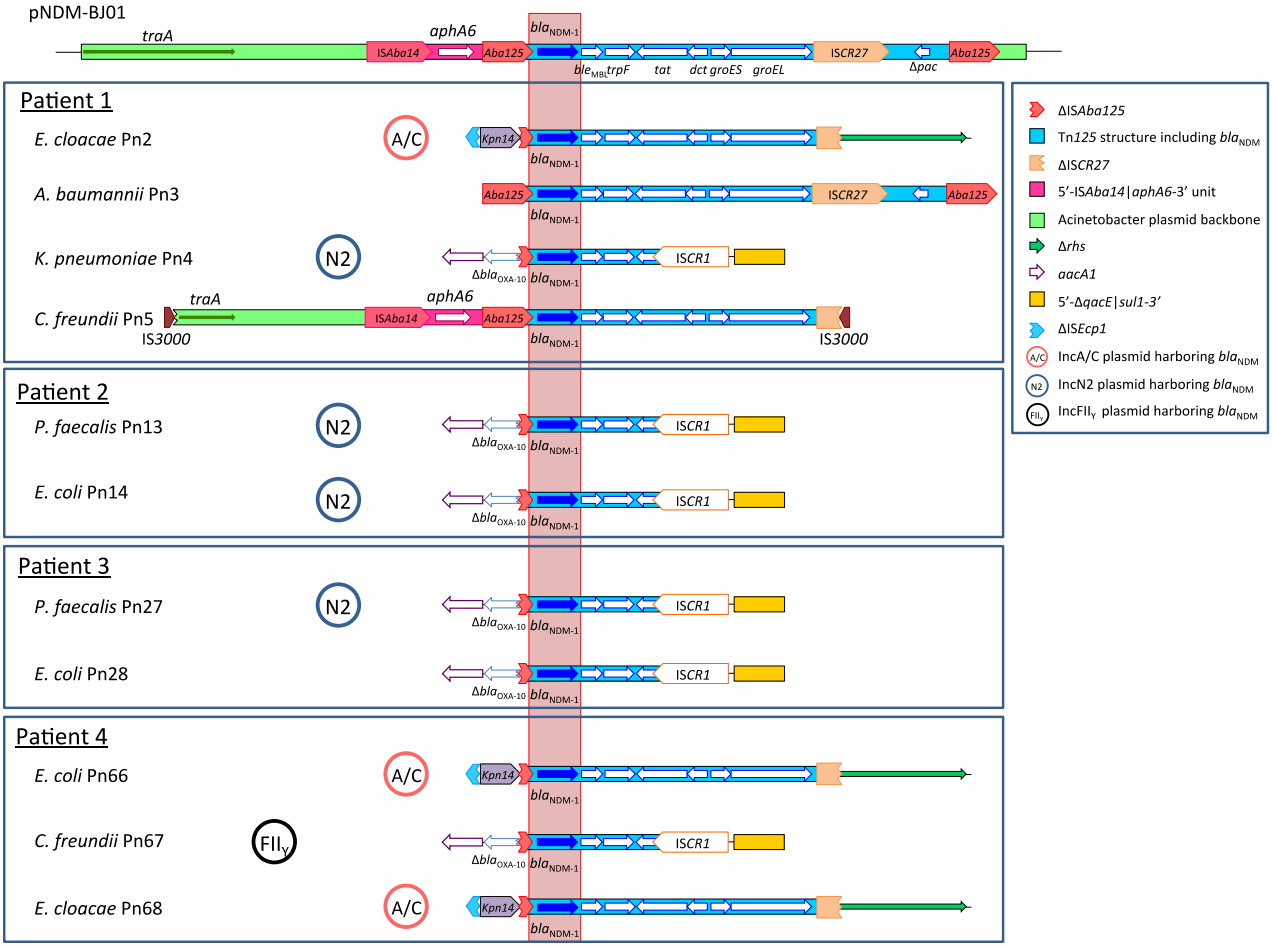

Figure 4. Schematic representation of all *bla*NDM genetic context within this study and reference sequence pNDM-BJ01 (Genbank Accession no. JQ001791)

*bla*NDM genetic contexts and their Genbank Accession number, Pn2: pPN2-ECL-NDM-1 (KP770024), Pn3: PN3-AB-NDM-1 (KP770025), Pn4: pPN4-KP-NDM-1 (KP770033), Pn5: PN5-CF-NDM-1 (KP770032); Pn13: pPN13-PCF-NDM-1 (KP770031), Pn14: pPN14-EC-NDM-1 (KP770030); Pn27: pPN27-PCF-NDM-1 (KP770029), Pn28: PN28-EC-NDM-1 (KP770023); Pn66: pPN66-EC-NDM-1 (KP770028), Pn67: pPN67-CF-NDM-1 (KP770027) and Pn68: pPN68-ECL-NDM-1 (KP770026). Δ, truncated gene. Insertion Sequence (IS) elements are represented as block arrows.

The molecular and *in silico* analysis results of each NDM-1 producing strain are summarized in Table 12. These results also include for each strain, clinically significant antimicrobial resistance determinants found within each strain, ST type, and the replicon type (Inc) of the plasmid carrying *bla*$_{\text{NDM-1}}$ (determined via PBRT of successful transconjugants). *In silico* analysis of each contig with *bla*$_{\text{NDM-1}}$ initially identified a truncated Tn*125* structure harboring *bla*$_{\text{NDM-1}}$ (ΔTn*125*). The sizes of ΔTn*125* were variable (Fig. 4). Different genes and IS elements were identified flanking the ΔTn*125* structure (Fig. 4). The combination of the different ΔTn*125* structure sizes and flanking contexts identified four distinct *bla*$_{\text{NDM-1}}$ genetic contexts.

The most common *bla*$_{\text{NDM-1}}$ genetic context 5'-*aacA1* Δ*bla*$_{\text{OXA-10}}$ ΔTn*125*-3' (identified in 5 of 11 strains and in all four patients) contained a 2,341 bp ΔTn*125* structure with *aacA1* conferring aminoglycoside resistance and a truncated narrow-spectrum β-lactamase, *bla*$_{\text{OXA-10}}$ upstream, and was determined to be on IncN2 type plasmids (except in Pn28 and Pn67). This *bla*$_{\text{NDM-1}}$ genetic context was found within three different bacterial species, *K. pneumoniae* Pn4 (pPN4-KP-NDM-1), *P. faecalis* Pn13 and Pn27 (pPN13-PCF-NDM-1, pPN27-PCF-NDM-1), *E. coli* Pn14 and Pn28 (pPN14-EC-NDM-1, PN28-EC-NDM-1) and *C. freundii* Pn67 (pPN67-CF-NDM-1), and is similar to an *E. coli* isolated from Japan (direct submission with Genbank accession no. AB769140), and two IncN3 plasmids, pLK75 (Genbank Accession no. KJ440076) of *E. coli* and pLK78 (Genbank Accession no. KJ440075) of *K. pneumoniae* isolated from Taiwan (203). Transmission of *bla*$_{\text{NDM-1}}$ may have occurred via conjugation of an IncN2 plasmid with this genetic context, as suggested by our successful conjugation experiments involving strains carrying IncN2 plasmids. These conjugation events would subsequently result in the different bacterial species producing NDM-1 within three of the four patients.

Table 12. Strains and their respective sequence type, antimicrobial resistance profile and Plasmid Replicon Typing results

Patient	Strain characteristics			Plasmid replicon types via Plasmidfinder (Inc)						PBRT (Inc)		Antimicrobial resistance determinants via Resfinder																							
												Beta-lactamases (*bla*)						Aminoglycoside resistance determinants										Quinolones resistance determinants			Other resistance determinants								
	Strain	Bacterial Species	MLST (ST)	A/C$_2$	N2	HI1	HI2	F types	Others	Plasmid carrying *bla*$_{NDM}$[a]	Other plasmids present strain	NDM-1	CTX-M-15	OXA-1	OXA-10	TEM-1B	Other	*aac(6')-Ib-cr*[b]	*rmtC*	*armA*	*strA & strB*	*aac(3)-IIa*	*aadA1*	*aadA16*	*aac(3)-IId*	*aph(3")V1a*	Other	*qnrB1*	*qnrS1*	Other	*catB3*	*catA1*	*sul1*	*sul2*	*dfrA14*	*dfrA27*	*tet(A)*	*ARR-3*	Additional resistance determinants
1	Pn2	*Enterobacter cloacae*	171			FIA (HI1)	HI2A			A/C$_2$							ACT-7 CMY-6										*aadA4*											
	Pn3	*Aceinteobacter baumannii*	113	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A						OXA-64																					
	Pn4	*Klebsiella pneumoniae*	101					FIB$_K$ FII	R	N2							SHV-1										*aadA5*			*qnrB66 oqxA oqxB*									*mph(A) dfrA17*
	Pn5	*Citrobacter freundii*	20[c]				HI2A	FIB		-																				*qnrB34*								*mph(E) msr(E) floR tet(C) dfrA5*
2	Pn13	*Pseudo-citrobacter faecalis*	N/A				HI2A	FIB$_K$		N2																												
	Pn14	*Escherichia coli*	2598				HI2A	FIB$_K$ FIB FIA	I1 Col$_{156}$	N2	I1 FIA FIB																												
3	Pn27	*Pseudo-citrobacter faecalis*	N/A				HI2A	FIB$_K$		N2																												
	Pn28	*Escherichia coli*	1431			HI1A HI1B	HI2A	FII	Col (BS512)	-	A/C HI1						CMY-4 OXA-9																					
4	Pn66	*Escherichia coli*	10					FIB$_K$	Y	A/C$_2$	Y						CMY-6										*aadA4*											
	Pn67	*Citrobacter freundii*	21[c]					FII(pMET) FII(Yp)		FII$_Y$							CMY-73													*qnrB54*								*mph(A)*
	Pn68	*Enterobacter cloacae*	171			FIA (HI1)	HI2A			A/C$_2$							ACT-7 CMY-6										*aadA2 aacA4*											

Note: Shaded boxes – Indicates the replicon types and genes present in each strain determined by Plasmidfinder and Resfinder, N/A - Indicates no positive Plasmid Replicon Typing results for *Acinetobacter* species and no MLST scheme for *P. faecalis,* [a] - PBRT was performed on transconjugants carrying the plasmid harbouring bla_{NDM}, [b] - this enzyme is responsible for aminoglycoside and quinolone resistance. [c] - Novel *Citrobacter freundii* Sequence Types via MLST scheme.

The IncN2 *bla*$_{NDM-1}$ genetic context was also found in *C. freundii* Pn67 in the fourth patient; however, it was identified on an IncFII$_Y$-type plasmid. This genetic context was also identified in *E. coli* Pn28, believed to be located on the chromosome or on a non-conjugative plasmid, as conjugation experiments were unsuccessful after multiple attempts. The *bla*$_{NDM-1}$ genetic contexts in Pn28 and Pn67 are highly similar to the IncN2 plasmids of our study as well as in the literature however were not located on IncN2 plasmids. It may be speculated these "IncN-like" *bla*$_{NDM-1}$ genetic contexts of Pn28 and Pn67 may have diverged from the IncN2 plasmids of our study through such events as homologous recombination (60). Alternatively Pn28 and Pn67 could have been the source from where the IncN2 plasmids acquired their *bla*$_{NDM-1}$ genetic context prior to horizontal gene transfer amongst the different species. Further investigation including full plasmid construction will be required to clarify the nature of these two isolates and the potential of *bla*$_{NDM-1}$ cassette transfer between plasmid backbones, IncN2 and IncFII$_Y$.

Another *bla*$_{NDM-1}$ genetic context observed, in 3 of the 11 isolates, involved a longer 6,332 bp ΔTn*125* structure. In comparison to the aforementioned IncN2 ΔTn*125* structure, this longer ΔTn*125* structure was flanked by an IS*Kpn14* and truncated IS*Ecp1* upstream, and a truncated Type IV secretion protein *rhs* downstream (Fig. 4). This IncA/C *bla*$_{NDM-1}$ genetic context was determined to be on IncA/C type plasmids and found in two patients, patient 1: *E. cloacae* strain Pn2 (pPN2-ECL-NDM-1) and patient 4: *E. coli* strain Pn66 (pPN66-EC-NDM-1) and *E. cloacae* strain Pn68 (pPN68-ECL-NDM-1). Similar to the aforementioned IncN2 *bla*$_{NDM-1}$ genetic contexts, the IncA/C *bla*$_{NDM-1}$ genetic context was also observed within two different species within the same patient (patient 4). Further it is very similar to the most frequently reported IncA/C *bla*$_{NDM-1}$ genetic context amongst the *Enterobacteriaceae* family (175, 201, 205). The combination of IncA/C *bla*$_{NDM-1}$ genetic

context identification (Figure 4), the previously reported clonal spread of *E. cloacae* and *E. coli* within these hospitals and the identification of IncA/C plasmids as the most prevalent plasmid type (206), might suggest the contribution of this specific IncA/C plasmid to carry and transmit carbapenem resistance among *Enterobacteriaceae* within this clinical setting.

Out of the 11 isolates, two unique genetic contexts for *bla*$_{\text{NDM-1}}$ were found in PN3-AB-NDM-1 (*A. baumannii* Pn3) and PN5-CF-NDM-1 (*C. freundii* Pn5) in patient 1. PN3-AB-NDM-1 carried a context composed of the longest Tn*125* structure (7,962 bp), which is very similar to the Tn*125* lengths frequently described within the *Acinetobacter* genus i.e. composed of a full Tn*125* structure extending from the left hand IS*Aba125* to the right hand IS*Aba125* (184, 200, 214, 215). PN5-CF-NDM-1 contained a 7,288 bp ΔTn*125* structure flanked by two IS*3000* (truncated left-hand IS*Aba125* to truncated IS*CR27*) with plasmid backbone elements in close proximity such as *traA*. This context has not been previously reported. Both *bla*$_{\text{NDM-1}}$ genetic contexts, PN3-AB-NDM-1 and PN5-CF-NDM-1, may potentially be located on a non-conjugative plasmid or the chromosome as suggested by unsuccessful transfers in conjugation experiments. To note, both PN3-AB-NDM-1 and PN5-CF-NDM-1 were found in patient 1 which carried four different NDM-1 producing species, i.e. *E. cloacae*, *A. baumannii*, *K. pneumoniae* and *C. freundii*, each with a different *bla*$_{\text{NDM-1}}$ genetic context (Fig. 4).

The characterized *bla*$_{\text{NDM-1}}$ genetic contexts of 11 clinical isolates retrospectively may suggest interspecies transmission of antimicrobial resistance at an *in vivo* level via plasmids. This has been previously described with *bla*$_{\text{KPC}}$ (216). Initial characterization of our isolates identified different plasmid replicon profiles with multiple species within the same patient. Genetic characterization in combination with other molecular typing methods has described,

clarified and provided an insight into the spread and acquisition of *bla*$_{\text{NDM-1.}}$ Apart from the non-conjugative *bla*$_{\text{NDM-1}}$ found on Pn3, Pn5 and Pn28, and the single IncFII$_{Y}$ plasmid in Pn67, the remaining strains have become carbapenem resistant by acquiring IncN2 and IncA/C type plasmids with the specific aforementioned *bla*$_{\text{NDM-1}}$ genetic contexts.

It must be noted however, that due to the nature of PBRT as an assay that targets specific regions of a plasmid type (e.g. replicons), information that can influence plasmid transfer such as the number of plasmids within each clinical strain cannot be determined. Many plasmids can carry multiple replicons but also can form co-integrates with other plasmids to assist with conjugation. Transconjugants may also contain more than one plasmid. PFGE with S1 nuclease and southern blotting would have to be applied to both donors and respective transconjugants to determine the number of plasmids present.

The prevalence of highly similar *bla*$_{\text{NDM-1}}$ genetic contexts within different species and amongst different patients highlights the possible role plasmids are able to provide in inter-species transmission of carbapenem resistance. We suggest that genetic characterization of plasmids for *bla*$_{\text{NDM-1}}$ could be considered as a tool similar to the Multi-Locus Sequencing Typing (MLST) approach to investigate the clonal epidemiology of antibiotic resistant bacteria, which utilizes the typing of conserved regions within the genome for comparison (42, 217, 218). By considering genetic context characterization in combination with standard molecular methods (bacterial species identification, resistance mechanism profiling and clonality via MLST) during epidemiological studies, sophisticated epidemiological links between patients and genetically unrelated bacterial species can be clarified in the case of *bla*$_{\text{NDM-1}}$ and potentially other plasmid mediated antimicrobial resistance determinants that have genetic context variation, such as *bla*$_{\text{CTX-M-type}}$ (219, 220) or *bla*$_{\text{CMY-type}}$ (221). Such

investigation will assist in clarifying whether or not within a specific geographical region and facility, antimicrobial resistance acquisition by species has occurred via a particular plasmid with a specific genetic context.

Here we have characterized the genetic nature in which $bla_{\text{NDM-1}}$ resides in different NDM-1 producing bacterial species co-existing in multiple patients, while identifying IncA/C and IncN2 plasmids as the platform providing carbapenem resistance to otherwise diverse and unrelated species of *Enterobacteriaceae* within this clinical setting of two Pakistan hospitals. The combination of genetic plasmid characterization and epidemiological molecular investigation methods presented here not only highlights the ability of plasmids to transmit and provide antimicrobial resistance determinants to various bacterial species and genera, but also has provided a novel approach for investigating the underlying mechanisms of $bla_{\text{NDM-1}}$ related spread associated with hospitalized patients.

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2.3 Chapter summaries and conclusions.

This chapter demonstrated a combined molecular/genetic approach able to assess and provide a recent account of the basic mechanisms involved in strain acquisition of plasmid-mediated *bla*$_{NDM}$. In this case, multiple species of *Enterobacteriaceae* became carbapenem resistant by the acquisition of two plasmid types (IncA/C and IncN2) harbouring the *bla*$_{NDM}$ gene. This analysis also describes possible inter-genera and inter-patient spread of *bla*$_{NDM}$. That is, the possibility of intera-genera spread with the conjugation of an IncA/C plasmid between *E. coli* and *E. cloacae* within a single patient (Patient 4). The inter-patient transmission between two patients may also be possible as both patients (Patients 1 and 4) carried an *E. cloacae* ST171 harbouring an IncA/C plasmid with the same NGC. Inter-genera and inter-patient spread was also possible with an IncN2 plasmid. Highly similar NGCs were observed on the IncN2 plasmids, and an IncFII$_Y$ plasmid carried by *C. freundii* Pn67; indicating a potential for gene exchange between plasmid backbones (IncN2 and IncFII$_Y$). This chapter provided a recent account of the primary mechanisms (IncA/C and IncN2 plasmid types), and their underlying interplay involved for *bla*$_{NDM}$ to spread between different genera and patients within a single healthcare facility.

Chapter 3: Characterisation of NDM plasmids within *Enterobacteriaceae* in Australia

3.1 Chapter Introduction

The combined molecular/genetic approach presented in Chapter 2 and the account of *bla*$_{NDM}$ dissemination via IncA/C and IncN_2 presented, was restricted by small sample size but also restricted to a local geographical region and health-care facility. Characterisation of NDM-producing *Enterobacteriaceae* on a national level is sequentially required to understand if this approach is applicable to assess and provide a recent account of plasmid-mediated *bla*$_{NDM}$ transmission nation-wide. This chapter investigates 12 NDM producing *Enterobacteriaceae* referred to the reference laboratory at University of Queensland Centre of Clinical Research between 2012 and 2014. The patients who carried these strains, were admitted by Australian hospitals in different states, and had a mixture between those without travel history and with international travel history. One strain had its whole genome announced as the first uropathogenic *E. coli* carrying the NDM-5 variant in Australia (Appendix A (21)).

This investigation was summarised and presented in a manuscript format published in *Antimicrobial Agents and Chemotherapy*:

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The University of Queensland requires the presentation of the submitted or accepted article. The numbers of the figures, tables and references of the presented article have been amended to suit the structure of this thesis. The article remains in American English spelling enforced by journal guidelines.

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3.2 Chapter 3 – Original article: Genomic characteristics of NDM-producing *Enterobacteriaceae* in Australia and their *bla*$_{NDM}$ genetic contexts

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Abstract

bla$_{NDM}$ has been reported in different *Enterobacteriaceae* species and on numerous plasmid replicon types (Inc). Plasmid replicon typing in combination with genomic characteristics of the bacterial host (e.g. sequence typing) is used to infer the spread of antimicrobial resistant determinants between genetically unrelated bacterial hosts. The genetic context of *bla*$_{NDM}$ is heterogeneous. In this study, we genomically characterized twelve NDM-producing *Enterobacteriaceae* isolated in Australia between 2012 and 2014: *Escherichia coli* (*n*=6), *Klebsiella pneumoniae* (*n*=3), *Enterobacter cloacae* (*n*=2) and *Providencia rettgeri* (*n*=1). We describe their *bla*$_{NDM}$ genetic context within Tn*125* providing insights into the acquisition of *bla*$_{NDM}$ into *Enterobacteriaceae*. IncFII type (*n*=7) and IncX3 (*n*=4) plasmids were the most common plasmid type found. IncHI1B (*n*=1) plasmid was also identified. Five different *bla*$_{NDM}$ genetic contexts were identified, indicating five particular plasmids with specific *bla*$_{NDM}$ genetic contexts (NGCs), three of which were IncFII plasmids (FII A to C). Of note, the *bla*$_{NDM}$ genetic context of *P. rettgeri* was not conjugative to sodium azide-resistant *E. coli* J53Azi^{r}. Epidemiological links between our NDM producing *Enterobacteriaceae* were established by their acquisition of these five particular plasmids. The combination of different molecular and genetic characterization methods, allowed us to provide an insight into the spread of plasmids transmitting *bla*$_{NDM}$.

Introduction

Plasmids have received increased attention in the last decade due to their ability to acquire genes conferring antibiotic resistance and transfer them between different bacterial hosts. Plasmids of the *Enterobacteriaceae* family have been categorized into replicon (Inc) types via PCR-based replicon typing (PBRT) (58, 222, 223). PBRT in combination with other characteristics of the bacterial host, such as serotype, sequence type via Multi-locus sequence typing (MLST) and resistance gene profiles are used to demonstrate the spread of antimicrobial resistance determinants between genetically unrelated bacterial hosts (40).

New Delhi Metallo-β-lactamase gene or *bla*$_{NDM}$-harboring plasmids have been extensively characterized. Genetic variations in the accessory regions of plasmids have contributed to the complexity that underlies the spread of antimicrobial resistant determinants between bacterial hosts. Since its first report (2), *bla*$_{NDM}$ has been reported on various plasmid Inc types (20), including IncA/C (175, 201), IncF types (202), IncL/M (74), IncH (183), IncN types (125, 203, 224), IncX types (204) and IncHI1 types (171) of the *Enterobacteriaceae* family. However, it may be misleading to assume that each plasmid of the same replicon type is identical, especially amongst the IncA/C (94, 175, 177, 205) and IncFII plasmids (182, 202). For *Enterobacteriaceae* plasmids harboring *bla*$_{NDM}$, the variation in the genetic context of *bla*$_{NDM}$ generally involves two features. Firstly, *bla*$_{NDM}$ is frequently observed in the 10,099 bp transposon Tn*125* (with two flanking IS*Aba125* elements) within NDM producing species of the *Acinetobacter* genus (72, 73, 138, 200, 205). The *bla*$_{NDM}$ gene was hypothesized to originate in the *Acinetobacter* genus (70). In *Enterobacteriaceae*, the Tn*125* structure carrying *bla*$_{NDM}$ is frequently truncated (ΔTn*125*) at various lengths (205). Secondly, the sequence flanking the ΔTn*125* structure involves various mechanisms of gene acquisition including different IS*CR* elements (177), Class one integrons (94), flanking insertion

sequence (IS) elements (177), Miniature Inverted-Repeat Transposable Element (MITEs) (225) and singleton IS elements, present in close proximity (74, 201). The variations observed concerning these two features have contributed to the different *bla*$_{NDM}$ genetic contexts reported, even on the same plasmid type.

The *bla*$_{NDM}$ genetic context of NDM-producers from Singapore, Japan, Hong Kong, Thailand and Taiwan has been described (74, 125, 177, 203, 224). Additionally, NDM-producing *Enterobacteriaceae* have been reported in Australia (42, 226). Limited studies have described the plasmid features and genetic contexts of NDM-producers from Australia (22, 75, 227). Here, we analyze the *bla*$_{NDM}$ genetic contexts of 12 NDM-producing *Enterobacteriaceae* isolated from Australia between 2012 and 2014, for providing insights into their likely acquisition.

METHODS

Isolates

Twelve clinical or screening isolates producing NDM in this study were referred to University of Queensland Centre for Clinical Research for detailed molecular characterization from Queensland, Australian Capital Territory and Western Australia between 2012 and 2014. These isolates included *Escherichia coli* (*n*=6), *Klebsiella pneumoniae* (*n*=3), *Enterobacter cloacae* (*n*=2) and *Providencia rettgeri* (*n*=1) (Table 1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility and minimum inhibitory concentration (MIC) characterization was performed by E-test® (bioMerieux Marcy l'Etoile, France). Antimicrobial agents tested were: ceftazidime, cefotaxime, ceftriaxone, ceftazidime, cefepime, aztreonam, amikacin,

doripenem, ertapenem, meropenem, imipenem and tetracycline. Susceptibility results were interpreted according to 2015 EUCAST clinical breakpoint guidelines (228).

Plasmid experiments

Plasmid transfer experiment by conjugation and transformation was performed on all NDM-producers using previously described technique (23). Sodium azide-resistant *E. coli* J53 and *E. coli* Top10 were used as the recipients for conjugation and transformation experiments, respectively. The transconjugants and transformants acquiring bla_{NDM}-harbouring plasmids were examined phenotypically and confirmed by PCR for bla_{NDM}. PBRT was used to identify the plasmid Inc type carrying bla_{NDM} as previously described (58, 222, 223).

Whole genome sequencing

Paired-end libraries of whole genomic DNA of all 12 isolates were prepared and sequenced by Illumina HiSeq2000 (Illumina, San Diego, USA). All sequences were *de novo* assembled using CLC Genomic Workbench v7.5 (CLC Bio, Aarhus, Denmark). Re-annotated sequences from the Genbank database were used as a reference for manual annotation, which included pNDM-BJ01 (Genbank accession no. JQ001791) (73). CLC Genomic Workbench was further used to BLAST (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*), analyze and manually annotate the bla_{NDM-1} genetic context according to the aforementioned reference sequences. IS element identification within each context was achieved via IS finder (https://www-is.biotoul.fr/). Contigs containing bla_{NDM} from each isolate were named as follows: pCR7-EC-NDM-1 (*E. coli* CR7), pCR15-EC-NDM-4 (*E. coli* CR15), pCR16-ECL-NDM-1 (*E. cloacae* CR16), pCR37-ECL-NDM-7 (*E. cloacae* CR37), pCR38-KP-NDM-1 (*K. pneumoniae* CR38), pCR53-EC-NDM-4 (*E. coli* CR53), pCR58-PR-NDM-1 (*P. rettgeri* CR58), pCR63-KP-NDM-1 (*K. pneumoniae* CR63), pWA1-EC-NDM-4 (*E. coli* WA1),

pWA2-KP-NDM-7 (*K. pneumoniae* WA2), and pACT1-EC-NDM-1 (*E. coli* ACT1). pCR694-EC-NDM-5 (*E. coli* CR694) had previously been submitted to Genbank database (Genbank accession no. KP178355) (22). Contigs of the entire genome were submitted to the Center of Genomic Epidemiology (http://www.genomicepidemiology.org/) to identify the plasmid replicons, resistance genes of each clinical isolate as well as their ST via available MLST scheme. Specifically to databases, Plasmid finder 1.2 (210), Resfinder 2.1 (209) and MLST 1.7 (211) were used, respectively.

Nucleotide sequence accession number.

Contigs containing *bla*$_{NDM}$ from each isolate where annotated and deposited into the Genbank database with the following accession number: pCR7-EC-NDM-1: KP826713, pCR15-EC-NDM-4: KP826709, pCR16-ECL-NDM-1: KP826704, pCR37-ECL-NDM-7: KP826705, pCR38-KP-NDM-1: KP826710, pCR53-EC-NDM-4: KP826711, pCR58-PR-NDM-1: KP826706, pCR63-KP-NDM-1: KP826712, pWA1-EC-NDM-4: KP826707, pWA2-KP-NDM-7: KP826708 and pACT1-EC-NDM-1: KP826702.

RESULTS AND DISCUSSION

In comparison to other geographical regions such as the UK, China and the Indian subcontinent (16, 200, 229-231), there are relatively few reports of NDM producing *Enterobacteriaceae* from Australia. In the majority of the cases preceding 2014, patients had a travel history to high incidence countries (Table 13). Investigations of plasmid-mediated *bla*$_{NDM}$ involving the description of carbapenem resistant species within Australia have rarely included genetic context characterization. By utilizing genetic context characterization in our study, we provide insights into the acquisition of *bla*$_{NDM}$, through five groups of plasmid each carrying a specific NGC type.

Table 13. Specimens, sequence type, resistance determinants and plasmid types of *Enterobacteriaceae* strains which acquired plasmids harboring *bla*$_{NDM}$

Plasmid Group Number	*bla*$_{NDM}$ associated			Bacterial and host characterstics				Plasmid types determined via Plasmidfinder (Inc)											Antimicrobial resistance gene determinants via ResFinder: Beta-lactamases (*bla*)						Aminoglycoside & quinolone resistance determinants										Other resistance gene determinants							
	NGC type	NDM variant (*bla*)	Plasmid type (Inc)[1]	Strains & References	Bacterial Species	Specimen and patient gender & age[3]	ST[4]	F types: FII	FIA	FIB	FII Y	FII K	FIB K	FIA HI1	X types: X3	X4	H types	Other	CTX-M-15	CMY-42	OXA-1	TEM-1B	SHV-28	Other	*rmtB*	*rmtC*	*armA*	*aac(6')Ib-cr*[5]	*qnrB1*	*aad* variants	*aac(3)* variants	*aph(3') variants*	*strA* & *strB*	*oqxA* & *oqxB*	*sul1*	*sul2*	*catB3*	*dfr* variants	*tet* variants	*mph* variants	*msr(E)*	Remaining
1	FII-A	NDM-4	FII	WA1[2]	*E. coli*	R M (29)	167																							*aadA2 aadA5*	*aac-IIa*							*dfrA17 dfrA12*	*tet(A)*	*mph(A)*		
		NDM-1	FII	ACT1[2]	*E. coli*	U F (23)	410											I1 Q1												*aadA2*	*aac-IIa*							*dfrA14 dfrA12*		*mph(A)*		
		NDM-4	FII	CR53[2]	*E. coli*	B F (28)	4450											I1												*aadA5*									*tet(B)*	*mph(A)*		*erm(B)*
		NDM-1	FII	CR7 (232)	*E. coli*	U M (61)	410											A/C_2 Y I1						OXA-10						*aadA1 aadA2*	*aac-IIa*											
2	FII-B	NDM-1	FII_Y	CR16 (232)	*E. cloacae*	U M (52)	265										HI2A HI2							ACT-16 SHV-12						*aadA2*	*aac-IIa -IId*							*dfrA12*		*mph(E)*		
		NDM-1	FII_Y	CR63[2]	*K. pneumoniae*	U M (23)	45											Q1						SHV-1								*aph-Ia*						*dfrA14*				*fosA*

3	FII-C	NDM-1	FII_Y	CR38 (232)	*K. pneumoniae*	U M (30)	15											L/M						OXA-48							*aac-IIa*							*dfrA30*	*tet(A)*			
4	X3-A	NDM-4	X3	CR15 (232)	*E. coli*	S M (85)	101											R I1																				*dfrB4*		*mph(A)*		*catA1 qepA*
		NDM-7	X3	CR37 (232)	*E. cloacae*	B F (64)	127																	ACT-16							*aac-IIa*							*dfrA8*				
		NDM-7	X3	WA2[2]	*K. pneumoniae*	R F (30)	15											R												*aadA1*	*aac-IIa*	*aph-Ia*							*tet(A)*	*mph(A)*		
		NDM-5	X3	CR694 (233)	*E. coli*	U F (55)	147											I1																				*dfrA17*		*mph(A)*		*erm(B)*
5	HI1B-A	NDM-1	HI1B	CR58[2]	*P. rettgeri*	U M (51)	N/A										HI1B															*aph-Ia aph-Via*						*dfrA12*		*mph(A) mph(E)*		

[1] Isolates from this study; [2] Strains were firstly described in this study; [3] Abbreviations of the patient genders and specimens: M = male, F = female, R = rectal swab, U = urine, B = blood, S = swab; [4] ST: Sequence Type, determined by available MLST schemes, N/A = not available; [5] Responsible for aminoglycoside and quinolone resistance.

Phenotypic characterization of the NDM-producing *Enterobacteriaceae*

All isolates were non-susceptible to all tested carbapenems with MICs to meropenem, ertapenem, imipenem and doripenem of >32 μg/mL. All isolates were resistant to the 3rd and 4th generations of cephalosporins with MICs of >32 μg/mL to cefotaxime and ceftriaxone, and >256 μg/mL to ceftazidime and cefepime. Interestingly, MICs to aztreonam were generally >256 μg/mL, except in NDM-5-producing *E. coli* with MIC of 24 μg/mL. Variability of the MICs to amikacin was observed and correlated with the presence or absence of 16S rRNA methylase. The MICs to amikacin of NDM-producing *Enterobacteriaceae* possessing 16S rRNA methylase genes were >256 μg/mL. In contrast, isolates without 16S rRNA methylase genes had amikacin MICs between 1 and 2 μg/mL.

Genotypic characterization of the NDM-producing *Enterobacteriaceae*

In silico analysis of the molecular characteristics of the isolates, STs, antibiotic resistance determinant genes, plasmid replicons and bla_{NDM} genetic context were tabulated in Table 1. The ST of CR58 as there was no available MLST scheme for *P. rettgeri*. Common antimicrobial resistance determinants identified amongst these isolates included the following: four bla_{NDM} variants were described here, i.e. bla_{NDM-1} in 6 strains, bla_{NDM-4} in 3 strains, bla_{NDM-5} in 1 strain (21) and bla_{NDM-7} in 2 strains (Table 13). Each clinical isolate carried $bla_{CTX-M-15}$ except CR53, CR58 and CR694, and at least one aminoglycoside resistance genes including 16S rRNA methylase genes, *rmtB, rmtC, aac(6')Ib-cr* or *armA*. CR38 also co-harbored the carbapenemase gene, bla_{OXA-48}. There was no correlation between the bla_{NDM} variants and the replicon types. Among NDM-producers with FII plasmids, two variants of bla_{NDM-1} and $_{-4}$ were identified. Four variants, $bla_{NDM-1, -4, -5}$ and $_{-7}$ were identified on replicon type X3 bla_{NDM}-harbouring plasmids. Comparisons of plasmid replicon types and

their *bla*$_{NDM}$ genetic contexts enabled us to identify links between genetically unrelated bacterial species, regardless of their STs and resistance determinant profile.

Characterization of plasmids harboring *bla*$_{NDM}$

Six *bla*$_{NDM}$-harbouring-plasmids which underwent plasmid transfer experiment by transformation were successfully transferred into *E. coli* TOP10. These *bla*$_{NDM}$ plasmids of CR15, CR16, CR37, CR694, WA1 and WA2 were transferred successfully. Multiple attempts to transfer *bla*$_{NDM}$-harbouring plasmids by transformation to the rest of NDM-producing *Enterobacteriaceae* were not successful. In conjugation experiment, of the 12 NDM-producing *Enterobacteriaceae*, 10 *bla*$_{NDM}$-harbouring plasmids were transferred. Of note, the conjugation experiment of *K. pneumoniae* CR38 resulted in the transfer of *bla*$_{OXA-48}$-harboring plasmid into *E. coli* J53, but not *bla*$_{NDM}$-harboring plasmid. The *bla*$_{NDM}$ of *P. rettgeri* CR58 was not transferred by conjugation and transformation. This may indicate the potential location of *bla*$_{NDM}$ on a non-conjugative plasmid or potential chromosomal location of *bla*$_{NDM}$. The replicons of plasmids harboring *bla*$_{NDM}$ extracted from transformed *E. coli* TOP10 and *E. coli* J53 transconjugants acquiring *bla*$_{NDM}$-harbouring plasmids were listed on Table 13.

Utilizing the WGS data, *bla*$_{NDM}$ genetic context characterization of each strain identified a truncated Tn*125* (ΔTn*125*) structure carrying *bla*$_{NDM}$. pNDM-BJ01 was used as the reference sequence (234). The left hand IS*Aba125* of ΔTn*125* was truncated and the ΔTn*125* sequence ends in various lengths downstream of *bla*$_{NDM}$ (Fig. 5). The sizes of the ΔTn*125* ranged from 1,769 bp to 8,046 bp. Characterizing the flanking regions of each ΔTn*125* structure identified two recurrent genetic contexts repeated in two clinical isolates and three distinct genetic contexts each found in a separate clinical isolate. Five different types or groups of *bla*$_{NDM}$

genetic contexts (NGC) were determined. Each isolate was categorized into one of five different *bla*$_{NDM}$-harbouring plasmid type groups, according to the NGC the plasmid carried. There are three types of NGC within FII type plasmids (FII-A to C). The other two types were types X3-A, and HI1B-A (Fig. 5). The strains, NDM plasmid type and the NGC type of each group are described as follows.

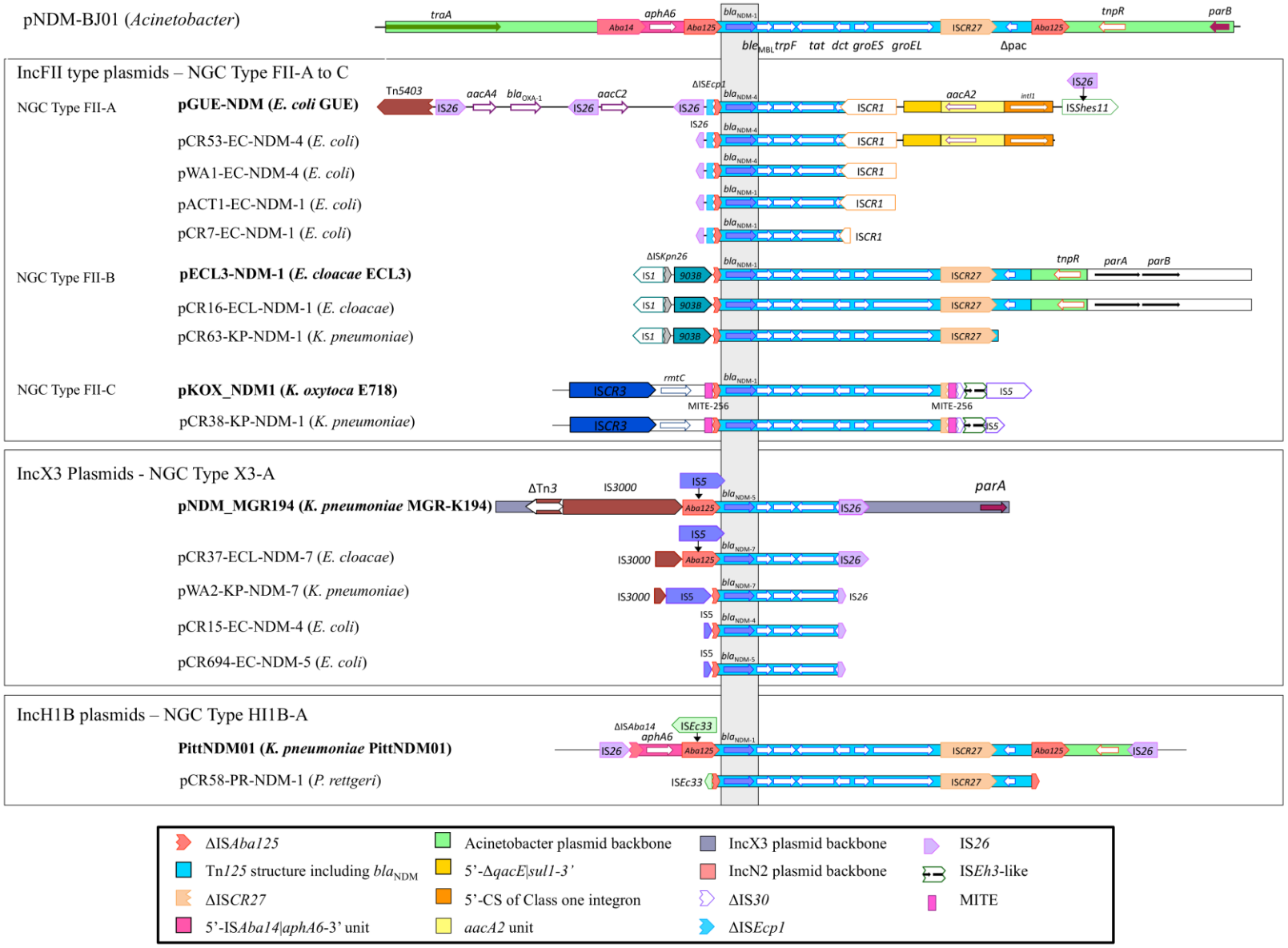

Figure 5. Schematic representation of all *bla*$_{NDM}$ genetic context (NGC) within this study and reference sequence pNDM-BJ01 (Genbank Accession no. JQ001791)

bla$_{NDM}$ genetic contexts and their Genbank Accession number, for IncFII plasmids with NGC type FII-A include pCR53-EC-NDM-4 (KP826711), pWA1-EC-NDM-4 (KP826707), pACT1-EC-NDM-1 (KP826702), pCR7-EC-NDM-1 (KP826713); with NGC type FII-B include pCR16-ECL-NDM-1 (KP826704), pCR63-KP-NDM-1 (KP826712); and with NGC type FII-C pCR38-KP-NDM-1 (KP826710), IncX3 plasmids with NGC type X3-A include pCR37-ECL-NDM-7 (KP826705); pWA2-KP-NDM-7 (KP826708), pCR15-EC-NDM-4 (KP826709), IncHI1B plasmids with NGC type HI1B-A include pCR58-PR-NDM-1 (KP826706). Δ – truncated gene. Insertion Sequence (IS) elements are represented as a block arrow. Black down arrows indicate insertion of IS element. Bolded names are reference sequence from Genbank for each genetic context, pGUE-NDM (Genbank Accession no. NC_019089), pECL3-NDM-1 (Genbank Accession no. KC887917), pKOX-NDM-1 (Genbank Accession no. JQ314407), pNDM_MGR194 (Genbank Accession no. KF220657), pTR3 (Genbank Accession no. JQ349086) and PittNDM01 (Genbank Accession no. CP006799). Gray box highlights *bla*$_{NDM}$ in each genetic context.

Strains harboring FII-type plasmids

IncFII type was the most frequent Inc type identified in 7 of the 12 plasmids harboring *bla*$_{NDM}$ (Table 13). Three of the five plasmid groups were NGC FII-type. The strains harboring IncFII types were categorized into three different FII groups according to the three different FII *bla*$_{NDM}$ genetic contexts (NGC type FII-A to C). The strains, their FII plasmid sub-type and corresponding NGC type were identified and described as follows: Those that carried a FII sub–type plasmid harboring NGC type FII-A were four *E. coli* strains CR7, CR53, WA1 and ACT1. The NGC type FII-A had a 3,328 bp ΔTn*125*, flanked upstream by a truncated IS*Ecp1* and the right end of IS*26* and downstream by an IS*CR1* element and is very similar the *bla*$_{NDM}$ genetic context on IncFII pGUE-NDM (Genbank Accession no. NC_019089) of an *E. coli* ST131 isolated in France (182) and IncFII pMC-NDM (Genbank Accession no HG003695) of an *E. coli* ST410 isolated in Poland (235).

The second plasmid group had strains with a FII$_Y$ plasmid with NGC type FII-B. Two strains *E. cloacae* ST265 strain CR16 and *K. pneumoniae* ST45 strain CR63 were included in this group. NGC type FII-B involved a 7,977 bp ΔTn*125* structure with IS*903B* and IS*1*-family element upstream and was very similar to pECL3-NDM-1 (Direct submission Genbank accession no. KC887917) of *E. cloacae* ECL3 isolated in Australia.

The third group carried a FII$_Y$ plasmid with NGC type FII-C with *K. pneumoniae* ST15 strain CR38. NGC FII-C type is a 5,947 bp ΔTn*125* structure, flanked by two identical 256 bp miniature inverted-repeat transposable elements (MITEs). The aminoglycoside resistance determinant *rmtC* was also identified upstream of the ΔTn*125* structure of NGC type FII-C and is very similar to IncFII pKOX_NDM1 (Genbank Accession no. JQ314407) of *Klebsiella oxytoca* isolated from Taiwan (225).

Strains harboring IncX3 and IncHI1B

Similar to the analysis of IncFII *bla*$_{NDM}$ plasmids, *bla*$_{NDM}$ genetic context groups were established with the remaining clinical strains which harbored IncX3 and IncHI1B plasmids. The fourth plasmid group composed of strains carrying an IncX3 plasmid with the NGC type X3-A. The four clinical isolates in this group are *E. cloacae* ST127 strain CR37, *E. coli* ST101 strain CR15, *E. coli* ST648 strain CR694 and *K. pneumoniae* ST15 strain WA2. NGC type X3-A involved a 3,167 bp ΔTn*125*, flanked by an IS*5* upstream and an IS*26* downstream and was similar to the IncX3 plasmid pNDM-MGR194 (Accession no. KF220657) of *K. pneumoniae* isolated from India (236).

The last remaining plasmid group carried an IncHI1B plasmid with NGC type HI1B containing *P. rettgeri* strain CR58. NGC type HI1B-A consists of a 8,046 bp ΔTn*125* sequence with a partial sequence of IS*Ec33* upstream and identical to IncHI1B pPKPN1 of *K. pneumoniae* strain PittNDM01 ST14 (Genbank accession no. CP006799) isolated in Pittsburgh, US (237).

Although this study had a small sample size, it could indicate further potential wide dissemination of *bla*$_{NDM}$ by IncFII type and IncX3 plasmids in Australia. Geographical specific dissemination of *bla*$_{NDM}$ by a certain group of plasmid types has been previously reported with five identical IncN2 plasmids harboring *bla*$_{NDM}$ was described in four *K. pneumoniae* and one *E. coli* ST131 in two countries in South East Asia (125, 224). The characterization presented here would indeed help to track the horizontal movement of *bla*$_{NDM}$ among the *Enterobacteriaceae* family.

While the mechanism and factors of how these genetic contexts originated and the nature (including the source and environment) in which these strains have acquired these plasmids remains unknown, the five groups of plasmids carrying these specific *bla*$_{NDM}$ genetic contexts within different bacterial species highlights the role of plasmids to transmit mechanisms of carbapenem resistance. Genetic context characterization was a method allowing us to refine an epidemiological links between strains, established by the acquisition of plasmids carrying a specific *bla*$_{NDM}$ genetic context. We suggest genetic context characterization as an additional tool in combination with other molecular methods such plasmids replicon typing and sequencing typing via MLST when conducting epidemiology studies involving NDM producers of the *Enterobacteriaceae* family and possibly other similar promiscuous antimicrobial resistant determinants.

In conclusion, we have identified five particular plasmids with specific *bla*$_{NDM}$ genetic contexts conferring carbapenem resistance in the *Enterobacteriaceae* family through genetic context characterization in combination with other epidemiological molecular methods. IncFII-type and IncX3 plasmids were the most frequent plasmids carrying *bla*$_{NDM}$ within our study, with three different *bla*$_{NDM}$ genetic contexts identified amongst the IncFII-type plasmids. By combining different molecular and genetic characterization methods, epidemiological investigations can provide a better insight into the spread of plasmids transmitting *bla*$_{NDM}$ and possibility of other similar promiscuous resistance mechanisms to genetically unrelated bacterial species.

Acknowledgement

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resistant *Enterobacteriaceae* and *Acinetobacter* spp. in Queensland). The funding for the whole genome sequencing was partially supported by Australian Infectious Diseases Research Centre. Part of this study was presented as a poster presentation at the Gram-negative Superbugs Gold Coast in 2013.

3.3 Chapter summaries and conclusions

The combined molecular/genetic approach was able to assess the dissemination of plasmid-mediated *bla*$_{NDM}$ with strains isolated nation-wide. For these strains to confer carbapenem resistance, this approach clarified the acquisition of plasmids carrying specific NGCs, primarily IncFII type and IncX3. The spread of plasmid-mediated *bla*$_{NDM}$ is able to be assessed both at local and national-wide geographical ranges through this approach. The characterisation of different NGCs and specific plasmid types spreading the *bla*$_{NDM}$ gene, IncA/C and IncN2 plasmid types in Pakistan (Chapter 2) and the IncFII type and IncX3 plasmid in Australia, implies a complicated combination of mechanisms involved for insertion of the *bla*$_{NDM}$ gene into a plasmid (inferred by the *bla*$_{NDM}$ different NGCs), plasmid type transfer between genera, and strain transfer between patients. Additionally, there appears to be gene exchange between plasmid types, with observations of highly similar NGCs on different plasmid types IncN2 and IncFII$_Y$ (Fig. 4; Chapter 2 (23)) and on the same plasmid type with IncFII$_Y$ carrying three different NGCs, *C. freundii* Pn67 harboured the first NGC (Fig. 4; (23)), *E. cloacae* CR16 and *K. pneumoniae* CR63 harboured a second NGC (Fig. 5; (22)) and *K. pneumoniae* CR38 harboured a third different NGC. The mechanisms identified in this chapter (observation of plasmid types harbouring additional NGCs) compliment and add to the mechanisms of acquisition and spread identified in Chapter 2. Collectively these suggest a complex combination of mechanisms involved for antimicrobial resistance acquisition and spread.

Chapter 4: Complete NDM plasmid backbone analysis and mechanisms for *bla*$_{NDM}$ capture

4.1 Chapter Introduction

Thus far, mechanisms of inter-genera transfer of the *bla*$_{NDM}$ gene and possible strain transfer between patients has been investigated. In the previous chapters, we observed various combinations of different NGCs and plasmid types, including the same NGC across different plasmid types and the same Inc type carrying three different NGCs. Plasmids can be considered important vehicles for capture and accumulation of AMR genes, in addition to their role in AMR gene spread. The initial gene insertion onto a plasmid allows the possibility for a gene to mobilise and spread to multiple genera, patients and health-care facilities, similar to situations described in Chapters 2 and 3. Numerous transposable elements facilitate gene insertion, to give rise to the NGCs previously described, and to facilitate the gene transfer between plasmid types. These are important concepts to acknowledge if insights into acquisition and spread of *bla*$_{NDM}$ genes is to be achieved.

This chapter constructed and analysed the full sequence of four plasmids harbouring a *bla*$_{NDM}$ gene (two IncA/C$_2$ and two IncFII$_Y$). The mechanisms leading to insertion of *bla*$_{NDM}$ into different parts of highly similar plasmid backbones were investigated. Comparison of these four plasmids with complete plasmids described in the literature was included to define and characterise mechanisms known at the time of analysis.

This investigation was summarised and presented in a manuscript format which has been accepted with minor review at *Antimicrobial Agents and Chemotherapy*. The numbers of the figures, tables and references of the presented article have been amended to suit the structure of this thesis.

4.2 Chapter 4 – Original article: Mechanisms involved in acquisition of *bla*$_{NDM}$ genes by IncA/C$_2$ and IncFII$_Y$ plasmids

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Running Head: *bla*$_{NDM}$ acquistion by IncA/C$_2$ and IncFII$_Y$ plasmids

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ABSTRACT

bla$_{NDM}$ genes confer carbapenem resistance and have been identified on transferable plasmids belonging to different incompatibility (Inc) groups. Here we present the complete sequences of four plasmids carrying a *bla*$_{NDM}$ gene, pKP1-NDM-1, pEC2-NDM-3, pECL3-NDM-1 and pEC4-NDM-6, from four clinical samples originating from four different patients. Different plasmids carry segments that align to different parts of the *bla*$_{NDM}$ region found on *Acinetobacter* plasmids. pKP1-NDM-1 and pEC2-NDM-3, from *Klebsiella pneumoniae* and *Escherichia coli*, respectively, were identified as type 1 IncA/C_2 plasmids with almost identical backbones. Different regions carrying *bla*$_{NDM}$ are inserted in different locations in the antibiotic resistance island known as ARI-A and IS*CR1* may have been involved in acquisition of *bla*$_{NDM-3}$ by pEC2-NDM-3. pECL3-NDM-1 and pEC4-NDM-6, from *Enterobacter cloaceae* and *E. coli*, respectively, have similar IncFII$_Y$ backbones but different regions carrying *bla*$_{NDM}$ are found in different locations. Tn*3*-derived Inverted-repeat Transposable Elements (TIME) appear to have been involved in acquisition of *bla*$_{NDM-6}$ by pEC4-NDM-6 and the *rmtC* 16S rRNA methylase gene by IncFII$_Y$ plasmids. Characterisation of these plasmids further demonstrates that even very closely related plasmids may have acquired *bla*$_{NDM}$ genes by different mechanisms. These findings also illustrate the complex relationships between antimicrobial resistance genes, transposable elements and plasmids and provide insights into the possible routes for transmission of *bla*$_{NDM}$ genes amongst species of the *Enterobacteriaceae* family.

In Gram-negative bacteria, especially the *Enterobacteriaceae* family, β-lactamases are a major mechanism of resistance against β-lactams. A group of β-lactamases known as carbapenemases are becoming the most troublesome for antimicrobial therapy, as they can confer resistance to the carbapenems, the major last-line antimicrobial. The NDM carbapenemase was first reported in 2009, produced by a *Klebsiella pneumoniae* isolated from a Swedish patient recently returned from India (2). There are currently 16 known NDM variants (http://www.lahey.org/Studies/other.asp#table1, accessed February 2016) and *bla*$_{NDM}$ genes have now been reported in strains sourced from every inhabitable continent and in multiple species of *Enterobacteriaceae,* including *Escherichia coli*, *K. pneumoniae* and *Enterobacter cloacae* (20).

Plasmids are important vehicles for the capture, accumulation and spread of various antimicrobial resistance determinants. Several different types of plasmids associated with the *Enterobacteriaceae* family have been reported to harbor *bla*$_{NDM}$ genes including IncA/C, IncFII sub-types, IncH types, IncL/M, IncN (20, 40, 205), and IncX (238). Some of these plasmids co-harbour additional antimicrobial resistance genes, including the 16S rRNA methylase genes *armA* and *rmtC* (conferring high-level aminoglycoside resistance), quinolone resistance genes (*qnrB1* and *qnrS1*) and/or other β-lactamase genes (such as *bla*$_{CMY-2}$ and variants, *bla*$_{CTX-M-15}$) (239).

The original source of *bla*$_{NDM}$ is not known, but *Acinetobacter* spp. may have acted as an intermediate between this organism and the *Enterobacteriacae* family (70, 240, 241). In *Acinetobacter* spp. *bla*$_{NDM}$ genes have often been observed within the composite transposon Tn*125*, a 10,099 bp region bounded by two copies of IS*Aba125* (70, 72, 184, 242). The *bla*$_{NDM}$ gene starts 93 bp downstream of the right-hand end (IR$_R$) of IS*Aba125*, which provides the -35 region of a promoter (103, 185), and is followed by several genes, including *ble*$_{MBL}$ (bleomycin resistance), *trpF* (involved in tryptophan biosynthesis), and the mobile

element IS*CR27*. In several *Acintetobacter* spp. plasmids (e.g. pNDM-BJ01; GenBank accession no. JQ001791 (73)), IS*Aba14* and the *aphA6* gene (amikacin resistance) are present upstream of the IS*Aba125* adjacent to *bla*$_{\text{NDM-1}}$ (Fig. 6A). In plasmids from the *Enterobacteriaceae*, *bla*$_{\text{NDM}}$ genes are generally found in the same immediate genetic context, with at least a fragment of IS*Aba125* containing the -35 region present upstream, within different length fragments matching *Acinetobacter* plasmids and associated with different mobile elements (22, 23, 74, 205, 225, 243, 244).

We previously reported locally-identified *K. pneumoniae* (15) and *E. cloacae* (189) clinical isolates carrying *bla*$_{\text{NDM-1}}$, *E. coli* carrying *bla*$_{\text{NDM-3}}$ (G283A, Asp95Asn) (189) and *E. coli* carrying *bla*$_{\text{NDM-6}}$ (C698T, Ala233Val) (101). The *bla*$_{\text{NDM}}$ gene could be transferred from all four isolates by transformation and/or conjugation, indicating a plasmid location in each case, but replicon types were not determined (15, 101, 189). In this study, we present the complete sequences of these four plasmids and a comparison of the genetic contexts of *bla*$_{\text{NDM}}$ with those in closely related plasmids.

MATERIALS AND METHODS

Bacterial isolates and plasmids. *K. pneumoniae* KP1 (15) and *E. cloacae* ECL3 carrying *bla*$_{\text{NDM-1}}$ (189) were isolated in Australia, as was *E. coli* EC2 carrying *bla*$_{\text{NDM-3}}$ (189) while *E. coli* EC4 carrying *bla*$_{\text{NDM-6}}$ (previously designated ARL10/167 (101)) was isolated in New Zealand. All isolates were from patients recently returned from India. Transconjugants in sodium-azide resistant *E. coli* J53Azi^{r} were available and/or were obtained as previously described (23).

DNA preparation and sequencing. Genomic DNA (gDNA) was extracted from KP1, ECL3 and EC4 using the UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, California, USA) and sequenced by Illumina HiSeq 2000 technology (Illumina, San Diego, USA). Illumina sequences were *de novo* assembled using CLC genomic workbench

v8.0 (CLC Bio, Aarhus, Denmark). Initial annotation of contigs was performed using RAST (245). IS finder (https://www-is.biotoul.fr/) and the Repository of Antibiotic-resistance Cassettes (RAC; http://rac.aihi.mq.edu.au/rac/) were used to identify IS and integron components, respectively. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi*) searches were used to identify related plasmids carrying bla$_{NDM}$ to guide* PCR-based gap closure and Sanger sequencing (Macrogen, Korea) to assemble contigs into complete plasmids.

gDNA from EC2 was sheared using a g-TUBE (Covaris®) into fragment sizes targeted at 20 Kb. Following purification, SMRTbell template libraries were prepared using the commercial Template Preparation kit (Pacific Biosciences Inc., Menlo Park, California, USA) and sequenced on a Pacific Biosciences (PacBio) RSII instrument (University of Queensland Centre for Clinical Genomics; UQCCG) using the P6 polymerase and C4 sequencing chemistry. The raw PacBio sequence data were assembled *de novo* using the hierarchical genome assembly process (HGAP version 2) and Quiver (246) from the SMRT Analysis software suite (version 2.3.0; http://www.pacb.com/devnet/) with default parameters and a seed read cut-off of 17,000 bp. Following assembly, contigs were examined for overlapping 5' and 3' ends (a characteristic feature of the HGAP assembly process) using Contiguity (https://peerj.com/preprints/1037/) and were manually trimmed to generate circular contigs. Raw sequence reads were then mapped back onto the assembled circular plasmid contig (BLASR (247) and Quiver) to validate the assembly and resolve any remaining errors.

RAST, IS finder, RAC, CLC genomic workbench v8.0, Geneious R9 (Biomatters Ltd, New Zealand, including Mauve (248)) and BLAST *were used for manual annotation, alignment,* SNP detection, and other analysis and comparisons of complete plasmid sequences.

Nucleotide sequence accession numbers. Existing GenBank entries for partial sequences of all four plasmids were updated to include the complete sequences, as follows: pKP1-NDM-1,

KF992018; pEC2-NDM-3, KC999035; pECL3-NDM-1, KC887917; pEC4-NDM-6, KC887916.

RESULTS AND DISCUSSION

General features of plasmids carrying *bla*$_{NDM}$. Isolates KP1, EC2, ECL3, EC4 each transferred a plasmid carrying *bla*$_{NDM}$ to *E. coli* J53Azi^{r} by conjugation. Plasmids carrying *bla*$_{NDM}$ assembled from whole genome sequences (at least 50 fold coverage) were designated pKP1-NDM-1, pEC2-NDM-3, pECL3-NDM-1 and pEC4-NDM-6, respectively. pKP1-NDM-1 (137,552 bp) and pEC2-NDM-3 (160,989 bp) were identified as type 1 IncA/C$_2$ (Table 14). The backbones of pKP1-NDM-1 and pEC2-NDM-3 are very closely related to those of several other type 1 IncA/C$_2$ plasmids (Appendix B; Table S1) and include characteristic IncA/C$_2$ core regions, such as the conjugative transfer (*tra*) region and *parA-parB* required for plasmid partitioning (249). They have identical replication regions, with a *repA* gene and fourteen 19 bp direct repeat sequences (iterons), which are binding sites for the RepA protein (249). pKP1-NDM-1 and pEC2-NDM-3 both have the same IS*Ecp1* transposition unit carrying a *bla*$_{CMY-2}$ variant, in this case *bla*$_{CMY-6}$, inserted in the same location as in many other type 1 IncA/C$_2$ plasmids, between *traA* and *traC*, flanked by 5 bp direct repeats (DR). Neither carries Tn*6170* present in some type 1 IncA/C$_2$ plasmids (250).

pECL3-NDM-1 (99,435 bp) and pEC4-NDM-6 (110,786 bp) are both IncFII$_Y$ type plasmids (Table 14) carrying two replicons, classified as Y4 (*repA*) and FIB36 (*repB*) by the replicon sequence typing (RST) scheme (223). The backbones of both plasmids are closely related to those of other IncFII$_Y$ plasmids carrying *bla*$_{NDM}$ (Table S2), which have not been well studied but include a conjugation (*tra)* region and stability (*psi, parAB*) and maintenance (*ccdAB*) genes (225, 243).

Table 14. General features of IncA/C$_2$ and IncFII$_Y$ plasmids studied here and close relatives

Plasmid[a]	NDM	Size (bp)	Species	ST[b]	Country[c]	Year[b]	Source[b]	GenBank accession no.	Reference
A/C$_2$									
pKP1-NDM-1	1	137,552	*K. pneumoniae*	147	India/Australia	2010	Human	KF992018.2	This study
pEC2-NDM-3	3	160,989	*E. coli*	443	IndiaAustralia	2010	Human	KC999035.2	This study
pNDM-EcoGN568	1	166,750	*E. coli*	1289	India/Canada	na	Human	KJ802404.1	(251)
pNDM-PstGN576	1	147,886	*P. stuartii*	N/A	India/Canada	na	Human	KJ802405.1	(251)
pNDM102337	1	165,974	*E. coli*	na	Canada	na	na	NC_019045.2	-
pNDM10505	1	166,744	*E. coli*	na	Canada	na	na	NC_019069.1	-
pNDM10469	1	137,813	*K. pneumoniae*	na	Canada	na	na	NC_019158.1	-
pNDM-KN	1	162,746	*K. pneumoniae*	14	Kenya	2009	Human	JN157804.1	(80, 175)
pNDM-US	1	140,825	*K. pneumoniae*	11	India/USA	2010	Human	CP006661.1	(201)
pNDM-US-2	1	140,821	*K. pneumoniae*[d]	na	-[d]	-	-	KJ588779.1	-
FII$_Y$									
pECL3-NDM-1	1	99,435	*E. cloacae*	265	India/Australia	2011	Human	KC887917.2	This study
pEC4-NDM-6	6	110,786	*E. coli*	101	India/New Zealand	2010	Human	KC887916.2	This study
pKOX_NDM1	1	110,781	*K. oxytoca*	na	China/Taiwan	2010	Human	NC_021501.1	(225)
pNDM1_EC14653	1	109,353	*E. cloacae*	177	China	2014	Human	KP868647.1	(252)
pNDM-EclGN574	1	110,786	*E. cloacae*	na	India/Canada	na	Human	KJ812998.1	(251)
pP10164-NDM	1	99,276	*L. adecarboxylata*	N/A	China	2012	Human	KP900016.1	(243)
pRJF866	1	110,786	*K. pneumoniae*	11	China	2011	Human	NC_025184.1	(253)
pYDC644	1	106,844	*K. pneumoniae*	na	Iran	na	na	KR351290.1	-

[a] Plasmids with names in bold typeface were sequenced in this study.
[b] na, not available.
[c] Travel history is given if available e.g. India/Australia indicates isolation in Australia from a patient recently returned from India.
[d] GenBank accession no. KJ588779 implies that pNDM-US-2 was extracted in China from the same strain, ATCC BAA-2146, as pNDM-US.

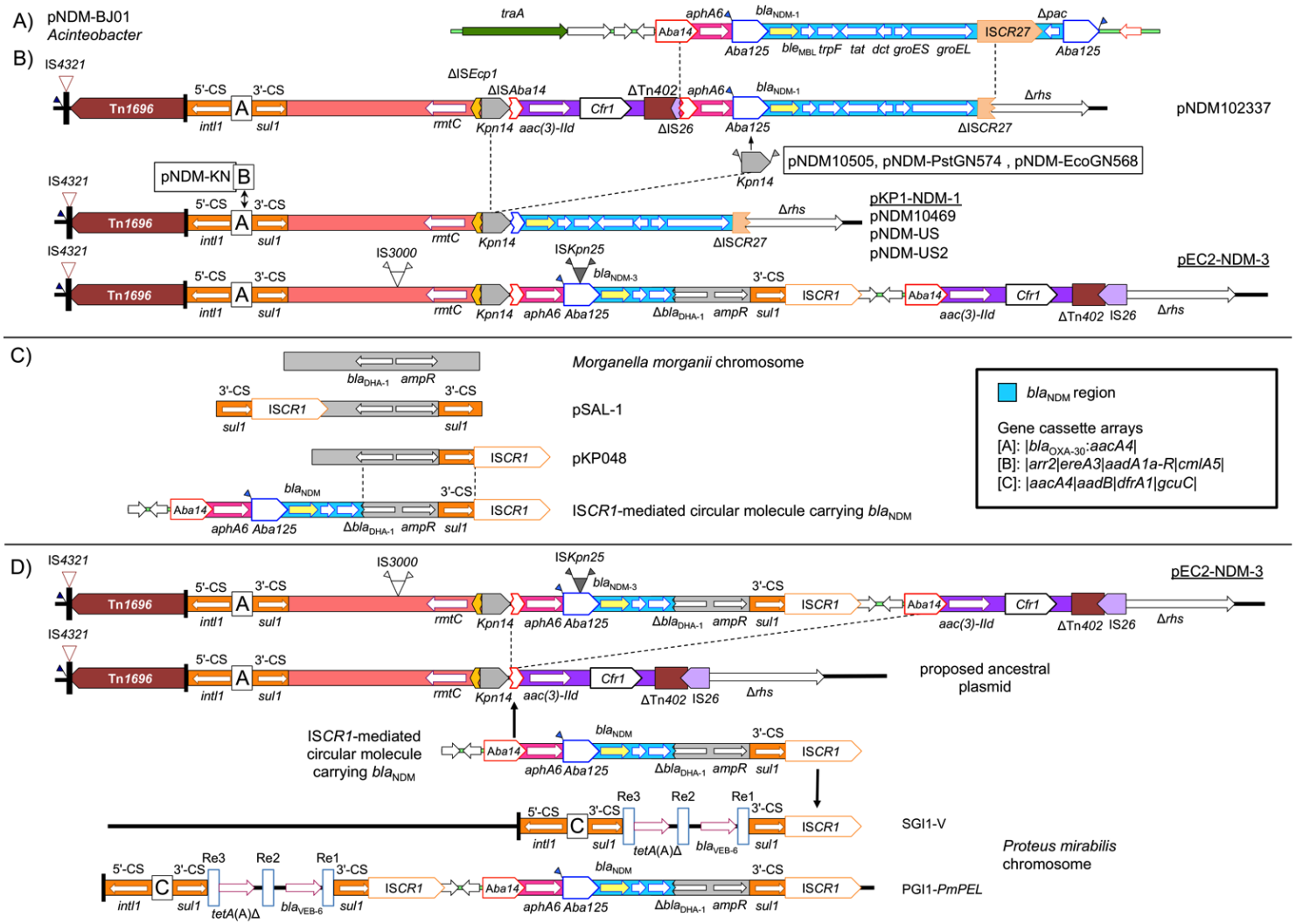

Figure 6. ARI-A of type 1 IncA/C$_2$ plasmids carrying *bla*$_{NDM}$, and potential routes for *bla*$_{NDM}$ insertion

IS are shown as block arrows labelled with their name or number. DR are represented by flags of the same colour. Triangles indicate the insertion sites of IS elements flanked by DR. Vertical black bars represent the transposon IR of ARI-A and IRi of class 1 In/Tn. Horizontal green and black lines represent *Acinetobacter* and IncA/C$_2$ plasmid backbones, respectively. Vertical dotted lines indicate boundaries of closely related sequences. Vertical black arrows

and diagonal dotted lines indicate possible deletion and insertion events. (A) Tn*125* in *Acinetobacter lwoffii* plasmid pNDM-BJ01. (B) ARI-A of type 1 IncA/C_2 plasmids closely related to pKP1-NDM-1 and pEC2-NDM-3. (C) Possible derivation of the circular molecule inserted in pEC2-NDM-3. (D) Insertion of circular molecular carrying *bla*$_{NDM}$ into pEC2-NDM-3 and a *P. mirabilis* genomic island. The sequences used to draw these diagrams are from GenBank accession numbers listed in Table 14, plus: pNDM-BJ01, NC_019268; pSAL-1, AJ237702; pKP048, NC_014312 ; SGI1-V, HQ888851; PGI1-*Pm*PEL, KF856624

Both IncA/C_2 plasmids carry *bla*$_{NDM}$ in antibiotic resistance island ARI-A. In both IncA/C_2 plasmids sequenced here the *bla*$_{NDM}$ gene is located within an antibiotic resistance island known as ARI-A that is common to type 1 IncA/C_2 plasmids. The prototype ARI-A, found in pRMH760, is a complex hybrid transposon structure bounded by 38 bp inverted repeats (IR) interrupted by IS*4321* and is inserted upstream of the *rhs* gene (unknown function) flanked by 5 bp DR (TTGTA) (250, 254). ARI-A in pRMH760 carries a class 1 In/Tn with IS*26*-*aphA1*-IS*26* interrupting the Tn*402 tni* region. Various other resistance genes and islands carrying *bla*$_{NDM}$ appear to be derived from this structure (205). In pNDM102337 (Table 14; Fig. 6B) nucleotides 1-1,616 of the 3′-CS of the class 1 integron are followed by a 3,562 bp region carrying a type III restriction-modification system and the *rmtC* 16S rRNA methylase gene, then 224 bp of the IR_R end of IS*Ecp1*. IS*Ecp1* is truncated by IS*Kpn14*, which is followed by a 198 bp fragment of IS*Aba14*, then a region found on a number of different plasmids that contains the *aac(3)-IId* (gentamicin resistance) gene and IS*Cfr1* (60). The adjacent fragment of the Tn*402 tni* region has the same boundary with IS*26* as in ARI-A of pRMH760, but only 217 bp of IS*26* is present. This is followed by an 8,913 bp region matching *Acinetobacter* plasmids such as pNDM-BJ01, which includes 662 bp of the right end of IS*Aba14*, *aphA6*, one copy of IS*Aba125*, *bla*$_{NDM-1}$ and a fragment of IS*CR27*. pNDM10505, pNDM-PstGN576 and pNDM-EcoGN568 (Table 14) have a variant of the pNDM102337 ARI-A with a second IS*Kpn14* inserted 130 bp upstream of the left end of IS*Aba125* (Fig. 1B). IS*Kpn14*-mediated deletion may have been responsible for creating the ARI-A variant present in the other closely-related type 1 IncA/C_2 plasmids pNDM-US, pNDM-US-2, pNDM-KN and pNDM10469, which lack the *aac(3)-IId* region (Table 14; Fig. 6B) (205). pKP1-NDM-1 sequenced here has an almost identical ARI-A except that only 89 bp of IS*Aba125* are present adjacent to IS*Kpn14* upstream of *bla*$_{NDM}$. This difference was confirmed, has been seen in other partial sequences (23, 255) and IS*Kpn14*, which is ~89%

identical to IS*1*, known to cause adjacent deletions (60). All of these type 1 IncA/C_2 plasmids except pNDM-KN have the same cassette array, consisting of single fused cassette comprised of the first 87 bp of the *bla*$_{OXA-30}$ cassette and position 17 to the end of the *aacA4* cassette, overlapping by a single A (61). The mechanism(s) responsible for insertion of the *bla*$_{NDM}$ region into the proposed pNDM102337-like progenitor plasmid are unclear, but it is possible that they involved IS*CR27* and/or IS*26* and subsequent deletion(s).

The backbone of pEC2-NDM-3 is almost identical to the pNDM102337-like plasmids described above (Appendix B; Table S1) but IS*Ec23* is inserted 222 bp upstream of ARI-A, flanked by 8 bp DR characteristic of this element. ARI-A of pEC2-NDM-3 includes the same *rmtC* region as described above except that IS*3000* is inserted upstream of *rmtC*, flanked by characteristic 5 bp DR. The region containing *bla*$_{NDM}$, however, is different from the one in the other IncA/C_2 plasmids and is inserted between IS*Kpn14* and the *aac(3)-IId*/IS*Cfr1*/*tni*$_{402}$ region. The region matching pNDM-BJ01 encompasses 198 bp of IS*Aba14*, *aphA6*, one copy of IS*Aba125*, *bla*$_{NDM}$, *ble*$_{MBL}$ and *trpF*. IS*Kpn25*, carrying a restriction-modification system, is inserted in IS*Aba125* upstream of the -35 promoter region, flanked by characteristic 8 bp DR (Fig. 6B). The *bla*$_{NDM}$ gene has the single nucleotide change giving *bla*$_{NDM-3}$ rather than *bla*$_{NDM-1}$ and *trpF* is followed by a truncated *bla*$_{DHA}$ gene and the associated *ampR* gene, nucleotides 180-1,313 of the 3'-CS and IS*CR1*. This region is separated from a complete IS*Aba14* by 934 bp matching the region upstream of IS*Aba14* in pNDM-BJ01. ARI-A in pEC2-NDM-3 ends with the *aac(3)-IId*/IS*Cfr1*/*tni*$_{402}$ region but a complete copy of IS*26* truncates the *rhs* gene in the IncA/C_2 backbone. The only other known location of the *bla*$_{NDM-3}$ variant is on an IncFII plasmid (256) associated with IS*CR1* but not with the truncated *bla*$_{DHA}$/*ampR* region present in pEC2-NDM-3.

This context in pEC2-NDM-3 suggests insertion of *bla*$_{NDM}$ from a circular molecule mediated by IS*CR1*. IS*CR1* is proposed to transpose by a rolling-circle mechanism, similar to the

related IS*91* family elements (64), in which replication proceeds from the *ori*IS end, located downstream of *rcr* (rolling circle replicase gene), towards the *ter*IS upstream and can continue into and capture an adjacent region. IS*CR1* has generally been found associated with class 1 integrons, after position 1,313 of the 3′-CS, suggesting integration of circular molecules by recombination in either the 3′-CS or an existing IS*CR1* (64). IS*CR1* has previously been suggested to be associated with movement of bla_{NDM} (257) and was recently shown to be responsible for mobilising a region containing bla_{NDM} and part of the 3′-CS, but without the bla_{DHA}Δ/*ampR* region, between plasmids (244).

IS*CR1* appears to have been responsible for capturing the bla_{DHA}Δ/*ampR* region from the *Morganella morganii* chromosome and inserting it into a class 1 integron (258) (Fig. 6C). Generation of a circular molecule by recombination between the two flanking 3′-CS and reintegration at IS*CR1* could create the arrangement seen in e.g. pKP048 (GenBank accession no. NC_014312), with IS*CR1* downstream of the bla_{DHA}Δ/*ampR* region and the 3'-CS, and the usual 3'-CS/IS*CR1* boundary (Fig. 6C). IS*CR1* may then have mobilised this 3'-CS segment and the bla_{DHA}Δ/*ampR* region and inserted them downstream of bla_{NDM}, before picking up the bla_{NDM} region as part of a circular molecule (Fig. 6C).

The complete IS*Aba14* in pEC2-NDM-3 has the same boundary with the *aac(3)-IId* region as the IS*Aba14* fragment in pNDM102337, suggesting that homologous recombination between the complete and partial copies of IS*Aba14* could have been responsible for the insertion of this circular molecule into pEC2-NDM-3 (Fig. 6D). The same circular molecule carrying bla_{NDM} also appears to have inserted in a *P. mirabilis* genomic island to create PGI-*Pm*PEL (257) but in this case by recombination in IS*CR1* (Fig. 6D), supporting the proposed mechanism of IS*CR1*-mediated capture of bla_{NDM}. Regions containing the same IS*CR1*, 3'-CS, bla_{DHA}Δ/*ampR* region, but adjacent to shorter fragments of the bla_{NDM} region, are found in the original bla_{NDM-1} plasmid pKpANDM-1 (FN396876.1) (2) and in plasmids of other Inc

types (205) (e.g. the IncL/M plasmid pNDM-HK) (74)), suggesting capture of shorter *bla*$_{NDM}$ regions and/or subsequent deletions.

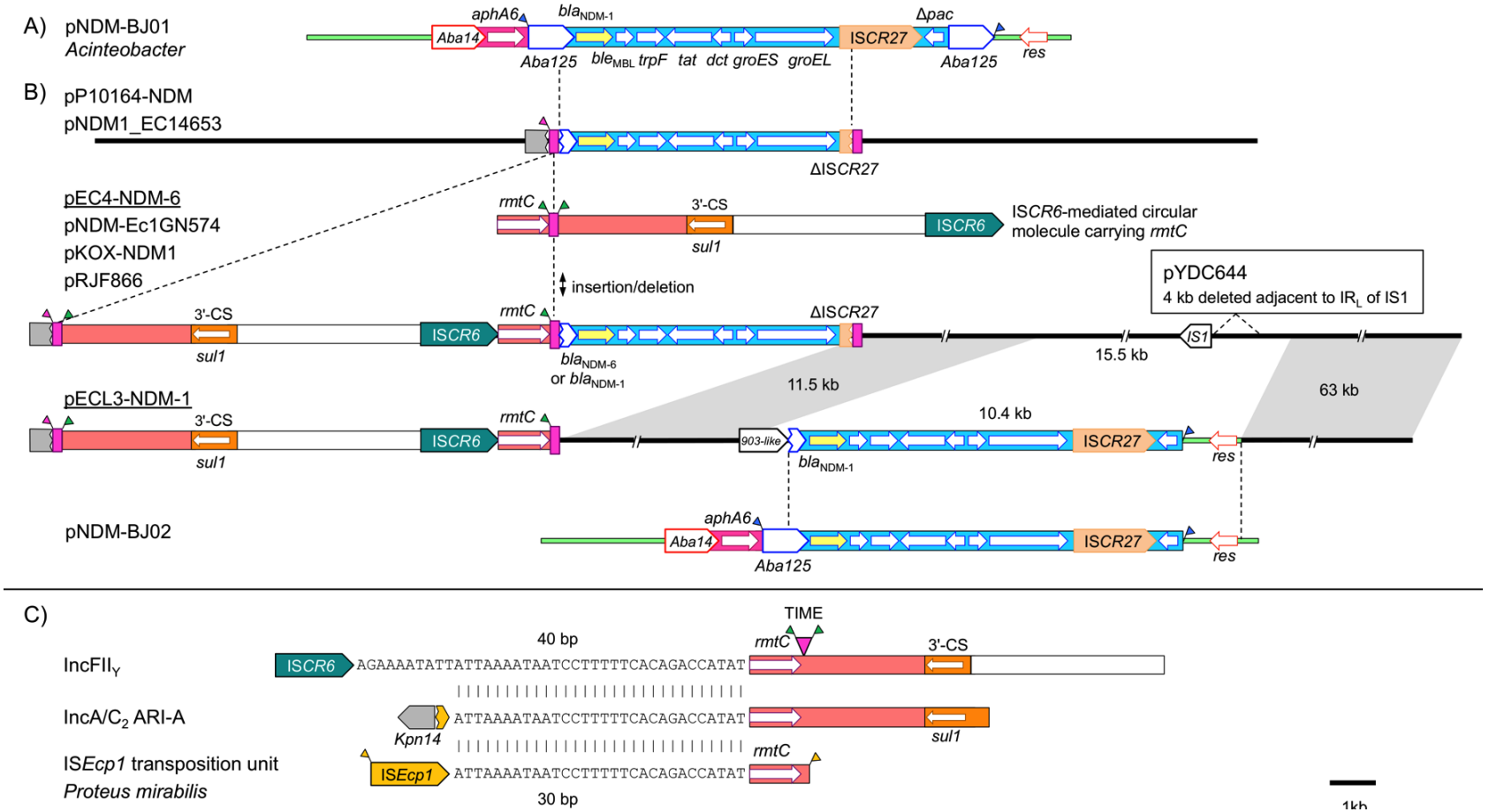

Figure 7. Contexts of *bla*$_{NDM}$ on IncFII$_Y$ plasmids

Features are generally shown as in Fig. 6. Solid black lines represent IncFII$_Y$ plasmid backbone. Grey shaded areas indicate matching plasmid backbone regions, with their sizes given. (A) Tn*125* in *Acinetobacter lwoffii* plasmid pNDM-BJ01. (B) Comparison of IncFII$_Y$ plasmids. (C) Comparison of *rmtC* contexts in IncFII$_Y$, plasmids, IncA/C$_2$ ARI-A and *Proteus mirabilis*. The sequence shown is the spacer between *rmtC* and the associated transposable element. The pink triangle indicates the insertion site of the TIME. The sequences used to draw these diagrams are from GenBank accession numbers listed in Table 14 plus: pNDM-BJ01, NC_019268; pNDM-BJ02, NC_019281.1; IS*Ecp1* transposition unit in *P. mirabilis*, AB194779.

IncFII$_Y$ plasmids carry *bla*$_{NDM}$ flanked by TIMEs. Several very closely related IncFII$_Y$ plasmids carrying a *bla*$_{NDM}$ gene have now been identified (Table 14). They all have almost identical backbones with the same insertions of multiple IS elements in the same places and minor sequence differences (Appendix B; Table S2), although pYDC644 alone appears to have a deletion adjacent to one copy of IS*1* (Fig. 7B). In all of these plasmids *bla*$_{NDM}$ lies within a 5,945 bp region matching Tn*125* that includes 101 bp of IS*Aba125* and a fragment of IS*CR27*. This region is flanked by two copies of a 256 bp Tn*3*-derived Inverted-repeat Transposable Element (TIME), each bounded by inversely oriented 38 bp IRs (259). These TIMEs, previously described as MITEs (Miniature Inverted-repeat Transposable Element), may have been responsible for capturing the *bla*$_{NDM}$ region from a pNDM-BJ01-like plasmid (225, 243, 252). pEC4-NDM-6 is very closely related to these plasmids (Appendix B; Table S2) but has the single nucleotide change giving *bla*$_{NDM-6}$ (260) rather than *bla*$_{NDM-1}$, suggesting mutation in this context.

In most of these IncFII$_Y$ plasmids carrying *bla*$_{NDM}$, an 11,029 bp region that includes the *rmtC* gene and an IS*CR6*-like element separates the TIME upstream of *bla*$_{NDM-1}$ from a third copy of this TIME. TIME create 5-6 bp DR on transposition like the Tn*3* transposons from which they appear to be derived (259). In these plasmids the 5 bp sequences adjacent to the "inside" of each TIME flanking the *rmtC* region are identical (TATAA). This configuration could be explained by insertion of a circular molecule, consisting of this region plus one copy of the TIME (flanked by these 5 bp sequences as DR), into the TIME upstream of *bla*$_{NDM-1}$ (Fig. 7B). Gain and loss of the *rmtC* region in this way is supported by the sequences of the IncFII$_Y$ plasmids pP10164-NDM and pNDM-EC14653 (Table 14; Fig. 7B), which lack the *rmtC* region. Removing the TIME and one DR of this circular molecule also gives a region that matches the *rmtC* region found in ARI-A of the IncA/C$_2$ plasmids, also supporting this hypothesis. *rmtC* was originally identified in a transposition unit flanked by DR with a

complete copy of IS*Ecp1* that also matches part of this structure (Fig. 7C) (261). The same 30 bp separate *rmtC* from this complete IS*Ecp1* and the IS*Ecp1* fragment in IncA/C_2 plasmids, while an additional 10 bp are present between IS*CR6* and *rmtC*. While these contexts are clearly related, without additional examples of *rmtC* contexts it is difficult to say exactly how each arose.

pECL3-NDM-1 carries the same *rmtC* region as the other IncFII$_Y$ plasmids but its backbone has a number of confirmed nucleotide differences (Appendix B; Table S2) and a different region carrying *bla*$_{NDM-1}$ has been inserted in a different location (Fig. 7B). This region matches pNDM-BJ02, which lacks the copy of IS*Aba125* downstream of *bla*$_{NDM}$ (205), rather than pNDM-BJ01, and also includes 1,369 bp of pNDM-BJ02 backbone. An IS*903*-like element truncates IS*Aba125*, leaving 83 bp upstream of *bla*$_{NDM-1.}$ This 10,411 bp region replaces a 15,560 bp region present in the other IncFII$_Y$ plasmids and it is possible that the IS*903*-like element was involved in the insertion of this *bla*$_{NDM}$ region into pECL3-NDM-1.

Conclusions. In summary, the analysis presented in this study supplements and complements the catalogue of previously characterised IncA/C_2 and IncFII$_Y$ plasmids carrying *bla*$_{NDM}$. All four plasmids studied here carried segments that align to different parts of the *bla*$_{NDM}$ regions found on *Acinetobacter* plasmids. Different mechanisms appear to have been responsible for independently transferring different segments of Tn*125* into ARI-A in the same IncA/C_2 plasmid backbone (giving pKP1-NDM-1-type plasmids or pEC2-NDM-3). Other less closely-related type 1 IncA/C_2 plasmids e.g. pNDM-1_Dok01 from *E. coli* (177) and pMR0211 from *Providencia stuartii* (94), also carry segments matching different parts of Tn*125* and adjacent *Acinetobacter* plasmid backbone in ARI-A, illustrating further variation in the ways in which *bla*$_{NDM}$ genes appear to have been acquired by similar plasmids. Different mechanisms also appear to have transferred different segments matching *bla*$_{NDM}$

contexts found in *A. baumannii* to slightly different IncFII$_Y$ backbones (giving pEC4-NDM-1-type plasmids or pECL3-NDM-1).

At least theoretically, transfer of *bla*$_{NDM}$ segments between *Acinetobacter* and *Enterobacteriaceae* plasmids could have occurred in either the *Acinetobacter* or in one or more of the *Enterobacteriaceae*. Transfer of *Acinetobacter* plasmids carrying *bla*$_{NDM}$ into *E. coli* J53 by conjugation has been demonstrated (103, 242) and recently a pNDM-BJ01-like plasmid (p3SP-NDM) was found in an *Enterobacter aerogenes* isolate (234). IncA/C plasmids have also been reported in a few *A. baumannii* clinical isolates on the basis of PCR (262). While independent transfer from *Acinetobacter* plasmids to different types of plasmids found in the *Enterobacteriaceae* is possible, it may be more likely that *bla*$_{NDM}$ regions have subsequently moved between these plasmids in the *Enterobacteriaceae.*

The four plasmids in this study were carried by clinical isolates from Australia or New Zealand, from different patients recently returning from India. We have also recently reported partial sequences of *bla*$_{NDM}$ contexts matching pKP1-NDM-1 (with the 89 bp IS*Aba125* fragment) in IncA/C plasmids harboured by isolates from a hospital in Pakistan (23) and those matching pECL3-NDM-1 or pEC4-NDM-6 in IncFII$_Y$ plasmids in isolates from multiple Australian healthcare facilities (22). The other related IncA/C$_2$ and IncFII$_Y$ plasmids harbouring *bla*$_{NDM}$ genes discussed here were also isolated in several different countries (Table 14). This distribution illustrates the geographical spread of *bla*$_{NDM}$ genes on these particular plasmid types.

There appears to be an underlying complex network of interactions between *bla*$_{NDM}$, different mobile elements and different plasmids, but without access to the sequences of additional intermediate and progenitor plasmids it is difficult to fully understand the contributions that different factors have to the transmission of *bla*$_{NDM}$ genes. The different mechanisms observed here to capture relevant genes onto different plasmid types emphasizes the

capability of *Enterobacteriaceae* to adapt to their environment, especially where antimicrobial pressure is present.

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4.3 Chapter summaries and conclusions

This chapter described the different transposition mechanisms involved for insertion of the *bla*$_{NDM}$ gene into plasmids of the same Inc type. Mechanisms identified were IS*26* and/or IS*CR27* and IS*CR1* (rolling-circle mechanism (64)) for *bla*$_{NDM}$ insertion into ARI-A on type 1 IncA/C$_2$ plasmids and TIMEs and IS*903*-like elements into IncFII$_Y$ plasmids. These mechanisms involved the insertion of *bla*$_{NDM}$ into plasmids described in Chapter 2 (IncA/C of strains Pn2, Pn66 Pn68; (23)) and Chapter 3 (IncFII$_Y$ of strains CR16, CR38 and CR63; (22)) which had highly similar NGCs. Identical regions carrying an AMR gene were also observed on two different plasmid types, *rmtC* region in IncA/C$_2$ and IncFII$_Y$ (Similar to Chapter 2; NGC on IncN2 and IncFII$_Y$). Suggesting gene exchange between plasmid types or alternatively acquisition by both plasmid types from a common source. Also noted and discussed were the different fragment lengths matching the Tn*125* region found on *Acinetobacter* spp. plasmids, which may be remnants of *bla*$_{NDM}$ transfer from *Acinetobacter* plasmids and those in the *Enterobacteriaceae*. Theoretically, the transfer of these *bla*$_{NDM}$ segments between *Acinetobacter* and *Enterobacteriaceae* plasmids could occur either in *Acinetobacter* or in one or more *Enterobacteriaceae*. Discussion of these observations has provided an insight into mechanisms of *bla*$_{NDM}$ acquisition and spread suggesting a complex relationship between antimicrobial resistance genes, transposable elements and plasmids.

Chapter 5: The prevalence of plasmids within the *Enterobacteriaceae* family

5.1 Chapter Introduction

Previous chapters have described numerous NGCs, the transposable element mechanisms involved for insertion of *bla*$_{NDM}$ genes into backbones of different plasmid types, and the gene exchange of regions between plasmid types, as well as between *Enterobacteriaceae* and *Acinetobacter* plasmids. This collectively suggested complex interactions between the *bla*$_{NDM}$ gene, transposable elements and plasmids types. The *bla*$_{NDM}$ gene and other additional AMR genes have the potential to be acquired by and transferred between each plasmid type. The interaction between plasmid types and different genera regardless of AMR gene carriage is investigated to complement these discussions, and place the plasmid types carrying *bla*$_{NDM}$ into a greater context of AMR acquisition and spread. This interaction has implications that could affect the direct transfer of *bla*$_{NDM}$ between different genera.

This chapter describes the plasmid content (via *in silico* plasmid typing) of 1683 *Enterobacteriaceae* isolated from 53 countries across seven continents, involving both clinical and environmental samples, starting from early 1980s until 2013. PCA of the shared plasmid sub-types harboured across different species was also conducted to describe specific routes of gene transmission between species and genera, facilitated by plasmids.

This investigation was summarised and presented in a manuscript format submitted to *Microbial Genomics.* The numbers of the figures, tables and references of the article have been amended to suit the structure of this thesis.

5.2 Chapter 5 – Original article: Surveying plasmid content of the *Enterobacteriaceae* family: a retrospective study

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Abstract

Treatment options for infections caused by members of the bacterial family the *Enterobacteriaceae* are increasingly becoming more limited due to the increase in antimicrobial resistance. This increase in resistance is partly facilitated by the rapid horizontal spread of genes carried on mobile genetic elements that confer resistance to therapeutic antimicrobials. Bacteria can acquire AMR genes from other bacteria via plasmids which are self-replicating extrachromosomal DNA molecules. Plasmids can be typed into different Incompatibility (Inc) groups and some of which have been associated with the dissemination of different AMR genes. However, the general distribution of AMR-specific plasmids remains largely undefined. Here, we assay the plasmid content of a broad, avaliable collection of *Enterobacteriaceae* isolate genomes, ranging from *Klebsiella* spp. to *Shigella* spp., by assessing the plasmid content of 1683 isolates using an *in silico* plasmid replicon typing method. Twelve major plasmid types were identified and allocated into three levels of occurence: Common, IncF (~65% of strains); Intermediate, IncHI, IncI, IncR (8-10%); and Rare, IncA/C, B/O/K/Z, L/M N, O, P, Q, X, and Y (0.5-3%). PCA identified specific plasmid sub-types to represent possible routes of gene exchange between different species/genera, with species clustering into two primary groups according to their shared plasmid content. Our findings provide insights into the distribution of enterobacterial plasmid types and an underlying network of transmission between bacterial species and genera.

Introduction

Bacterial pathogens from the *Enterobacteriaceae* family cause a number of serious infections including pneumonia, urinary tract, intra-abdominal and blood stream infections, as well as asymptomatic colonisation (263, 264). Treatment options for these infections are diminishing with isolates frequently reported as resistant to major last-line antimicrobials, such as carbapenems and colistin (22, 23). The genes conferring resistance to major classes of antimicrobials are often carried on extrachromosomal DNA elements, known as plasmids, which can act to disseminate these genes amongst the broader bacterial population (2, 39, 40, 198). Plasmids are widespread amongst members of the *Enterobacteriaceae* and have significantly contributed to the global dissemination of antimicrobial resistance (AMR) genes, acting as a vehicle to capture, accumulate and transmit AMR genes between different strains, species and genera (1, 2, 20, 198). A recent example of this is the plasmid-mediated AMR gene, *mcr-1* (confers resistance to colistin), identified in *Escherichia coli* isolated from food and food-producing animals in China (198). The plasmid carrying *mcr-1* was shown to be transferable to and maintained by other Gram-negatives, *Klebsiella* spp. and *Pseudomonas* spp. This initial description was followed by reports of *mcr-1* found in Asia, the Netherlands, France, parts of Africa and South America. Since then other plasmids harbouring *mcr-1* in *Salmonella enterica* serovar Typhimurium have been reported (265-267).

In the 1970s, plasmids associated with the *Enterobacteriaceae* family were categorised into different incompatibility (Inc) groups, based on the finding that certain plasmid combinations are unable to stably propagate and coexist within the bacterial same cell, hence two plasmids were considered to be “incompatible”. Later, this phenomenon was clarified as the inability for two plasmids with the same replication system to be co-retained within the same cell line (40, 56). Incompatibility (Inc) groupings were translated into a molecular Inc typing scheme in 2005 known as PCR-based replicon typing (PBRT) (58). PBRT used multiplex primer sets

specific for 18 major plasmid types which allowed plasmids to be categorized into sequence groups belonging to known Inc families. This typing scheme was based on the premise that the backbone of each plasmid has regions responsible for core functions that are highly conserved within Inc groups such as replication (known as the replicon) and/or partitioning. Identification of these regions would therefore represent the backbone for a plasmid type. Regions containing additional, non-core functions such as multi-drug resistance regions and transposable elements inserted into the plasmid backbone can be considered as accessory regions. These regions can be transferred between plasmid Inc types as well as act as a target point for additional AMR gene insertion (60), highlighting the potential for each plasmid type to acquire AMR genes. Recently, as whole genome sequencing (WGS) data has become more readily available, investigations have used *in silico* BLAST more routinely to characterise the plasmid type using sequence identity matching against typed plasmids in public sequence databases (210).

The distribution of enterobacterial plasmid Inc groups has been briefly investigated during the development of the PlasmidFinder database, used for *in silico* Inc typing, which categorised 559 finished plasmid sequences from the NCBI database (210). Published plasmid types carrying AMR genes have also been reviewed via literature compilation (39, 40). The presented study aims to gain an initial estimation of the Inc group distribution within *Enterobacteriaceae* using, for the first time, a large set of WGS short-read sequence data. Here, we perform an investigation of 1683 isolates of different species within the *Enterobacteriaceae* using an opportunistic and non-selective approach of existing sequencing data from several global and regional studies (268-274). Three studies were designed to capture the global diversity of *Klebsiella pneumoniae* (271), the *Yersinia* genus (273), and Enterotoxigenic *E. coli* (ETEC) (274) were included, as well as five regional studies looking

at *S.* Typhimurium in Africa (272), *Shigella flexneri* outbreak in the U.K. (268), *Shigella sonnei* movement in Vietnam(270), *Klebsiella pneumoniae* associated septicemia in Nepal (269), and AMR pattern diversity amongst *K. pneumoniae* and *Enterobacter cloacae* in an U.K hospital. A survey of the plasmid content from this broad collective of samples, will provide a generalised snapshot of *Enterobacteriaceae* plasmid types and also insights into their transmission between different species and genera.

Methods

Isolates. Isolates included in this study involve various species of the *Enterobacteriaceae* collected for eight different studies (268-274). Each study lies within a 'global' or 'geographical/region specific' scope according to the list of countries covered by their strain collection (Appendix C; Table S3). Global studies include a 'Global *Klebsiella pneumoniae*' (271), '*Yersinia* spp. study' (273) and 'Enterotoxigenic *E. coli* (ETEC) study' (274), spanning a minimum of four continents. 'Global *K. pneumoniae*' investigated the diversity of animal and human *K. pneumoniae* isolates (n=247) from four continents. The '*Yersinia* spp. study' investigated the evolution of pathogenicity in pathogenic and non-pathogenic *Yersinia* spp. collected from environmental, animal and human sources (n=214) from countries across each continent. 'ETEC study' investigated the phylogeny and evolution of ETEC by analysis of 353 ETEC isolates collected across 20 different countries from Africa, Asia North, Central and South America.

Geographically narrow/regional studies where isolates were collected from less than 3 countries, included 'Nepal *K. pneumoniae*' (269), 'U.K. *K. pneumoniae Enterobacter cloacae*' (Ellington M., Unpublished), 'MSM *Shigella flexneri*' (268), 'Vietnam *Shigella sonnei*' (270) and 'Africa/UK *Salmonella* Tyhimurium' (272). 'Nepal *K. pneumoniae*' characterised a hospital *K. pneumoniae* outbreak in Nepal through the WGS of 87 isolates.

'U.K. *K. pneumoniae E. cloacae*' analysed 160 *K. pneumoniae* and 133 *E. cloacae* routinely collected clinical isolates to describe the genetic diversity causing human infection within the region. All isolates of 'Nepal *K. pneumoniae*' and 'U.K. *K. pneumoniae E. cloacae*' were included in our analysis. The 'MSM *Shigella flexneri*' study investigated 171 *S. flexneri* isolates to characterise factors driving the shigellosis U.K. outbreak in men who have sex with men (MSM). 'Vietnam *S. sonnei*' investigated the evolution of *S. sonnei* in Vietnam over a 15-year period. 223 *S. sonnei* isolates were included in our study. 'Africa/U.K. *Salmonella* Typhimurium' investigated the population structure of 215 *S.* Typhimurium in Africa with comparison to global *S.* Typhimurium populations, compared the population structure of 73 African *S.* Typhimurium strains with 22 U.K. *S.* Typhimurium strains.

The isolates from each study included in our analysis were composed of Illumina data sequenced at the WTSI and were previously published in the European Nucleotide Archive. Contigs from each isolate were obtained as previously described (268-274). A collective total of 1683 isolates passed QC based on total alignment length and number of contigs. Assemblies that were not of expected genome size for that species, indicative of multiple genomes (2x) were excluded as well as assemblies with more than 1000 contigs. Plasmid Inc types present in each strain were identified by comparing all contigs against the PlasmidFinder 1.3 database (accessed: April 14 2015) using the map_resistome script as described previously (275). >95% identity and >98% length match filter parameters were applied to reduce redundant positives and false positives.

Plasmid Inc sub-typing. The PlasmidFinder database is comprised of sequence probes targeting replicon loci specific. However, the plasmid incompatibility type and sub-type in which these probes belong was not available. To report the Inc sub-type of the plasmids

carried by isolates, each sequence probe of the PlasmidFinder database was revised with their correct plasmid Inc sub-type (Appendix C; Table S3). To achieve this, each sequence of the PlasmidFinder database was submissed to the an online database pMLST (http://pubmlst.org/) for plasmid Multi-Locus Sequence Typing (MLST) (213), and literature reviewed (223). Sequence probes which could not be sub-typed were noted as 'not defined'.

PCA plot analysis. Principal component analysis (PCA) was performed on samples to identify and visualize specific patterns of plasmid carriage. Plasmid sub-types were used for this analysis instead of the major plasmid families identified, because analysis of the major plasmid groups would only describe an ancestral linkage between species that existed prior to the divergence of plasmid sub-types. IBM® SPSS® Statistics 20.0 was used to perform to perform this analysis.

Results

General features of the combined enterobacterial data set. A total of 1683 whole genome sequences were gathered from three focused studies looking at global diversity of *K. pneumoniae*, the *Yersinia* genus, and ETEC, and two regional studies looking at *S.* Typhimurium in Africa and the movement of *S. sonnei* in within specific human populations in Vietnam (Appendix C; Table S3). Isolates from a studies characterising, the emergence and flux of multidrug resistant *Klebsiella* associated septicemia in Nepal, the genetic diversity of *K. pnuaemoniae* and *E. cloaceae* causing human infection within a single U.K. hospital, and the U.K. *S. flexneri* outbreak in men who have sex with men (MSM), were also included (see Chatper 5.2 Methods for more details; Appendix C; Table S3). All isolate genomes were screened for their plasmid content using *in silico* PBRT (see Chapter 5.2 Methods; Appendix C; Table S4). In total from all collections, our dataset comprised of *K.*

pneumoniae (n=494), *S. sonnei* (n=223), *Yersinia* spp. (n=214), *S. flexneri* (n=171), *E. coli* (n=353), *E. cloacae* (n=133) and *S.* Typhimurium (n=95). Isolates were collected from a range of sources including different animal hosts from the environment, human clinical samples, the community and the environment. Human clinical samples included blood, urine and stool samples while environmental samples were mainly isolated from water. Geographically isolates were originally collected from 53 countries. None of the seven studies used plasmid content as a selection criterion although the U.K. *K. pneumoniae E. cloacae* study aimed to understand the diversity of AMR patterns seen over time in this hospital.

To report the Inc type of the plasmids detected, each replicon sequence probe of the PlasmidFinder database was revised to provide an plasmid Inc type and sub-type (refer to Chapter 5.2 methods; Appendix C; Table S4). Using this revised PlasmidFinder database we were able to detect the presence of at least one plasmid in 75.1% (1267/1683) of all isolates included in this study. Our total collection of plasmid replicons identified by the PlasmidFinder database were identified to reside within 12 major Inc groups, included A/C, B/O/K/Z, F, HI, I, L/M, N, P, Q, R, X, Y. These Inc groups described 99.63% of all plasmids detected. Although a further 10 plasmid replicons were detected, these replicons have not been assigned to an Inc group, so were denoted ‘novel Inc groups’ (Appendix C; Table S2). Over a quarter of all isolates (26.6%; 448/1683) showed the presence of multiple Inc groups with only 406/1683 isolates lacking any known Inc-type. The distribution of the Inc types across all species in our collection is summarised in Table 15. The ‘general *Enterobacteriaceae* plasmid type distribution’ of the 12 plasmid types could be categorised into three main levels of occurence according to the percentage of isolates carrying the plasmid type: Common, the IncF plasmid group at 64.6%; Intermediate, IncHI, IncI and IncR

at ~9-11%; and Rare, the remaining plasmid types harboured by approximately 0.5-3% of isolates (Fig. 8).

Since these isolates were not sequenced for this study and to understand if the bacterium or the study was more important to the distribution of the Inc types, we performed a PCA analysis of all isolates and revealed that the major determinant for the carriage of specific plasmid types was the species of the isolate (Appendix C; Fig. S1), not the study itself, suggesting that study variables such as isolation technique did not confound our analysis and allows for comparison across studies.

Table 15. Number of detected plasmid types across bacterial species of this study

Species	No. isolates	Plasmid type (Inc)*												
		F	**HI**	**I**	**IR**	**A/C**	**B/O/K/Z**	**L/M**	**N**	**P**	**Q**	**X**	**Y**	**Novel**
Klebsiella pneumoniae	494	323	129	3	139	15	1	7	23	4	13	10	0	1
Enterobacter cloaceae	133	45	36	0	14	4	0	1	1	1	0	1	0	0
Shigella flexneri	171	169	0	0	0	0	0	0	0	0	0	0	0	0
Salmonella enterica serovar Typhimurium	95	89	6	4	0	0	0	1	0	1	28	0	0	0
Yersinia spp.	214	60	0	0	0	0	0	0	5	0	1	2	0	6
Shigella sonnei	223	91	0	85	0	1	0	0	0	0	0	0	0	0
Escherichia coli	353	310	8	56	4	0	15	0	4	30	8	19	9	3

* Plasmid Incompatibility (Inc) type determined by *in silico* plasmid typing using the PlasmidFinder database (see methods).

Three levels of occurence represent the general *Enterobacteriaceae* plasmid type distribution.

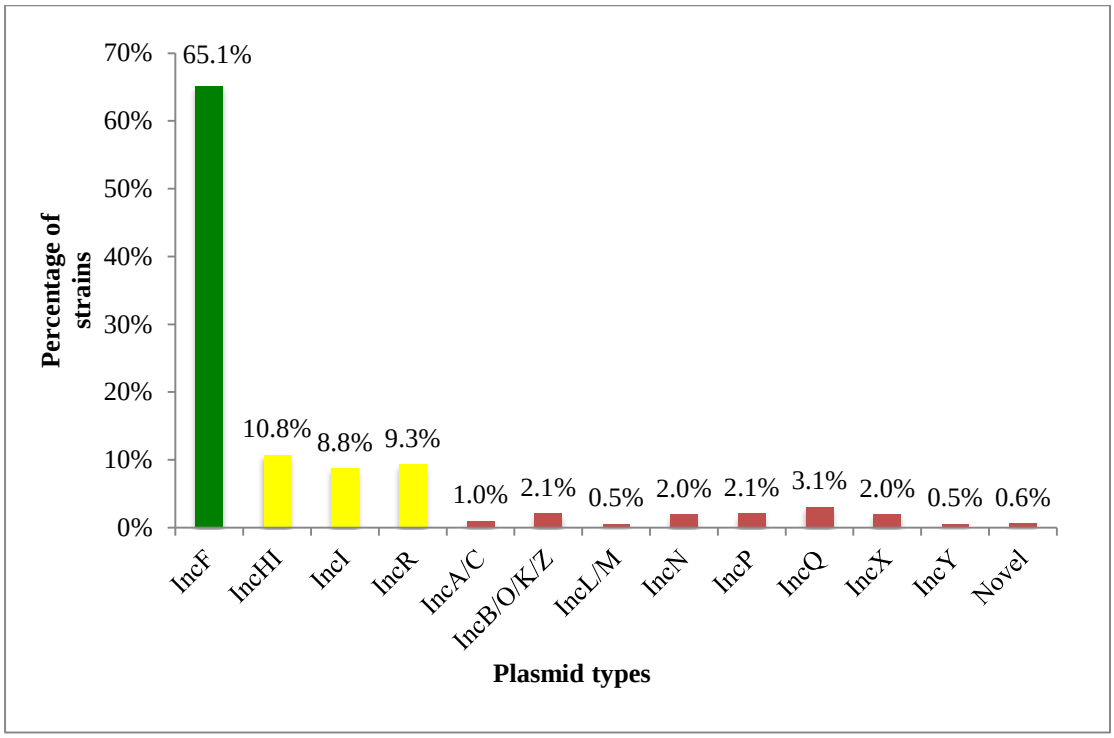

Figure 8. Distribution of the 12 major plasmid Inc groups and novel plasmids across 1683 isolates.

Distribution is represented as percentage of strains harbouring each plasmid Incompatibility (Inc) type. Common, intermediate and rare occurence plasmid types are represented by green, yellow and red respectively.

Common Inc types

The IncF group (FIA, FIB, FII) were the most frequently identified in our collection, harboured by 64.6% of all isolates tested (1087/1683) (Fig. 8). IncF plasmids can carry one to three replicons FIA, FIB and FII, and can be termed "multireplicon". IncFII plasmid (primarily carrying the FII replicon; varies in co-harbouring FIA and FIB replicons) was the most frequent type amongst IncF plasmids, harboured by over half of all isolates (54.9%; 925/1683). IncF plasmids in several instances have become restricted to different genera and maintained by most members of that genera. The plasmids of this type usually also carry genes essentially for virulence of lifestyle and are referred to as 'virulence plasmids'. The

IncF virulence plasmids in our collection include the $IncFII_K$ for *Klebsiella* spp., $IncFII_S$ for *Salmonella spp.* and $IncFII_Y$ for *Yersinia* spp. (223). The FII 'virulence' sub-type plasmids for *Salmonella* spp., and *Yersinia* spp. were only observed in their native species. $IncFII_S$ was observed in 93.7% of *S.* Typhimurium (89/95), and $IncFII_Y$ was observed in 24.8% of *Yersinia* spp. (53/214). $IncFII_K$ was observed in 38.5% of its native species, *K. pneumoniae* (190/494), however were observed in 8/133 *E. cloacae* isolates. The remaining IncFII plasmids are not associated with any one genera and are disseminated across multiple different genera (39, 40, 223). The FII replicon of the $IncFII_2$ plasmid sub-type was the most frequently acquired plasmid replicon, identified in 30.6% of plasmid carriers (388/1267) and harboured by *K. pneumoniae*, *E. coli, S. flexneri, S. sonnei* species but not in *E. cloacae, S.* Typhimurium and *Yersinia* spp.

IncHI, IncI and IncR represent the Intermediate occurrence Inc types. Intermediate occurance Inc types included IncHI, IncR and IncI types which were detected in between 8-10% of isolates (Fig. 8). The IncHI group is split into subtypes IncHI1 and IncHI2 both associated with emerging resistance genes due to their large plasmid backbone enabling ample space for gene integration (276). The multireplicon IncHI plasmids are detected using the replicons, HI1A and HI1B with a non-essential FIA replicon (276), and HI2 (for IncHI2s only). The IncHI plasmid group was observed in 10.6% of strains (179/1683) including *S.* Typhimurium, *K. pneumoniae, E. cloacae* and *E. coli*. IncR was detected in 9.3% (157/1683) of isolates being found most commonly in *K. pneumoniae*, *E. cloacae* but seldom in *E. coli*. IncI types were observed in 8.8% (148/1683) of isolates, but largely in *E. coli* and *Shigella* spp. but were also in *K. pneumoniae*, *S.* Typhimurium and *Yersinia* spp. in isolated cases (Table 15).

Rare Inc types are widely distributed. Plasmids Inc types A/C, B/O/K/Z, L/M, Q, N, P, X, Y, and plasmids within the novel group were detected in 0.5-3.1% of isolates (Fig. 8). *K. pneumoniae* harboured all of the rare Inc types except IncY (only found in *E. coli*). IncX was the highest shared plasmid type harboured by *K. pneumoniae, E. cloacae*, *Shigella* spp., *E. coli* and *Yersinia* spp. but these were in isolated cases (Table 15). Plasmids Inc types A/C, B/O/K/Z, N, X, Y were not detected in *S.* Typhimurium.

Certain "portal" plasmid sub-types act as a connection between genera. Each major Inc type was observed across multiple genera. Principal component analysis outlined a network of bacterial species/genera linked by specific Inc-types we called these promiscuous Inc-types. These promiscuous Inc-types likely represent "portal" plasmids, offering possible routes of gene exchange between genera facilitated by plasmid transfer (Appendix C; Fig. 9). These promiscuous Inc-types were defined as being present in more than 1% of a particular genus as well as at least two other bacterial genera. The plasmid sub-type network details a comparatively simple and recent account of possible exchange events between species. Divided according to the general Inc type distribution, IncF replicons in addition to being the most abundant were detected in all six genera we included in this study: $IncFII_2$ were found in *E. coli*, *S. sonnei* and *S. flexneri* and $IncFII_{20}$ in *E. coli* and *S. flexneri*. Of the intermediate and low occurrence Inc types: IncHI1 sub-type were detected in: *E. coli*, *S.* Typhimurium, *E. cloacae* and *K. pneumoniae*. IncR and $IncA/C_2$ were detected in *K. pneumoniae* and *E. cloacae*, while both $IncI_1$ and $IncI_2$ were present in *E. coli* and *S. sonnei*. IncQ were found in *E. coli*, *S.* Typhimurium, and *K. pneumoniae*. *Yersinia* spp. had comparatively fewer Inc types shared with other genera, with only one plasmid type (IncN1) present outside of the *Yersinia*, also found in *K. pneumoniae*. This may be expected considering *Yersinia* spp. is the

most genetically diverse species amongst this data set (Appendix C; Fig. S2). *Yersinia* spp. mostly harboured its own genera-specific plasmid type IncFII$_Y$.

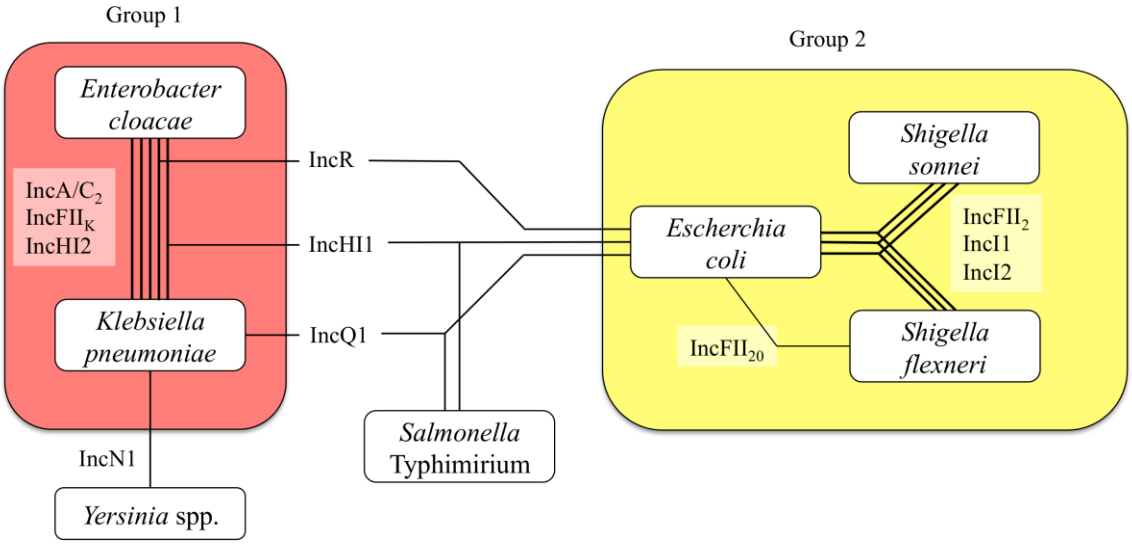

Figure 9. A network of bacterial species connected by plasmid sub-types.
Principal component analysis identified 'portal' plasmid Incompatibility (Inc) sub-types representing potential gene transmission routes between bacterial species. Species clustering according to shared plasmid content identified two groups. Group 1: *Enterobacter cloacae* and *Klebsiella pneumoniae*; Group 2: *Escherchia coli*, *Shigella sonnei* and *Shigella flexneri*.

The clustering of species into two primary groups based on their shared plasmid sub-type content was observed by PCA (Fig. 9; Appendix C Fig. S1). These groups were Group 1: *K. pneumoniae* and *E. cloacae*, encompassed plasmid sub-types A/C$_2$, FII$_K$, HI1, N1, R and an undefined IncF plasmid; and Group 2: *E. coli*, *S. sonnei* and *S. flexneri* with plasmid sub-types FII$_C$, FII$_1$, FII$_2$, FII$_{15}$, FII$_{33}$, FII$_{20}$, I1, I2 and P. Species clustered together were also observed to be phylogenetically related (Appendix C; Fig. S2) (277, 278).

Plasmid types reside across multiple countries and their general distribution remains the same in both a global scale and local regions. IncF plasmids types were observed in 41 countries (out of total of 53; Appendix C; Fig. S3). The intermediate Inc types IncHI, IncI and IncR were found in 15, 17 and 12 countries, respectively. Although infrequently detected the Rare Inc types were detected in between five and thirteen countries. The eight studies in which isolates were originally collected were categorised according to them having a 'global' or 'regional' scope (Appendix C; Table S3). 87.1% of isolates from the global studies were plasmid carriers and 34.8% carried more than one type. 66.6% of isolates collected within a regional studies carried at least one plasmid and 20.9% carrying more than one type. The plasmid type distribution of both types of study was similar to the general Inc type distribution. For the global and regional studies, 77.0% and 55.9% isolates carried IncF types, followed by ~8-14% and ~9-10% for intermediate IncHI, IncI and IncR plasmids types. Less than 4% of isolates in either the global or regional studies carried a low prevalence plasmid.

Discussion

This study using existing published genome sequence data sets to bench mark the relative abundance of different plasmid Inc types carried as part of their hosts genomes. Plasmid Inc types are used as a proxy for different plasmid backbones, and so unlike most studies which focus on the cargo genes carried by plasmids, for example antimicrobial resistance genes, we have provided insight into one of the most important vehicles of the spread of antimicrobial resistance in *Enterobacteriaceae*. We showed there were three major levels of abundance with IncF being highly abundant, IncH, IncI and IncR showing intermediate abundance and ; Inc types A/C, B/O/K/Z, L/M Q, N, O, P, X, Y and some novel Inc types, being rare in our collection (Fig. 8).

IncF replicons were the most abundant Inc types amongst the *Enterobacteriaceae* included in this study (39, 40, 210). This in consistent with previous work on IncF plasmids which can carry multiple replicons FIA, FIB and FII. This is thought to represent an advantage, compared to single replicon plasmids, because it avoids plasmid incompatibility with other plasmids replicating in the same cell, by having alternative replicons (223). The multireplicon status is also associated with a higher level of sequence divergence in the FII replicon through functional redundancy (279, 280) and a broader host range. Intermediate plasmid type IncHI also carry multiple replicons. No multi-replicon plasmids were described as rare Inc types. Geographically, all plasmid types were observed in bacterial isolates collected from multiple countries (except IncY) and present in at least three different genera. The latter are likely to define important plasmid sub-types able to shuttle genes across various species and genera (Fig. 9). This is consistant with the different genera described to carry these plasmid sub-types (22, 39, 40, 125, 249, 276).

It is also clear that the relative abundance of Inc types observed in this study are somewhat consistent with previous studies looking at plasmids associated with AMR (39, 40). For example the common Inc type, IncF, and rare plasmids type IncB/O, IncK and IncT have been previously described as most prevalent and rare, repectively, when associated with AMR (39, 40). In constrast, intermediate level IncHI types and IncI are described as the one of the most prevalent plasmid families (http://pubmlst.org/plasmid/; (39, 40)). The IncR plasmids described here at intermediate levels, are seldomly reported carrying AMR genes until recently where they have emerged (circa 2010) as a prominent resistance plasmid type carrying various genes conferring resistance to the front line therapeutic antimicrobial carbapenem (39, 40, 210, 281-284). IncA/C, IncL/M, and IncN plasmid types also described at most prevalent when associated with AMR (20, 39, 40, 210), are found here at a low

abundance across our collection. These data give the first glimpses into the relative flux in the plasmid population and that an understanding of this flux may be important for identifying the barriers and the facilitators affecting the dissemination of genes associated with AMR which warrants further investigation.

As stated, this study highlights the value of reanalysis of existing sequencing data, to answer broader scientific questions. However, the nature of Illumina data, the PlasmidFinder database and the variability of non-standardised metadata collection, limits several aspects of this study. For example the PlasmidFinder database used in this study will inherently miss new plasmid types, and we could not directly associate Inc types to a comparable AMR gene or phenotype profile. To provide a comprehensive overview of the plasmid prevalence within the *Enterobacteriaceae* family requires samples with even distribution across countries, genera and species, including those within environmental and animal samples (especially within agriculture and farming), standardised meta data, full plasmid assembly as well as a complete plasmid replicon typing scheme including sub-typing of all plasmid Inc types. Many replicons have been identified but have yet to be assigned to an Inc group or sub-group, many of which remain undefined and noted as 'novel' (Appendix C; Table S4).

In summary, we provide an insight into the general distribution of plasmid Inc types across members of the *Enterobacteriaceae*, observed in three levels of abundance where IncF plasmid were most common followed by IncHI, IncI and IncR (intermediate). A handful of sub-types were identified with the potential to facilitate gene exchange between genera. There may be a limiting degree of compatibility between plasmid type and species influencing these interactions. These data show a complex network of interactions between the bacterial hosts, and the geographic and temporal distribution of plasmid Inc types. These data also highlights the need for a more detailed understanding of the factors that affect this flux and the need for a more systematic and detailed understanding of the drivers which

affect their relative distribution inorder for us to fully understand the dissemination of AMR genes globally.

Abbreviations

AMR, Antimicrobial resistance; Inc, Incompatibility.

5.3 Chapter summaries and conclusions.

The investigation of Chapter 5 observed 12 major plasmid types at three levels of occurence: Common, IncF; Intermediate, IncHI, IncI, IncR; and Rare, IncA/C, B/O/K/Z, L/M N, O, P, Q, X, and Y. Those that are frequently associated with AMR genes such as IncF, IncHI and IncA/C (39, 40), were observed at each level of occurence within this population, High: IncF; Int: IncHI; Low: IncA/C. 'Portal' plasmid sub-types e.g. IncHI types, were also identified to represent possible route of gene transmission between different genera. In retrospect, this is not unexpected considering there appears to be a degree of compatibility that was identified between plasmid sub-type and the species/genera of the strain. Two primary groups were identified according to their shared plasmid sub-type content, *E. cloacae* with *K. pneumoniae*, and *E. coli* with *S. flexneri* and *S. sonnei* (Fig. 9). Species of these groups were also observed to be phylogenetically similar (Appendix C; Fig. S2). This possible plasmid type/genera compatibility would indeed be a large factor with the inter-genera transmission of AMR genes. Conjugation would have to involve a plasmid type that complements the recipient strain based on their genera or possibly species, in order for the strain to retain the plasmid and particular plasmid-mediated AMR genes. Consequently this would suggest a requirement for an exchange of AMR genes between plasmid types.

Chapter 6: General Discussion: The *bla*$_{NDM}$ gene may have transferred through a network of AMR gene acqusition and spread

6.1. Overview

Plasmids have been identified to be present prior to the emergence of AMR genes (Appendix D; (285)). It is clear that investigations into the acquisition and spread of the *bla*$_{NDM}$ gene indicate an underlying network of interactions between antimicrobial resistance determinants, transposable elements, plasmid types and bacterial genera. This possible network of AMR gene acquisition and spread may have been existent prior to the transfer of the *bla*$_{NDM}$ genes from *Acinetobacter* spp. into *Enterobacteriaceae* plasmids (241). The *bla*$_{NDM}$ gene would transfer along this network following its interactions and limitations, leading to its association with numerous transposable elements, genetic contexts, different *Enterobacteriaceae* plasmids types and consequently the spread into the various species of the *Enterobacteriaceae* family (Chapter 1-4; (20, 22, 23)). The components of the proposed network and their interactions, have in part been previously characterised for other AMR genes (39, 40, 60, 62, 64, 286, 287), however, they will not be discussed in full here. The insights into plasmid-mediated *bla*$_{NDM}$ acquisition and spread described in this thesis raise many scientific questions and unknown avenues for further investigation. The following chapter will discuss the proposed network, its components, their interactions and compatibilities to explain and place into context, the mechanisms of *bla*$_{NDM}$ gene acquisition and spread observed within this thesis.

6.2 Interactions of Antimicrobial Resistance Determinants

Dissemination of AMR genes also transfers the immediate sequence flanking the target gene during their interaction with transposable elements. Regions frequently co-transferred with AMR genes, include regulatory genes, promoters and passenger genes that lie in close proximity. Regulatory genes can mediate AMR gene expression, for example the *ampR* gene, a negative regulator of expression for *bla*$_{DHA}$ gene (288). Promoters are small regions able to enhance the expression of the

AMR gene by promoting transcription initiation (289), and can be provided by an IS element upstream of an AMR gene. The IS*Aba125* upstream of *bla*$_{NDM}$ provided the -35 promoter region (185, 290) and IS*Ecp1* provided the overexpression of *bla*$_{CTX-M-55}$ (291) and *bla*$_{CMY-2-like}$ (37) genes. Passenger genes are co-transferred depending on the mechanism of the transposable element. The *trpF*, *dct*, *groEL*, *groES* genes appear to be co-transferred with *bla*$_{NDM}$ by the mechanism of Tn*125* transposon within *Acinetobacter* spp. These genes are also observed on occasion within *Enterobacteriaceae* plasmids carrying *bla*$_{NDM}$ genes (21-23, 205).

6.3 Interactions of Transposable Elements

Establishment of a transposable element in close proximity to an AMR gene provides an opportunity for dissemination to other plasmid types and chromosomal locations. Mobilisation of a region via transposable elements is dependent on multiple factors including separate recognizable start and stop sequences in its vicinity (60). The transposition of genes adjacent to a transposable element for example occurs when the enzyme of the IS element (e.g. transposase) recognises a sequence downstream, with a high similarity with its IR_R. The enzyme would recognized the IR_L and highly similar IR_R and mobilise the sequence between these two points, essentially between skipping its own IR_R. A well-known example of this mechanism is the one-ended mobilisation of *bla*$_{CMY-2}$ by IS*Ecp1* (60, 71). Another example of a transposition mechanism is the rolling circle mechanism via IS*CR1* mobilising large regions upstream (64). Numerous IS elements are associated with mobilisation of *bla*$_{NDM}$ genes within the *Enterobacteriaceae*. Comparison between Tn*125* on *Acinetobacter* plasmids e.g. pNDM-BJ01 (Genbank accession no. JQ001791; (73)) and the ΔTn*125* segments on *Enterobacteriaceae* plasmids was able to identify co-transferred regions, but also able to identify possible associated mechanisms, inferred from the elements truncating Tn*125* or the IS*Aba125* upstream of *bla*$_{NDM}$. Transposition mechanisms that mobilise large segments encompassing Tn*125*, such as IS*CR1*, were inferred with the co-presence of IS*CR1*, *Acinetobacter* plasmid backbone and Tn*125* on the *Enterobacteriaceae* plasmid (Chapter 4). This however has yet to be proven. This thesis identified eight different IS elements associated with

bla$_{NDM}$, inferring its mobilisation mechanisms. The transposable elements and their respective plasmid types are, IS*CR27* and/or IS*26* on type 1 IncA/C$_2$, IS*CR1* on IncN$_2$ (23), IncA/C$_2$ (Chapter 4) and IncFII$_2$ (22), IS*CR-6*-like (Chapter 4), IS*903*-like (22), and TIMEs on IncFII$_Y$ (22), IS*26* and/or IS*3000* on IncX3 (22), and IS*26* composite transposon on IncH1B (22). Alternatively, homologous recombination of the Tn*125* fragment into *Enterobacteriaceae* plasmids may also be a possible transfer mechanism, especially when DRs cannot be identified flanking the Tn*125* fragment.

To contrast, the other major AMR genes (identified prior to *bla*$_{NDM}$) are associated with the mechanisms of specific IS elements or transpositional units for mobilisation and insertion, because of their frequently observed genetic contexts and importantly new direct repeats of the target site found at either end of the transposable unit. The *bla*$_{CMY-2}$ (37), *bla*$_{CTX-M}$ types (292), and *bla*$_{OXA-181}$ (293) genes are frequently associated with the one-ended transposition of IS*Ecp1*. The *bla*$_{CTX-M}$ types are also associated with IS*CR1* and their insertion into class 1 integrons (294, 295). *bla*$_{IMP}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$ (296) frequently lie within a cassette of a class 1 integron, a Tn*3*-based transposon Tn*4401* (297) and its variants, and Tn*1999* bound by two IS*1999*, respectively. These associated transposable elements are significant for dissemination of AMR genes as they facilitate their transfer and insertion into different plasmid types (297-299). Examples of possible gene exchange between plasmids types via IS*CR* rolling-circle mechanism was also described during studies of this thesis; in Chapter 2 with a *bla*$_{NDM-1}$ genetic context of 3′-*aacA1*|Δ*bla*$_{OXA-10}$| ΔTn*125*-5′ on both IncN$_2$ and IncFII$_Y$ plasmid types (23), and in Chapter 4 with the same *rmtC* segment on both type 1 IncA/C$_2$ and IncFII$_Y$ plasmid types.

An additional factor for AMR gene insertion can also depend on whether the transposable element is able to recognise a suitable sequence for insertion. Common insertion sites lie within intergenetic regions, other transposable elements, class 1 integrons or antimicrobial resistance islands. These

insertion sites are catalogued in databases such as the IS finder database (https://www-is.biotoul.fr/).

6.4 Interactions of Plasmid Types

Plasmids facilitate the exchange of AMR genes between bacterial hosts but also interact with the transposable elements within the bacterial host itself. Each plasmid type has the potential to acquire an AMR gene. A common feature of those plasmid types is the establishment of a 'founder element'. That is the insertion of a mobile element into a location with no deleterious effect, which can act as a target for further insertions (60, 300). These have been described as an exceptionally rare event but the structures which derive from these are able to widely disseminate (60). An example of this is the antibiotic island ARI-A of IncA/C_2 plasmids (249), which has accumulated a range of different AMR genes (refer to Chapter 4). These islands will be more than likely continue to accumulate additional insertions of AMR genes via transposable elements. Other plasmid types such as IncX and IncFII$_Y$ that have recently become associated with AMR genes tend to have less complicated AMR regions (225, 238, 243, 252), suggesting relatively recent establishment of their founder element. Within this thesis, IncX and IncFII$_Y$ plasmid backbone was observed either side of the *bla*$_{NDM}$ genetic context (22), suggesting insertion of the *bla*$_{NDM}$ gene was either involved in the initial insertion of the founder element (e.g. TIMEs/MITEs, IS*903B* for IncFII$_Y$) or inserted sequentially afterwards.

6.5 Interactions of a Bacterial Host

The exchange of AMR genes between species and genera is primarily provided by plasmids. This interaction is partly dependent on various genes of a plasmid type such as those responsible for conjugation and replication. The interactions between plasmids, transposable elements, and AMR genes (including the genes within the genetic context) may potentially be governed and influenced by the bacterial strain, based on genus. The components of the network model may have an underlying degree of compatibility (as discussed in Chapter 5) on the interactions between bacterial

strain and plasmid type, and bacterial strain with transposable elements located on the plasmid. Those that are not compatible may be replaced by another compatible transposable elements or plasmid types.

6.5.1 Interaction and compatibility between Bacterial Strain and Plasmids Types

The genus of a strain was observed as a major determinant for carriage of specifc Inc type (Chapter 5), which may indicate a compatibility between the strain and plasmid type. This would have implications on plasmid-mediated gene exchange between different genera, where only certain plasmid sub-types are frequently shared between phylogenetic-related genera. Chapter 5 identified the clustering of two primary groups according to their shared plasmid content, Group 1: *E. cloacae* and *K. pneumoniae* and Group 2: *E. coli*, *S. sonnei* and *S. flexneri*. Group 1 encompassed plasmid sub-types A/C_2, FII_K, HI1, N1, R and an undefined IncF plasmid; Group 2 encompassed plasmid sub-types FII_C, FII_1, FII_2, FII_{15}, FII_{33}, FII_{20}, I1, I2 and P. Although it must be noted there may be missing information in this *in silico* study, as it is dependant on the available species carrying certain plasmid types and detection of plasmid types. Nevertheless, species within each group were also observed to be phylogenetically related (Appendix C, Figure S2). The IncF 'virulence' sub-types in constrast were frequently carried by their native genera (223), *K. penumoniae* ($IncFII_K$), *Salmonella spp.* ($IncFII_S$), and *Yersinia* spp. ($IncFII_Y$) (Chapter 5). An exception was the $IncFII_K$ sub-type plasmids carried by *E. cloaceae* isolates, which was seen in the same cluster group as *K. pneumoniae* (Group 1). Compatibility between plasmid types and genera would limit the direct exchange of bla_{NDM} or any other AMR gene between genera that are phylogenetically different.

Gene exchange events between plasmid types would be a mechanism to avert the plasmid types/genera compatibility. This event would possibly occur after a strain received a plasmid type that cannot be retained, where the AMR gene and any adjacent sequence would be transferred from said incompatible plasmid onto a plasmid type already co-resident and established within the strain. Such events would result in the observation of the same genetic context on two different plasmid

types, such as the *bla*$_{NDM}$ genetic context on IncN$_2$ and IncFII$_Y$ (Fig. 4; Chapter 2), and *rmtC* genetic context on IncA/C$_2$ and IncFII$_Y$ (Fig. 7C; Chapter 4). Gene exchange between plasmid types may have been a mechanism involved to overcome the plasmid type/genera compatibility during transfer of *bla*$_{NDM}$ from its progenitor via *Acinetobacter* spp. as an intermediate species, into the *Enterobacteriaceae* family (241). The source of *bla*$_{NDM}$ would be a transferred *Acinetobacter* plasmid into an *Enterobacteriaceae* strain, such as the *Acinetobacter* pBJ01-NDM-1-like plasmid, p3SP-NDM, observed in *Enterobacter aerogenes* (234). Another possibility may include transfer into *E. coli*, as there are examples where plasmids carrying *bla*$_{NDM}$ have been transferred from *Acinetobacter* spp. to *E. coli* (72, 301, 302). Those strains that could not retain the *Acinetobacter* plasmid, would hypothetically transfer *bla*$_{NDM}$ into a stable *Enterobacteriaceae* plasmid via available transposable elements.

6.5.2 Interactions between Bacterial Host and Transposable Elements

Analysis of *Enterobacteriaceae* plasmids has highlighted numerous transposable elements can be involved in the transposition a single AMR gene. IS*Aba125* and IS*Aba14* native to *Acinetobacter* spp. (303, 304) are frequently truncated by insertion of another IS element on *Enterobacteriaceae* plasmids (21-23, 205), by numerous IS elements including IS*903B*, IS*Kpn14*, IS*5*, and IS*26*. The *bla*$_{NDM}$ genes reported in *Acinetobacter* spp. to constrast, are frequently observed within Tn*125* (184, 200, 241). It may be speculated that transposable elements have a degree of compatibility within a strain depending on their genera. Truncation of an IS element sequence for example could respresent the inability of that genera to transcribe the DNA sequence of that transposable element. The DNA sequence of these incompatible elements may be regarded as foreign DNA, available for insertion of a compatible transposable element when transfer of DNA regions is required. Additionally, if transposable elements have an influence on the length of the target sequence to be transferred, for example the recognition of a similar IR$_R$ other than their own (similar to the mechanism of IS*Ecp1*), this may partially explain the different lengths of truncated Tn*125* structure observed on *Enterobacteriaceae* plasmids.

6.6 Interactions and compatibilities for other AMR genes

The acquisition and spread of other AMR genes such as the *bla*$_{KPC}$ genes appears to follow the discussed network, and the interactions and limitations described. The *bla*$_{KPC}$ genes are associated but not limited to *K. pneumoniae* ST258 and the transposable element/genetic context of Tn*3*-based transposon Tn*4401* and other slight variants/isoforms (*a*, *b*, *c*, *d* and *e*) (305-308). *bla*$_{KPC}$ genes (within the Tn*4401* isoform) are frequently carried on IncFII$_K$ plasmids as well as other plasmid types to a lesser extent IncI2, IncA/C$_2$, IncR, IncL/M, IncN, and IncX (309-311). Plasmid types reported to carry a Tn*4401* isoform in *K. pneumoniae* are, IncFII$_K$ (the most frequent), IncI2, IncN, and IncX (311, 312), and in *E. cloacae* are, IncHI2 and IncN plasmids types. In context of the proposed network, this indicates IncFII$_K$, IncI2, IncN, IncL/M and IncX can be retained by *K. pneumoniae*, and Tn*4401* is a compatible transposable element within *K. pneumoniae* and other *Enterobacteriaceae* such as *E. cloacae*. The network components associated with acquisition and spread of the *bla*$_{KPC}$ genes and other prominent AMR genes have less variability than the *bla*$_{NDM}$ genes, where the plasmid types are the most variable component for *bla*$_{KPC}$ genes. This distinction between the *bla*$_{NDM}$ genes and other prominent AMR genes e.g *bla*$_{KPC}$, may be due to the compatibility described, and also the difference in their progenitors.

The degree of variation in associated genetic contexts, transposable elements and plasmid types for the *bla*$_{NDM}$ gene is not observed for other prominent AMR genes within the *Enterobacteriaceae*. These AMR genes have constant genetic contexts only with slight variations, IS*Ecp1* transpositional unit (*bla*$_{CMY\text{-}2\text{-}like}$ and *bla*$_{CTX\text{-}M\text{-}15}$ as well as many other antimicrobial resistant determinants) and Tn*1999* (*bla*$_{OXA\text{-}48\text{-}like}$). Their progenitors have been identified within the *Enterobacteriaceae*, *Citrobacter freundii* (*bla*$_{CMY\text{-}2\text{-}like}$ (313)), *Kluyvera ascorbata* (*bla*$_{CTX\text{-}M\text{-}15}$ (314)) and *Shewanella* spp. (*bla*$_{OXA\text{-}48}$ (315) and *bla*$_{OXA\text{-}181}$ (316)). This would suggest these transposable elements were involved during their early mobilisation from their chromosomal

location onto *Enterobacteriaceae* plasmids and were not required to change, as well as indicating a compatibility within the *Enterobacteriaceae*. The progenitor for the *bla*$_{KPC}$ genes has yet to be identified, however its constant genetic context Tn*4401* was determined as likely to be at the origin of *bla*$_{KPC}$ mobilisation (299). AMR genes highly associated with plasmid types, e.g. *bla*$_{CMY\text{-}2\text{-}like}$ carriage on IncA/C plasmids, are usually upon plasmid types that are compatible with the genera of the strain and would not require gene exchange between plasmid types. Observations of plasmid-mediated AMR genes and their associated transposable elements, genetic contexts, plasmid types and species provide pieces of information that if collectively placed together can form this proposed underlying network of AMR acquisition and spread.

6.7 Antimicrobial Pressure on the Network Model and other Factors

There are numerous factors that are outside of the proposed network and cannot be identified specifically for each AMR gene capture event. These factors however are important to characterise in future investigations to clarify several discrepancies identified during this thesis. Two examples include plasmids types IncFII$_Y$ and IncA/C. The IncFII$_Y$ plasmid sub-type was observed in different species (*C. freundii* (Chapter 2 (23)), *E. cloacae* and *K. pneumoniae* (Chapter 3 (22)), *Yersinia* spp. (Chapter 5) and has acquired *bla*$_{NDM}$ in different locations in its backbone via separate events (Chapter 3 and 4). The IncFII$_Y$ plasmid sub-type was observed and reported as highly prevalent amongst *Yersinia* spp. (Chapter 5) (223). This plasmid sub-type was carried by species outside of the Yersinia genus only when the plasmid type harboured the *bla*$_{NDM}$ gene (225, 243, 251-253). In contrast, the IncA/C plasmid type is highly associated with many AMR genes (39) and has acquired *bla*$_{NDM}$ via different mechanisms (Chapter 4) (205). However, like many AMR associated plasmid types, it was observed as a low prevalent plasmid type (1%) amongst 1683 strains (Chapter 5). These discrepancies cannot be explained here, however candidates for influencing factors such as antimicrobial pressure may be suggested. The presence of antimicrobials has been observed to induce the SOS response and affect the rate of transposition and conjugation (317). There are limited studies investigating the influence of antimicrobial presence on transposition of *bla*$_{NDM}$ or

its associated transposable elements. Plasmids carrying *bla*$_{NDM}$ however, have been observed to conjugate at a rate ~40 fold higher (from 9.1 x 10^{-4} to 3.9 x 10^{-2} transconjugants per recipient) in the presence of sub-MIC concentrations of ciprofloxacin (Wailan 2012, Unpublished data, Appendix J). The presence of antimicrobials may have played a role in the transmission of plasmid types or the transposition of AMR genes, or even both. International travel including medical tourism, community acquisition and stool colonisation are also factors involved in the international dissemination of AMR genes, facilitating the spread of strains carrying plasmids as discussed in Chapter 1 (20, 46, 49).

6.8 Future directions

6.8.1 Approaches to access the network

The bioinformatic analysis of *Enterobacteriaceae* plasmids presented in this thesis is retrospective and has limited sample size. It does not necessarily reflect how widespread the *bla*$_{NDM}$ genes, other AMR genes and plasmid types have disseminated. A survey of samples of the environment and community/general public would be recommended as reports for plasmid types, AMR genes and *bla*$_{NDM}$ are generally limited to medical health care facilities and outbreaks, representing the tip of the iceberg and not the underlying dissemination of these genes via the proposed network. In order to evaluate the complex dissemination of plasmid-mediated AMR genes for epidemiological purposes, the combined molecular/genetic approach demonstrated in Chapters 2 and 3, at this present time should be incorporated as a screening method. That is, combining the information of clonal molecular characterisation that includes bacterial species identification, Sequence Typing via MLST, PCR-based plasmid typing and resistance determinant characterisation with the WGS characterisation of the target antimicrobial resistance determinant. Through this approach, the plasmid types providing *bla*$_{NDM}$-related carbapenem resistance and perhaps other plasmid-mediated AMR genes can be surveyed and traced across a population, in multiple medical facilities (Chapters 2, 3) and potentially within community and environmental reservoirs. Sequentially, as WGS

becomes increasingly cost effective, construction of complete plasmid sequences and their comparative analysis as demonstrated in Chapter 4, would compliment and provide further resolution of the network used for AMR genes to be acquired and spread throughout a bacterial population.

6.8.2 Additional investigations

The insights presented here for acquisition and spread of the *bla*$_{NDM}$ genes, provide other avenues for further investigation. The interactions between AMR genes, transposable elements, plasmid types and bacterial species/genera require further definition. This includes the factors that influence them, such as the degree of compatibility between them and the presence of antimicrobials. Also S1 nuclease PFGE should be implemented to complete molecular plasmid analysis for description of plasmids replicon numbers and sizes. The combination of detailed surveillance, molecular typing, whole genome sequencing and in-depth bioinformatics analysis of *Enterobacteriaceae* and other Gram-negatives with a standardised meta-data, would complement and build upon those components characterised within this thesis and the literature. Also as novel plasmid types, resistance determinants and even transposition elements are described and updated in databases, it is important for investigators to also reassess previously published isolate collections to also provide the most comprehensive description available for novel resistance determinants, plasmid types and transposition element. This is important as investigators have to be aware that databases such as resfinder and plasmidfinder which are reasonable for strain and plasmid characterisation, always have a chance in not identifying novel plasmids types and resistance determinants. Further, a database of the components of the network encompassing AMR genes, transposable elements, plasmid types and bacterial strains (including environmental, community and clinical samples), detailing their mechanisms, interactions and the factors which affect these interactions will begin to clarify the details of this proposed network. A network which *bla*$_{NDM}$ and other AMR genes have passed through to be acquired by various *Enterobacteriaceae*.

6.9 Thesis conclusion

The detailed bioinformatic analysis of *Enterobacteriaceae* plasmids provided insights into the acquisition and spread of *bla*$_{NDM}$ genes amongst the *Enterobacteriaceae*. This thesis characterised numerous combinations of species, plasmid type, genetic context and transposable elements associated with the *bla*$_{NDM}$ gene, as well as an indication of compatibility between plasmid types and the bacterial host, based on genus. The insights provided by these investigations, collectively identified an underlying network encompassing interactions between AMR genes, transposable elements, plasmid types and the bacterial host. The *bla*$_{NDM}$ gene appears to have transferred along this network following its interactions, compatibilities and limitations, resulting in numerous species of *Enterobacteriaceae* carrying their *bla*$_{NDM}$ gene in different plasmid types, NGCs and associated with numerous transposable elements. A database listing the network's components and their interactions is required for proper definition of the proposed network. Surveillance of *Enterobacteriaceae* strains with the combined genetic/ molecular approach and subsequently full plasmid construction and analysis demonstrated in this thesis are recommended as approaches to continue compilation for this database and define this complex network. A complex network that may have been used for the rapid spread of carbapenems resistance amongst the *Enterobacteriaceae* family.

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Appendices

Appendix A: Supplementary Original Manuscript related to Chapter 3

The following appendix, published manuscript, has been provided to supplement Chapter 3. This manuscript described the genome of an NDM-5 producing *E. coli* isolated in Australia.

***Wailan AM**, Paterson DL, Caffery M, Sowden D, Sidjabat HE*. Draft Genome Sequence of NDM-5-Producing *Escherichia coli* Sequence Type 648 and Genetic Context of $bla_{\text{NDM-5}}$ in Australia. Genome Announc. 2015 Apr 9;3(2). PMID: 25858833

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Draft genome sequence of NDM-5-producing *E. coli* ST648 and genetic context of *bla*$_{NDM-5}$ in Australia

Genome Announcements

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ABSTRACT

We report the draft genome of uropathogenic *E. coli* ST648 possessing *bla*$_{\text{NDM-5}}$ from a 55-year-old female in Australia with travel history to India. The plasmid-mediated *bla*$_{\text{NDM-5}}$ was in a genetic context nearly identical to the GenBank entry of IncX3 *bla*$_{\text{NDM-5}}$ plasmid previously reported from India (*K. pneumoniae* MGR-K194).

MANUSCRIPT

The Indian sub-continent has been reported as a geographical reservoir for acquisition of NDM-producing *Enterobacteriaceae* (1). A 55-year-old female with chronic diarrhoea had a carbapenem-resistant *Escherichia coli* isolated from her urine in January 2014. She travelled to India in late 2013 and developed diarrhoea without admission to medical facility. Upon her return to Australia, ongoing diarrhoea prompted multiple hospital admissions. She was diagnosed with Crohn's Disease. During admission, a mid-stream urine sample was collected wherein the carbapenem-resistant *E. coli* CR694 was identified.

Whole genomic DNA of *E. coli* CR694 was prepared using the Nextera XT DNA sample preparation kit (Illumina, USA) and sequenced using the Illumina HiSeq 2000 (Illumina) at the Australian Genome Research Facility. De novo assembly was performed using CLC genomic workbench version 7.5 (CLC Bio, Denmark). The draft genome consisted of 5,523,407 base pairs. Contigs were initially annotated using RAST (http://rast.nmpdr.org/). BLAST analysis and manual annotation utilized previously re-annotated reference sequences and IS finder (http://www-is.biotoulfr). Databases MLST, ResFinder and PlasmidFinder (http://www.genomicepidemiology.org/) were used to characterize Sequence Typing (ST), antibiotic resistance mechanisms and plasmid Inc types of *E. coli* CR694, respectively. ST 648, plasmid Inc types of IncFII, IncFIB, IncX3, IncI1 and IncX4 and $bla_{\text{NDM-5}}$, $bla_{\text{CMY-42}}$, *aac-6-Ib-cr*, *aadA5*, *erm*(B) and *mph*(A), *sul1*, *tet*(B) and *dfrA17* were identified.

Additionally, the annotation through RAST identified type 1 fimbriae fimA-H, virulence determinants relevant for urinary tract adhesion (2). Further, five other types of fimbriae identified as a membrane transport type VII protein secretion system, namely (i) htrE fimbriae cluster, (ii) stf fimbriae cluster, (iii) alpha-fimbriae, (iv) colonization factor antigen I fimbriae (CFA/I fimbriae) and (v) sfm fimbrial cluster. Cluster responsible for Curli production or type VIII secretion was

identified. Siderophore enterobactin, aerobactin and other hemin transport systems for iron acquisition were identified. In addition, type IV pilus and IncF conjugal transfer system were identified. Gene for serum survival (iss) was also identified. The identified virulence determinants may have contributed to the infection and or colonization in the urinary tract of CR694 (2).

The contig pCR694-EC-NDM-5 carried the *bla*$_{\text{NDM-5}}$ genetic context. blaNDM has been reported to reside within a 10,099bp transposon known as Tn*125* (3). *bla*$_{\text{NDM-5}}$ on pCR694-EC-NDM-5 was located within a truncated 3,167bp Tn*125* structure, flanked by an IS*5* upstream and an IS*26* downstream. pCR694-EC-NDM-5 was identical to an NDM-5 IncX3 plasmid, pNDM-MGR194 (as direct submission with GenBank accession no. KF220657). Both *bla*$_{\text{NDM-5}}$ genetic contexts did not possess Tn*125* genes *groES*, *groEL* and IS*CR27*. Both pCR694-EC-NDM-5 and pNDM-MGR194 were also highly similar to NDM-1 IncX plasmid, pKPN5047 (GenBank accession no. NC_020811), containing a longer Tn125 structure where groES, groEL and ISCR27 were present.

The *bla*$_{\text{NDM-5}}$ genetic context of pCR694-EC-NDM-5 has not been reported within E. coli and within Australia. NDM-5-producing *Enterobacteriaceae* have been reported in Japan, Algeria, United Kingdom and India, of which an *E. coli* ST648 harbored *bla*$_{\text{NDM-5}}$ in both aforementioned United Kingdom and Japan reports (4-7). This case of a NDM-5-producing typical uropathogenic *E. coli* highlights further inter-continental acquisition of carbapenemase-producing Enterobacteriaceae through travel to geographical reservoirs.

Nucleotide sequence accession numbers. This project is registered as BioProject PRJNA268254 and BioSample SAMN03217331. The *bla*$_{\text{NDM-5}}$ genetic context, pCR694-EC-NDM-5 was submitted to the GenBank database and assigned the following accession number KP178355. The draft genome of NDM-5-producing *E. coli* ST648 GenBank accession number is JTGI00000000.

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Appendix B: Supplementary Material related to Chapter 4

The following supplementary material is complementary information for the manuscript presented in Chapter 4. This material lists the differences between sequences of IncA/C_2 (Table S1) and IncFII$_Y$ (Table S2) plasmids harbouring *bla*$_{NDM}$.

TABLE S1 Differences between closely-related IncA/C type 1 plasmids carrying *bla*$_{NDM}$[a]

Position[b]	Location[c]	pNDM 10505	pNDM-PstGN574	pNDM-EcoGN568	pNDM 102337	pNDM -US	pNDM-US-2	**pKP1-NDM-1**[d]	pNDM 10469	pNDM-KN	**pEC2-NDM-3**[d]
Method		not stated	Illumina	Illumina	not stated	mixed[e]	not stated	Illumina	not stated	Illumina	PacBio
955-7	HP	3xC	3xC	2xC	3xC	3xC	3xC	3xC	3xC	3xC	3xC
3898-905	HP	8xT	8xT	8xT	8xT	8xT	8xT	8xT	8xT	7xT	8xT
8746	peptidase	C	C	C	C	C	C	C	C	C	T
9613	HP	T	T	T	T	T	T	T	T	T	C
19333	-	C	T	T	C	C	C	C	C	C	C
38111	HP	C	C	C	C	T	T	C	C	C	C
52042-8	-	7xT	7xT	7xT	7xT	7xT	7xT	7xT	7xT	6xT	7xT
57990-1	IS*Ecp1* IR$_R$	2xG	2xG	2xG	2xG	2xG	2xG	2xG	2xG	3xG	2xG
60543-4	*bla*$_{CMY-6}$ TU	-	-	-	-	-	-	-	-	IS*Ec23*	-
94439-40	HP	-	-	-	-	-	-	-	-	-	IS*Ec23*
95218-96931	IS*4321*-Tn*1696*	-	-	-	-	-	~88% identity	-	-	-	-
95959	IS*4321*	A	A	A	A	A	G	A	A	A	G
97817-23	Tn*1696* *tnpA*	2xGCGT AGCG	2xGCGTAG CG	2xGCGTA GCG	2xGCGTA GCG	1xGCGTA GCG	1xGCGTA GCG	2xGCGTA GCG	2xGCGTA GCG	2xGCGTA GCG	2xGCGTAG CG
100771	Pc TGN	G	G	G	G	G	G	G	G	C	G
102934	*sul1*	T	T	T	T	T	T	T	T	A	T
103029-30	3′-CS	2xC	2xC	2xC	2xC	2xC	2xC	2xC	2xC	3xC	2xC
104316-7	*dcm*	-	-	-	-	-	-	-	+217 bp	-	-
104534-5	*dcm*	2xC	2xC	2xC	2xC	2xC	1xC	2xC	2xC	2xC	
105989-90	-	-	-	-	-	-	-	-	-	-	IS*3000*
107113-4	IS*Kpn14* IR	2xG	2xG	2xG	2xG	2xG	2xG	2xG	2xG	3xG	2xG
107881-117385	*aac(3)-II* region	-	6 indels	-	-	not present	not present	not present	not present	not present	-
117386-118153	IS*Kpn14*	IS*Kpn14*	IS*Kpn14*	IS*Kpn14*	not present	not present	not present	not present	not present	not present	not present
118154-118383	IS*Aba125*	130 bp	130 bp	130 bp	130 bp	130 bp	130 bp	89 bp	124 bp	129 bp	83 bp
128810	-	T	T	T	T	T	T	T	T	T	A
128916-147793	Tn*6170*+5 bp DR	Tn*6170*	-	Tn*6170*	Tn*6170*	-	-	-	-	Tn*6170*	-
150874	-	A	A	A	A	A	A	A	A	A	C
152608-9	-	2xATTA TCGTA	2xATTA TCGTA	2xATTA TCGTA	2xATTA TCGTA	2xATTA TCGTA	2xATTA TCGTA	2xATTA TCGTA	3xATTA TCGTA	2xATTA TCGTA	-
152560-1	-	-	-	-	-	IS*3000*	IS*3000*	-	-	-	-
161161	*traG*	G	G	G	A	G	G	G	G	G	G
162413-4	-	2xC	2xC	2xC	2xC	1xC	1xC	2xC	2xC	2xC	2xC

[a] Accession numbers and references are given in Table 1 in the main manuscript.

[b] Position in pNDM10505. Differences from pNDM10505 are highlighted in red and at least some (e.g. different numbers of bases in homopolymer regions) may be errors. There are no differences between any parts of the ΔIS*Aba14*-*aphA6*-ΔIS*Aba125*-*ble*$_{MBL}$-*trpF*-*tat dct groESL*-IS*CR27*Δ region found in different plasmids, except for the G to A change giving *bla*$_{NDM-3}$ in pEC2-NDM-3.

[c] HP, hypothetical protein; -, intergenic region.

[d] Any differences in plasmids sequenced here (names in bold) have been confirmed by checking the raw sequence data.

[e] Combination of Illumina and PacBio.

TABLE S2 Differences between IncFII$_Y$ plasmids carrying *bla*$_{NDM}$[a]

Position[b]	Location[c]	**pEC4-NDM-6**[d]	pKOX-NDM-1	pRJF866	pNDM-EclGN574	pYDC644	pP10164-NDM	pNDM1_EC14653	**pECL3-NDM-1**[d]
Method		Illumina	454	ABI Sanger	Illumina	454	Illumina	Illumina	Illumina
2834	IS*Sen4*	T	T	T	T	T	T	C	T
2905	IS*Sen4*	G	G	G	G	G	G	A	G
3621	IS*Sen4*	A	A	A	A	A	A	G	A
4398	IS*5*	A	A	A	A	A	G	A	A
5211-5390	*ygbN*	-	-	-	-	-	180 bp Δ	-	-
6833-8441	*ccd*	-	-	-	-	55% identity	-	-	-
8329-8332	-	4xG	4xG	4xG	4xG	4xG	4xG	4xG	3xG
8976-8977	IS*1*	2xC	2xC	2xC	2xC	2xC	2xC	3xC	2xC
8992	IS*1*	T	T	T	T	T	C	T	T
13572-8	-	7xT	7xT	7xT	7xT	7xT	6xT	7xT	not present
15042-17318	*kpsSC*	-	-	-	-	-	94% identity	-	not present
18607-23459	IS*1* & adjacent	-	-	-	99% identity	4085 bp Δ	95% identity	98% identity	not present
20412-20418	HP	7xT	6xT	7xT	7xT	not present	7xT	7xT	not present
22951-22957	HP	7xT	6xT	7xT	7xT	not present	8xT	7xT	not present
23541-24596	IS*903*	-	-	-	-	not present	97.8%	-	not present
25447-25452	-	6xT	6xT	6xT	6xT	6xT	7xT	6xT	6xT
29072-3	*umuC*	-	-	-	-	-	-	+9,851 bp	-
31950	HP	C	C	C	C	C	C	T	C
36032-8	HP	7xG	7xG	7xG	7xG	6xG	7xG	7xG	7xG
42178-42648	HP	-	-	-	-	-	-	-	96% identity
43033-9	HP	7xA	6xA	7xA	7xA	7xA	7xA	7xA	7xA
48476	*traL*	T	G	G	T	T	G	G	T
51147-8	*traB*	2xG	2xG	2xG	2xG	2xG	1xG	2xG	2xG
57087	*traU*	T	T	T	T	G	T	T	T
62964-6	*traH*	3xG	3xG	3xG	3xG	3xG	2xG	3xG	3xG
64048-54	*traH*	7xG	7xG	7xG	7xG	6xG	7xG	7xG	7xG
67148-54	*traS*	7xT	6xT	7xT	7xT	7xT	7xT	7xT	7xT
70081-70188	*traD*	12x9 bp	12x9 bp	12x9 bp	12x9 bp	12x9 bp	7x9 bp	12x9 bp	12x9 bp
77281-5	*dsbA*	5xG	5xG	5xG	5xG	4xG	5xG	5xG	5xG
78081-4	-	4xC	4xC	4xC	4xC	3xC	4xC	4xC	4xC
80852-85111	-	-	-	-	-	-	-	-	18 differences
91483-91488	*copG*	6xC	6xC	6xC	6xC	5xC	not present	not present	6xC
93095	*sul1*	C	C	C	C	A	not present	not present	C
107822-9016	IS*5*	-	1 difference	-	-	-	7 differences	7 differences	2 differences
110735-40	-	6xG	6xG	6xG	6xG	5xG	6xG	6xG	6xG

[a] Accession numbers and references given in Table 1 in main manuscript.

[b] Position in pEC4-NDM-6. Differences from pEC4-NDM-6 are highlighted in red and at least some (e.g. different numbers of bases in homopolymer regions) may be errors. There are no differences in the ΔIS*Aba125-ble*$_{MBL}$*-trpF-tat dct groESL*-IS*CR27*Δ region common to all IncFII$_Y$ plasmids listed, except for the C to T change giving *bla*$_{NDM-6}$ in pECL3-NDM-1.

[c] HP, hypothetical protein; -, intergenic region.

[d] Any differences in plasmids sequenced here (names in bold) have been confirmed by checking the raw sequence data.

Appendix C: Supplementary Material related to Chapter 5

The following supplementary material is complementary information for the manuscript presented in Chapter 5. This material lists the PCA identifying species clustering between species according to plasmid content (Fig. S1), phylogenetic tree of the *Enterobacteriaceae* strains (Fig. S2), number of countries the 12 major plasmid types were isolated from (Fig. S3), and meta data tables of the *Enterobacteriaceae* strains (Table S3) and plasmid sub-types (Table S4) included in the study.

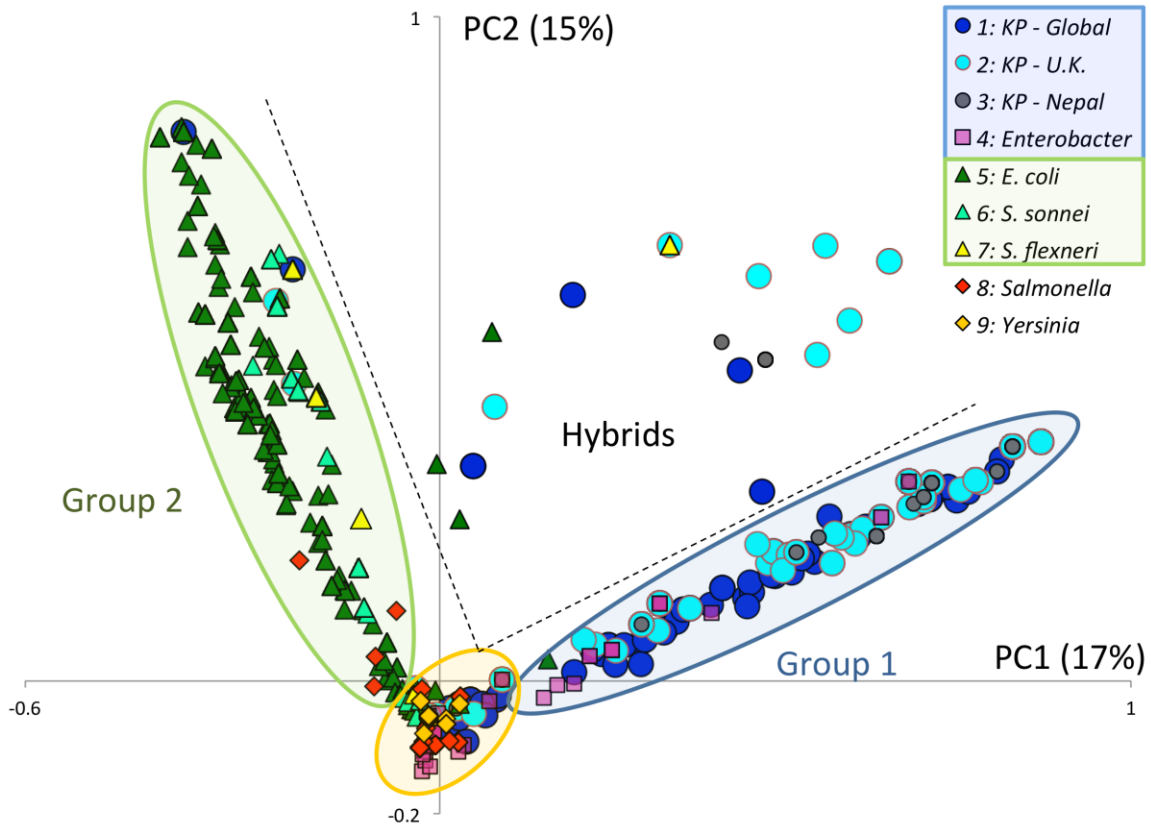

Figure S1. PCA plot analysis on the plasmid content of all *Enterobacteriaceae* species included in this study. PC1 (17%) and PC2 (15%), represented on the x- and y-axis respectively, highlight the existence of two groups of species which have a very high inter-species plasmid transmission. Group 1, which consists out of *Klebsiella pneumoniae* and *Enterobacter cloacae*, in depicted by the blue oval on the right and group 2, which consists out of *Escherichia coli*, *Shigella sonnei* and *Shigella flexneri*, is depicted by the green oval on the left. An orange oval on the bottom depicts those strains which either do not contain any plasmids, or which only contain rare plasmids, or which only contain plasmids which are very species specific such as is often the case when describing *Yersinia* spp. and (to a lesser degree) *Salmonella* typhimurium isolates. Plasmid exchange is however clearly not limited within these two groups as is depicted by the "hybrid" isolates in the upper right. These hybrid isolates are bacteria that have at least 2 plasmids; they have (a) plasmid(s) that is/are

commonly found within their own group but they also have a plasmid which common to the other group. Similarly of interest are the isolates belonging to species from group 1 but found in the circle of group 2, or vice versa. These represent samples that do not contain any plasmids common to their "own" group but instead carry 1 or more plasmids from the "other" group.

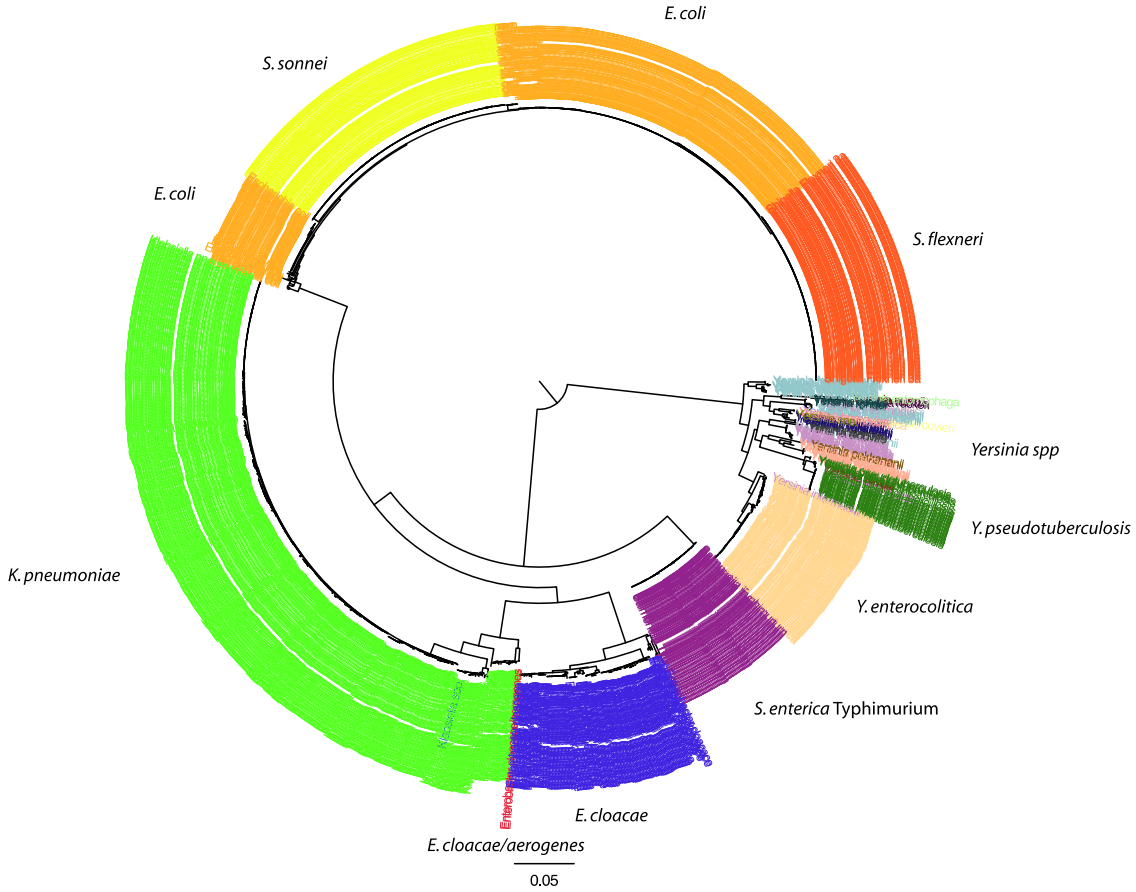

Figure S2. Phylogenetic tree of strains of the *Enterobacteriaceae* family based on rpoB.

Gene sequences corresponding to *rpoB* were extracted from the assembled genomes, and if necessary complemented with data from mapping to a *Klebsiella pneumoniae* isolate. The phylogeny was estimated using RAxML. Species are highlighted in different colours, and important groups are labelled.

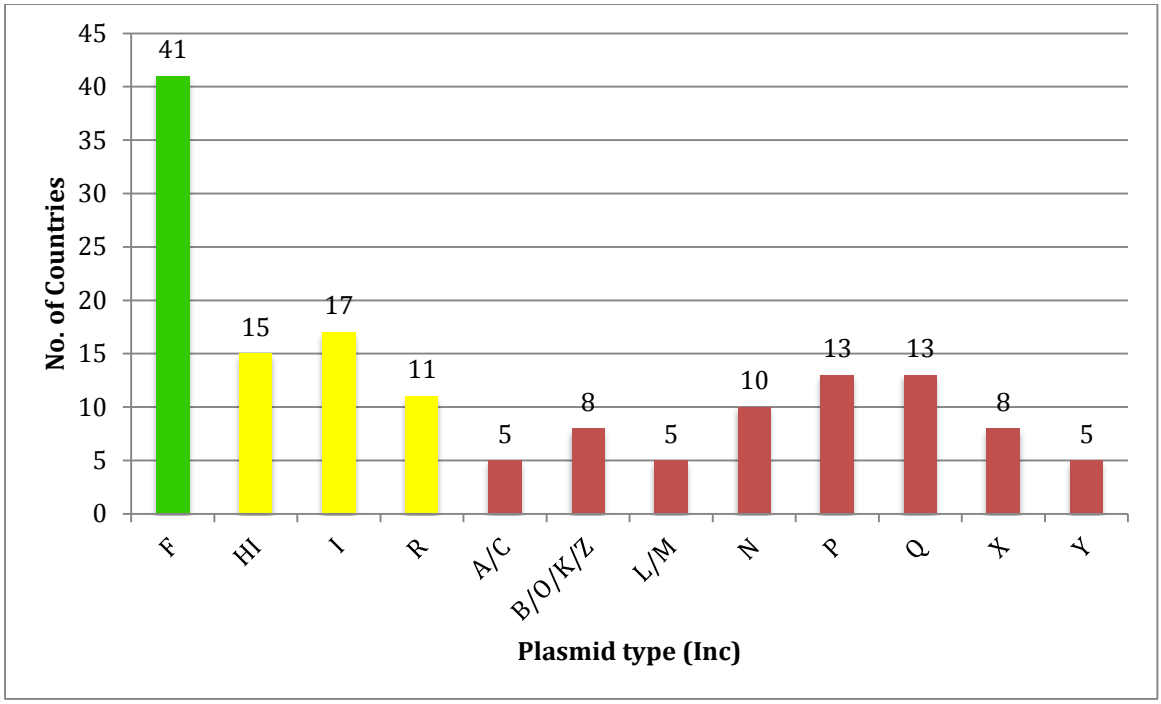

Figure S3. Number of countries each major plasmid Inc group originated. IncF type plasmid types were wide spread across 41 countries. Intermediate and low occurrence plamids types were observed within 5-17 countries. High, intermediate and low occurrence plasmid types are represented by green, yellow and red respectively.

Table S3 – Studies in which strains were originally collected.

Scope	Study name	WGS Strains		Species		Countries[a]		Ref.
		Publication	Analysis					
Global	Global *K. pneumoniae*	288	247	*Klebsiella pneumoniae*		Australia Loas Indonesia	Singapore U.S. Vietnam	(271)
	Yersinia study	241	214	*Yersinia* • *aldovae* • *aleksiciae* • *bercovieri* • *enterocolitica* • *entomophaga* • *frederiksenii* • *kristensenii* • *intermedia* • *nurmii* • *massiliensis*	• *mollaretii* • *pekkanenii* • *pestis* • *pseudo-tuberculosis* • *rohdei* • *ruckeri* • *similis*	Argentina Australia Belgium Brasil Canada China Czechoslovakia England Finland France Germany Greece Russia	Ireland Italy Japan Korea New Zealand Norway South Africa Spain Switzerland U.K. U.S. Poland	(273)
	Entero-toxigenic *E. coli* (ETEC)	362	355	*Escherchia coli*		Argentina Bangladesh China Egypt Guatemala	Indonesia Kenya Mexico	(274)
Regional	Nepal	90	87	*K. pneumoniae*		Nepal		(269)

K. pneumoniae					
U.K.	N.P.	294	*K. pneumoniae*	U.K.	N.P.
K. pneumoniae			*Enterobacter cloacae*		
E. cloaceae					
MSM *Shigella*	207	171	*Shigella flexneri*	U.K.	(268)
flexneri				Oceania	
Vietnam	263	223	*Shigella sonnei*	Vietnam	(270)
Shigella sonnei					
Africa/UK	129	95	*Salmonella enterica*	Africa	(272)
Salmonella			serovar Typhimurium	U.K.	
Tyhimurium					

N.P., not published;[a] designates countries where strains included in analysis were isolated.

Table S4 - Sub-typing of the PlasmidFinder database.

Replicon[a]	Assigned Inc sub-type	Replicon size	Target sequence
IncA/C_1__FJ705807	A/C_1	417	GAGAACCAAAGACAAAGACCTGGAGAAACTCGACGTTATCAAAGACTCAC
IncA/C2_1__JN157804	A/C_2	417	GAGAACCAAAGACAAAGACCTGGAGAAACTCGACGTAATCAAAGACTCAC
IncB/O/K/Z_1__CU928147	$B/O/K/Z_1$	151	TCCGGAAAGTCAGAAAACGGCAGGATGCGCCATAAGGCATTCAGGATGTA
IncB/O/K/Z_2_GU256641	$B/O/K/Z_2$	160	GCGGTCCGGAAAGCCAGAAAACGGCAGAATGCGCCATAAGGCATTCAGGA
IncB/O/K/Z_3_GQ259888	$B/O/K/Z_3$	152	TCCGGGAAGTCAGAAAATGGCAGGATGCGCCATAAGGCATTCAGGACGTA
IncB/O/K/Z_4_FN868832	$B/O/K/Z_4$	149	TCCGGGAAGTCAGAAAACGGCAGGATGCGCCATAAGGCATTCAGGACGTA
IncFIA_1__AP001918	FIIC1	388	TGTCTGTGACAAATTGCCCTTAACCCTGTGACAAATTGCCCTCAGAAGAA
IncFIA(HI1)_1_HI1_AF250878	HI1 ST5	388	CTTTCTGTGACAAGTTGCCCTTAACCCTGTGACAAATTGCCCTCAGGAAG
IncFIB(AP001918)_1__AP001918	FII_{C1}	682	CTGTTTATTCTTTTACTGTCCACAGGCAGAAGGCTTTCTGGAAAACGAAA
IncFIB(K)_1_Kpn3_JN233704	FII_{K1}	560	GTTTGGGGTTGCGATAATGCACGCTGAAATAGGTGGCTCGGCCACGTTTA
IncFIB(Mar)_1_pNDM-Mar_JN420336	H	439	TATCAAGAGCCTTAAGGCGAAGATAAACCTTATAGTCAATCTGATAGAGC
IncFIB(pB171)_1_pB171_AB024946	ND	643	GGACAAGGACAATCTGGACATAAAAAAGCTGTTTGAAGAGGTGGATAAAT
IncFIB(pCTU1)_1_pCTU1_FN543094	ND	809	CCGGCGAGGTGGTCACGCTGGTTCCCAACCGTAACAACACGGTGCAGCCG
IncFIB(pCTU3)_1_pCTU3_FN543096	ND	693	TCATGGAGACATACAACGTACCGGCAGGCATACTTTCGAAATAGACATAA
IncFIB(pECLA)_1_pECLA_CP001919	ND	560	GTTTCGGATTACGGTAATGCACGCTGAAGTAGGTGGCCCGGCCGCGTTTG
IncFIB(pENTAS01)_1_pENTAS01_CP003027	ND	560	GTTTTGAATTACGATAATGCACACTGAAGTATGTGGAGCGGCCACGCTTG
IncFIB(pENTE01)_1_pENTE01_CP000654	ND	560	GAATGAGAATCTAACCTCAGTCCACTGGTCAAACCTGCCTGCAGATGAGC
IncFIB(pHCM2)_1_pHCM2_AL513384	ND	875	CAAATGGTCTCTATGGACGCCTCTGCTGAACTCAAACAGCTGTCTCTGGC
IncFIB(pKPHS1)_1_pKPHS1_CP003223	ND	560	ATGCTGTCAGCGGTAAACGATATGCTGTCAGCGGTACGGTATATGCTGCC
IncFIB(pLF82)_1_pLF82_CU638872	ND	560	CAAATGGTCTCTATGGATGCCTCTGCTGAACTCAAACAGCTGTCTCTGGC
IncFIB(pQil)_1_pQil_JN233705	FII_{K2}	740	GAAGGTTATACAGACATCCGTATTACCGGCCCACGACTTTCGATGGAGAC
IncFIB(S)_1__FN432031	FII_{S1}	643	GGACAAGGACAATCTGGACATAAAAAAGTTGTTTGTAGAGGTGGATAAAT
IncFIC(FII)_1__AP001918	FII_{C1}	499	CACACCATCCTGCACTTACAATGCACAGAAGGAGTGAGCACAGAAAGAAG
IncFII_1__AY458016	FII_2	261	CACACCATCCTGCACTTACAATGCGCAGAAGGAGCGAGCACAGAAAGAAG
IncFII_1_pKP91_CP000966	FII_{K4}	230	TTTTGGTGTGCCACGCCGTAAGGTGGCAGGGAGCTGGTTTTGTGGATGTT
IncFII_1_pSFO_AF401292	FII_3	258	CTGATCGTTTAAGGAATTTTGTGGCTGGCCACGCCATAAGGTGGCAGGGA
IncFII(29)_1_pUTI89_CP003035	FII_{29}	259	CACACCATCCTGCACTTACAATGCGCAGAAGGAGCGAGCACAGAAAGAAG
IncFII(K)_1__CP000648	FII_{K1}	148	TCTTCTTCAATCTTGGCGGAAGGAAAAGATTAACGGGGCCTTCATAAACT

IncFII(p14)_1_p14_JQ418538	closest FII_{Y3}	262	TTTGAAGAATTCTGATGGCTGGCCACGCCGTAAGGTGGCAGGGAACTGGT
IncFII(p96A)_1_p96A_JQ418521	closest FII_{S3}	534	GTGACTGATGTTACACATTACCTACAGGTCAAAAATCCTAACCCTCAGTT
IncFII(pCoo)_1_pCoo_CR942285	FII_{15}	262	CACACCATCCTGCACTTACAATGCGCAGAAGGAGTGTGCACAGAAAGAAG
IncFII(pCRY)_1_pCRY_NC_005814	ND	593	TCAGGCAAAGGTTGGGGAGTAGTATTCCTGTTTGCGCTGGCGAGCTGCGT
IncFII(pCTU2)_1_pCTU2_FN543095	ND	577	GAGTAGTATTCCTTTTTGCGTTCACGAGCTGCCTGGCGCTTGGCTTGCAT
IncFII(pECLA)_1_pECLA_CP001919	ND	747	GAATGACAATCAGATTTCTGTTCACTGGTCAGATCTACCGAAAGATGAGC
IncFII(pENTA)_1_pENTA_CP003027	ND	560	GAGTGACATTCCATTATCCGTCCACTGGTCTGAACTGCCGGAAGATGAAC
IncFII(pHN7A8)_1_pHN7A8_JN232517	FII_{33}	260	CACACCATCCTGCACTTATGTTGCACAGAAGGAGTGAGCACAGAAAGAAG
IncFII(pKPX1)_AP012055	closest FII_{Y4}	577	GAGAAATATTCCCGTTTACGCTGGCGAGCTACGTCGCGCTTAGCTTTCAT
IncFII(pMET)_1_pMET1_EU383016	ND	577	GAGTAGTATTCCTGTTTACGCTGGCGAGCTACTTCACGCTTCGCCTTCAT
IncFII(pRSB107)_1_pRSB107_AJ851089	FII_1	261	CACACCATCCTGCACTTATGTTGCACAGAAGGAGTGAGCACAGAAAGAAG
IncFII(pSE11)_1_pSE11_AP009242	FII_{20}	264	CACACCATCCTGCACTTACAATGCGCAGAAGGAGTGAGCACAGAAAGAAG
IncFII(pseudo)_1_pseudo_NC_011759	ND	390	GGGGGTTTTTGCATTCAAGGCCCGCTGTTCTGGTACCTTTTCCCTTGAGC
IncFII(pYVa12790)_1_pYVa12790_AY150843	ND	674	AAAACTGAAAAGAAAAGAACGCTTCGGAAACGGGGTGAACACTCAACTGA
IncFII(S)_1__CP000858	FII_{S1}	262	CTAAAGAATTTTGATGGCTGGCCACGCCGTAAGGTGGCAGGGAACTGGTT
IncFII(SARC14)_1_SARC14_JQ418540	closest FII_{Y5}	445	GTGCATATGCGCACGAATGCTGGCCGTGAGCGCGATTTCCGACAGGAATT
IncFII(Serratia)_1_Serratia_NC_009829	closest FII_{K11}	278	GGGGTTTTGCTTTTGTATCTCCCGGCTAACTGCGCCGAAAGTCCCTAAAT
IncFII(Y)_1_ps_CP001049	FII_{Y1}	227	TGGCAGGGAACTGGTTCTGCTAAGGTGTTTACTTGGAACCAGAAAAGCAA
IncFII(Yp)_1_Yersenia_CP000670	FII_{Y3}	230	TGGTAGGGAACTGGTTCTGATGAGGTGTCTACCCGGGACCAGAAAAGCAA
IncHI1A_1__AF250878	ND	420	CGGATCACTGGTCTTAAGCTTTCGATGTCAACCGATTTTAAGTGTTGGCT
IncHI1A(CIT)_1_pNDM-CIT_JX182975	HI_1 ST12	420	CGGATCACTGGCCTTAAGCTTTCGATGTCCACGGACTTCAAGTGTTGGCT
IncHI1B_1_pNDM-MAR_JN420336	HI_1 ST 1 or ST2	570	CTGATTCTTTTCGAGACAGGGTCTTCAATATTTTTAAGTAAAGTCGGGTC
IncHI1B(CIT)_1_pNDM-CIT_JX182975	HI_1 ST12	538	ATTCCAGAAAACCGATCTCTTTAAGCTGGCCCAGCGCCTTTTTAACTGTG
IncHI1B(R27)_1_R27_AF250878	HI_1 ST5	540	ATTCCAGAAAACCGATCTCTTTAAGCTGGCCCAGCGCCTTTTTAACCGTG
IncHI2_1__BX664015	HI_2 ST1	327	TTTCTCCTGAGTCACCTGTTAACACCCGGTTTCTACGCTTTACTTCATTG
IncHI2A_1__BX664015	HI_2 ST1	630	AGATCGGAGGGTTATGACGACATCAAGATAACTGGTGTCAAACTATCTAT
IncI1_1_Alpha_AP005147	I_1 ST13	142	CGAAAGCCGGACGGCAGAATGCGCCATAAGGCATTCAGGAGAGATGGCAT
IncI2_1_Delta_AP002527	I_2	316	CAGGCTTGAACATCGTTGATCGATTGCGCCCATGCTGCTAAATTTGCAGG
IncL/M_1__AF550415	M_2	664	CCGCCGAATATGGCGGGTTTTTTGTGTATACTCAAGTGGTTATAGTCGTA
IncL/M(pMU407)_1_pMU407_U27345	L/M	739	GGATGAAAACTATCAGCATCTGAAGAGTAAACTGACAGACGCTGGATGGT
IncL/M(pOXA-48)_1_pOXA-48_JN626286	L	661	CCGCCTGAAAAGGCGGTTTTTTCATGTATACTCAGGTGGTTATAGTCGTA

IncN_1__AY046276	N_1	514	GTCTAACGAGCTTACCGAAGCTGCTTACTACCTCTCGCTAAAAGCAAAGC
IncN2_1__JF785549	N_2	477	GGGTGAAGATATGGCATTCACCCACTCCATTCTGTGCCAGGTCGGTTTGC
IncN3_1__EF219134	N_3	477	TTATAATAGCCTTCGGACAGGGTGAGTGTTCCCGGCCATAGCGTGAGCTG
IncP_1_alpha_L27758	Pα	534	CTATGGCCCTGCAAACGCGCCAGAAACGCCGTCGAAGCCGTGTGCGAGAC
IncP(6)_1__JF785550	P6	806	TGTGAAACAGGCTGATAAGCGTGTCGTTCTTGGGCGTGTAGAGCGGCGCA
IncP(Beta)_1_Beta_U67194	P-1β	582	CCGATTCGAGCCGGCCGATGCGTTGGGATGAGAACTGCATGGCCGACGCC
IncQ1_1__HE654726	Q_1	450	GTCATGCTCGACAGGTAGGACTGCCAGCGGATGTTATCGACCAGTACCGA
IncQ2_1__FJ696404	Q_3	450	GTCATCTTGGCGACGAAGCCGCACCAGCGGGCGTTGTCGATCAGTGCCGA
IncR_1__DQ449578	R	251	TCGCTTCATTCCTGCTTCAGCCAGCCACGGACGTTTAACTTCTTCAAACT
IncT_1__AP004237	T	749	TTGGCCTGTTTGTGCCTAAACCATCAAGGTCATCTGACTACAGCCCGATG
IncU_1__DQ401103	U	565	TCACGACACAAGCGCAAGGGGCTTTTTCTCGCTCCTGGTGCAATGGCCAA
IncW_1__EF633507	W	243	CCTAAGAACAACAAAGCCCCCGGCCATCGTATCAACGAGATCATCAAGAC
IncX1_1__EU370913	X_1	374	ATGGCTAAAATCTATCAATTCCCTCAGGGGGAAGAACGTGCTAAATTCAG
IncX1_2__CP003417	X_1	348	GCTAAAATCTTCAATTCCCTCAGGGGAAAACGTGCTAATTCAGGAAGAAT
IncX1_3__CP001123	X_1	373	ATGGCTAAAATCTATCAATTCCCTCATGGGAAGAACGCGGGAAATTCAGG
IncX1_4__JN935898	X_1	377	ATGGCTAAAATCTATCAATTCCCCCAGGGGGACGAACGCGGTAAATTCAG
IncX2_1__JQ269335	X_2	374	ATGAGACTCAAGGTCATGATGGACGTGAACAAAAAAACGAAAATTCGCCA
IncX3_1__JN247852	X_3	374	ATGCGGTTGTTGCTATCTTTAGATATGAAGATCCTCAGATCTTCATATCT
IncX3(pEC14)_1_pEC14_JN935899	ND	374	ATGAGACTTAAAGTTATGATGGATGTGAATAAAAAAACAAAGATTCGTCA
IncX4_1__CP002895	X_4	374	ATGAGAATGACGACAAATAAGACTTCCCTTTCTCGCTTAACAAAAGTGAG
IncX4_2__FN543504	ND	712	ATGGTCTTAAAGAATAATAAAAATAGCGACTGTAATGATGTTCAAAGTTT
IncX5_1__NC_015054	X_5	374	ATGTTCATCTACAGTGTATAAATTAAGTTCTTCTTTTAATTCATCGATAG
IncX6_1__AM942760	X_6	374	ATGAGAGTAACGATGAATAAAAAATCACTATCTCGGTTAACTAAAGTCAG
IncY_1__K02380	Y	765	AATTCAAACAACACTGTGCAGCCTGTAGCGTTGATGCGCTTGGGGGTATT
p0111_1__AP010962	ND	885	ATGCTGGAAGAAAATAAAGGCTTCCTTAGCGTTGAAGAAGTTGCAGGAAA
pADAP_1__AF135182	ND	540	TTACTGGTCTATTTTTCGACGTTGAGCCCGAAGTTTGATGGCTTCCAGCG
pEC4115_1__NC_011351	ND	706	ATGTTGCCAGTAGAGGTGAATACGCGAGAGGGGGAGTTAATATTTCAATC
pENTAS02_1__CP003028	ND	979	TTGAGTCACCGCAGTGGTGGTTAAACCGCCTGCGCCGTATCCATGCCCGG
pESA2_1__CP000784	ND	750	ATGCTTATTCATGACGTAACGGAAAGAAAAAAAGCGTCAGCAAGTAAAAT
pIP31758(p153)_1_p153_CP000719	ND	909	GTTTACATCGCTATGTTGAAAATCGATAGCAAAGTTAAATTAGAGAATGG
pIP31758(p59)_1_p59_CP000718	ND	918	CCACCTCTGGTATGCAGTTTCATACGGTGTTAAAGATGCTTTCTTTATTT
pIP32953_1__BX936400	ND	927	AACATATCTCGCGATGAGATTCGTTTTCTATTCCTGGCATTAACTAAAAT

pJARS36_1__NC_015068	ND	534	ATGAGGATTCCGAAGAGAAGGAAACTTGGTTGCCAGCCAGTAAGAAAAAT
pSL483_1__CP001137	ND	995	TAACACAAGGCAATCAGCTTATTGAAGGTAGTTACGATATTAACCTGGCT
pSM22_1__NC_015972	ND	623	ACCACCCAGCGCCGGATCTTTTTCAGTCGGTTCTTCAGCGAGTGCTTGCG
pXuzhou21_1__CP001927	ND	720	TAACACAAGGCAATCAGCTTATTGAAGGTAGTTACGATATTAACCTGGCG
pYE854_1__AM905950	ND	979	TTGATGCTCTTGGCGGCTTAACACCTTCCCAATACTGCCGATGGTTGTGG

ND, Not defined; [a] designates the replicon name in the ResFinder Database.

Appendix D: Supplementary Orginal Manscript related to Chapter 6

The following appendix, published manuscript, has been provided to supplement discussion of this thesis. Part of this manuscript observed a number of plasmid types present prior to the emergence of AMR genes.

Baker KS, Burnett E, McGregor H, Deheer-Graham A, Boinett C, Langridge G, **Wailan AM**, *Cain A, Thomson NR, Russel J, Parkhill J*. The Murray collection of pre antibiotic era *Enterobacteriaceae*: A unique research resource. Genome Med. 2015 Sep 28;7(1):97. doi: 10.1186/s13073-015-0222-7.

The Murray collection of pre-antibiotic era *Enterobacteriacae*

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Abstract

Studies of historical isolates inform on the evolution and emergence of important pathogens and phenotypes, including antimicrobial resistance. Crucial to studying antimicrobial resistance are isolates that predate the widespread clinical use of antimicrobials. The Murray collection of several hundred bacterial strains of pre-antibiotic era *Enterobacteriaceae* is an invaluable resource of historical strains from important pathogen groups. Studies performed on the Collection to date merely exemplify its potential, which will only be realised through the continued effort of many scientific groups. To enable that aim, we announce the public availability of the Murray collection through the National Collection of Type Cultures, and present associated metadata with whole genome sequence data for over half of the strains. Using this information we verify the metadata for the collection with regard to subgroup designations, equivalence groupings and plasmid content. We also present genomic analyses of population structure and determinants of mobilisable antimicrobial resistance to aid strain selection in future studies. This represents an invaluable public resource for the study of these important pathogen groups and the emergence and evolution of antimicrobial resistance.

Background

Antimicrobial resistance (AMR) in bacteria represents a global public health crisis, and AMR in Enterobacteriaceae is a particularly recognised threat [1, 2]. This bacterial family includes pathogenic genera (e.g. Salmonella, Shigella, Escherichia, Klebsiella) that are responsible for a significant proportion of the global diarrhoeal disease burden [3] as well as systemic and nosocomial infections, often associated with heightened virulence or AMR [4, 5]. To manage these pathogens, it is critical that we understand the emergence and the evolution of clinically relevant phenotypes. Pivotal to understanding pathogen emergence and evolution is the context in which it occurred, and historical isolates have greatly informed theories regarding the emergence, disappearance and primary reservoir hosts of the pathogens that cause plague, leprosy and tuberculosis [6-8]. More recently, isolates of Vibrio cholerae and Shigella flexneri sampled from before the widespread clinical use (and consequent evolutionary pressure) of antimicrobials, i.e. the 'pre-antibiotic' era, were used to examine the evolution of virulence and AMR in these pathogens [9, 10]. To expand these studies in our continued efforts to understand the emergence and persistence of AMR, historical isolates must be studied alongside their contemporary counterparts.

The Murray collection (the 'Collection') comprises several hundred bacterial strains (mostly Enterobacteriaceae) collected from diverse geographic locations largely in the pre-antibiotic era (between 1917 and 1954) [11]. The Collection was amassed by the late eminent microbiologist Professor Everitt George Dunne Murray over the course of his career [12], and was stored on Douglas digest agar slopes [13]. On E.G. D. Murray's death in 1964, the collection was passed on to his son, Robert Everitt George Murray, who was also an eminent microbiologist. In the early 1980s, R.E.G. Murray in collaboration with British

microbiologists, lyophilised and transferred subcultures of the Collection from The University of Western Ontario, Canada, to the National Collection of Type Cultures (NCTC) at Public Health England, where they are held today.

Use of the Collection to provide historical context has already yielded important insights regarding the state-of-play of enteric pathogens in the first half of the 20th century, and phenotypic shifts that have occurred since those times. Seminal work by scientists who coordinated the international transfer of the Collection showed that the machinery for the accumulation and plasmid-borne transfer of AMR (e.g. Incompatibility group types) [11, 14], were qualitatively similar to those of modern isolates, and this was also demonstrated for mercury resistance and Salmonella virulence determinants [15-17]. Other studies have demonstrated significant phenotypic shifts, including increased virulence and resistance to antimicrobials and antiseptics in Klebsiella sp. [18], and an increase in the magnitude and incidence of AMR in modern Escherichia isolates [19]. These studies however, merely exemplify the potential of the Collection. For example, its use to inform pathogen evolution through dating analyses remains entirely untapped, and enormous scope exists to further study the emergence and evolution of the pathogens, and their AMR and other traits.

In fact, the scale of the remaining work requires the coordinated expertise and effort of multiple microbiological research groups. Here, to serve that purpose, we announce the public release of the Murray collection isolates through formal accession of the 683 strains into the NCTC and provide the associated metadata. In addition to facilitating access to the physical strains, we verify the metadata by bacterial subtyping and analysis of whole genome sequencing data (also released here) generated for 370 of the strains. Finally, we present

preliminary phylogenetic and gene content analyses that will aid strain selection for future scientific studies.

Results and discussion

Collection composition and associated metadata

The Murray collection (as held by the NCTC) comprises 683 bacterial strains belonging to 447 equivalence groups (Table 1). Equivalence groups (Additional file 1: Table S1) included strains that were related in one of the following three ways: duplicate strains in the original collection with the same name and original date; colony variants detected during subculture in Canada before transfer to the UK; or derivatives (colony variants detected during receipt of strains at NCTC). The isolates were primarily Salmonella, Escherichia and Shigella (which are combined here), Klebsiella and Proteus (Table 1), and fell into variably diverse subgroups e.g. subspecies, serotypes beyond those designations (see Additional file 1: Table S2, Additional file 2: Figures S1, Additional file 3: Figures S2. Additional file 4: Figures S3, and Additional file 5: Figures S4). Bacteria outside of these four main genera (see Other, Table 1) were originally poorly designated e.g. coliform, Enterobacteriaceae, and were subsequently determined (see 'Confirming the collection' below) to belong to the main genera, or the following: Morganella, Rauotella, Aeromonas and Enterobacter (Tables 2, Additional file 1: Table S2).

Table 1. Summary of the collection contents by genus and time

	Collection			Sequenced		
	Total strains	Unique inc. equivalence groups	Years of isolation	Total strains	Unique inc. equivalence groups	Years of isolation
Salmonella	361	222	1917 - 1952	174	127	1917 - 1946
E. coli/ Shigella	256	174	1917 - 1954	140	121	1917 - 1954
Klebsiella	42	30	1920 - 1949	35	26	1920 - 1949
Proteus	18	16	1919 - 1940	14	12	1919 - 1940
Other sp.	6	6	1920 - 1940	7	6	1935 - 1940
Total	683	447 *		370	291 *	

* These totals affected by an equivalence group containing both *Klebsiella* (M45) and *Escherchia/Shigella* (M162)

Table 2. Assembly characteristics of the sequenced Murray collection isolates

		Assembly characteristics [mean (range)]			
Genus	No.	GC content	Total length (bp)	Contigs	N50 (bp)
Salmonella	174	52 (51 - 52)	4739744 (4450735 - 5136048)	44 (15 - 126)	316870 (70209 - 992086)
Escherichia/ Shigella	140	50 (50 - 51)	4679816 (3820214 - 5434207)	258 (63 - 567)	64933 (14204 - 369379)
Klebsiella	35	56 (55 -57)	5287110 (4980231 - 5582843)	172 (24 - 286)	117718 (58784 - 465957)
Proteus	14	39 (38 - 39)	3935672 (3823752 - 3991064)	35 (18 - 58)	313856 (201904 - 763476)
Morganella	3	51 (NA)	3842744 (3744830 - 3948322)	23 (19 - 29)	557210 (403231 - 664661)
Enterobacter	2	54 (NA)	5364204 (5291805 - 5436603)	58 (52 - 64)	341570 (341563 - 341 576)
Aeromonas	1	59	4494408	39	166907
Raoultella	1	55	5488300	33	336936

The demographic features (e.g. place, person, time) and clinical details of pathogen infection are often crucial in the interpretation of genotypic and phenotypic analyses on the isolated pathogen. Although many of these details are available for the Collection strains, this metadata is incomplete and somewhat imperfect. The diverse geographical origins of the collection "including Europe, Malta, the Middle East, northern Russia, India and North America" has been reported [11], but were not available for individual strains. Metadata held at the NCTC showed the strains originated from diverse clinical specimens, e.g. stool, urine, blood, antral washes, cerebrospinal fluid, but the clinical syndrome, e.g. meningitis, pneumonia, hepatitis and cholecystitis, or patient/supplier name were also alternatively recorded (Additional file 1: Table S2). This 'Origin' information was only available for approximately one quarter (n=150) of the strains. Contrastingly however, the large majority (92%, 628 of 683) of strains had a date or year noted on the original vial (Additional file 1: Table S2). When these dates were stratified by genus, a unique

time signature emerged, perhaps reflecting E.G.D. Murray's changing research interests over time (Figure 1a). Notably, these dates were presumed to be the date of isolation for the strains, but could also represent date of strain receipt, or some other event. Overall however, the novel analyses presented in this study largely support the original metadata demonstrating that it is, if imperfect, robust.

In addition to the published studies on conjugative plasmids that highlighted the importance of the collection for studying mobilisable-AMR [11, 14], efforts to comprehensively determine the full plasmid content of the collection were made in the late 1980s [20]. Using traditional plasmid preparation and gel electrophoresis techniques, this work determined the number and approximate sizes of plasmids contained in each of 489 Collection strain subcultures (from [14]). The findings showed that the strains contained between zero and seven plasmids each, and that certain genera contained more plasmids than others (Figure 1b, full results reproduced in Additional file 1: Table S2). Plasmids ranged in estimated molecular weight from 1 to 500 Md (though estimates $\geq$ 150Md were noted as likely to be inaccurate). Attempts to verify this plasmid content metadata among 271 strains that were made (see Additional file 6: Supplementary Material).

Confirming the collection

In order to confirm the genus designations in the Collection, modern laboratory and in silico tools were applied to a subset of strains. The subset included all ACPD Hazard Group 2 (HG2) organisms and excluded most known HG3 organisms (23 HG3 organisms were included), thereby excluding known Shigella dysenteriae and Salmonella enterica where the serovar was unknown (see Additional file 1: Table S2).

Of the total 683 isolates, 359 underwent MALDI-TOF analysis (of which 354 also underwent characterisation by 16s rRNA sequencing). Outside of the 'Other' genera discussed above (and see Table 1), the MALDI-TOF results were generally concordant, with the exception of three isolates (M108, M162, M144) originally designated as Klebsiella that were determined to be Escherichia/Shigella sp., and the misidentification of a Salmonella isolate (M179) as an Escherichia by 16s rRNA sequencing (Additional file 1: Table S2). Of the isolates that underwent MALDI-TOF analysis, 334 progressed to whole genome sequencing, alongside an additional 36 isolates not characterised by MALDI-TOF. Those revived isolates originally designated to be shigellae also underwent serotyping, and were largely confirmed (for 66 of 72 strains) to be either S. flexneri or S. sonnei as originally designated (Additional file 1: Table S2). Genus identification and in silico multi-locus sequence typing on whole genome sequencing data (Additional file 1: Table S2 and Additional file 7: Table S3) confirmed the MALDI-TOF designation, or the original genus designation in all cases.

Genomic analysis of the Murray collection

To verify the robustness of the Collection, as well as add value, provide further metadata, and facilitate the development of selection criteria for ongoing studies, 370 strains (representing 291 equivalence groups), mostly representative of the collection (Tables 1, ,2,2, Additional file 1: Table S2 and Additional file 7: Table S3) were whole genome sequenced. Some analyses of these genomes are briefly reported here, and more detail is given in the Additional file 6: Supplementary Material.

De novo assemblies created to facilitate core genome identification exemplified the unique genomic characteristics of each bacterial genus (Table 2, see Additional file 7: Table S3 for full results), which were similarly reflected in features of the core genomes including the discovery rate and final number and size of the core genome (Table 3, Fig. 2). For example, the Proteushad a lower GC content than the other genera (Table 2) and Salmonella strains had a larger core genome (Table 3) than Escherichia/Shigella, which had a larger accessory genome (Fig. 2).

Table 3. Core genome size for the main genera in the Collection

	Salmonella	Escherichia/ Shigella	Klebsiella	Proteus
Total isolates (inc. refs)	185	185	37	14
Core genes (≥ 95% isolates)	3002	1983	3296	2870
Core genes (100% isolates)	2159	1255	2966	2813
Core genome (CG) length (bp)	2195115	1381269	2881098	2775840
CG variant sites (bp)	136888	114723	64138	47079

To provide enhanced subgrouping information, core genome phylogenies were constructed from the variant sites in core genes for the main genera (Additional file 2: Figure S1; Additional file 3: Figure S2; Additional file 4: Figure S3 and Additional file 5: Figure S4). In addition to providing context for future strain selection, core genome phylogenies were used to verify the designation of equivalence groups within the Collection.

Antimicrobial resistance

Although no phenotypic studies of AMR were done here, AMR has been reported in the pre-antibiotic era Murray Collection strains, including tetracycline resistance in Proteus sp., ampicillin resistance in the Klebsiella and both ampicillin and kanamycin

resistance in Escherichia sp. [11, 18, 19]. To aid the future selection of isolates based on the potential presence and absence of AMR, the presence of antimicrobial resistance genes among the strains was determined (Table 4). This revealed many resistance genes (often known to be chromosomally encoded) that were present across all members of a genus, particularly across Salmonella, Escherichia/Shigella and Klebsiella whose profiles differed greatly, though unsurprisingly, from the more phylogenetically remote Proteus. Some genes however were differentially present among the genera with differing degrees of correlation to population structure (Table 4, Figure S3). For example, the tetC gene was present in nearly all Klebsiella isolates, but only a fraction of Escherichia/Shigella and Salmonella isolates, highlighting the potential of the Collection for studying the early horizontal transmission of AMR among Enterobacteriaceae.

Summary

This study comprehensively describes a large collection of diverse bacteria (primarily Enterobacteriaceae) from the pre-antibiotic era, now publicly available from the NCTC, and thus represents an invaluable resource for studying the evolution and emergence of AMR and Enterobacteriaceae. We also created a significant genomic resource for the scientific community in the form of freely available whole genome sequencing data for over half of the strains in the Collection. Using this data, we verified much of the metadata of the Collection including species identification, plasmid content and the existence of equivalence groups among the strains. Finally, we presented additional analyses to guide future scientific studies; defining the phylogenetic subgroups and genetic determinants of mobilisable AMR present in the Collection. The availability of these live isolates, associated sequencing data and

preliminary analysis to the scientific community will surely spark a spate of studies into the evolution and epidemiology of these pathogens and their antimicrobial resistances.

Availability of supporting data

The strains in the collection are available at the NCTC under the Murray Collection Identifiers, and accession numbers shown in Table S1. The whole genome sequencing data is available at the European Nucleotide Archive at (http://www.ebi.ac.uk/ena/data/view/PRJEB3255), according to the strain-specific accession numbers shown in Table S2.

Legends

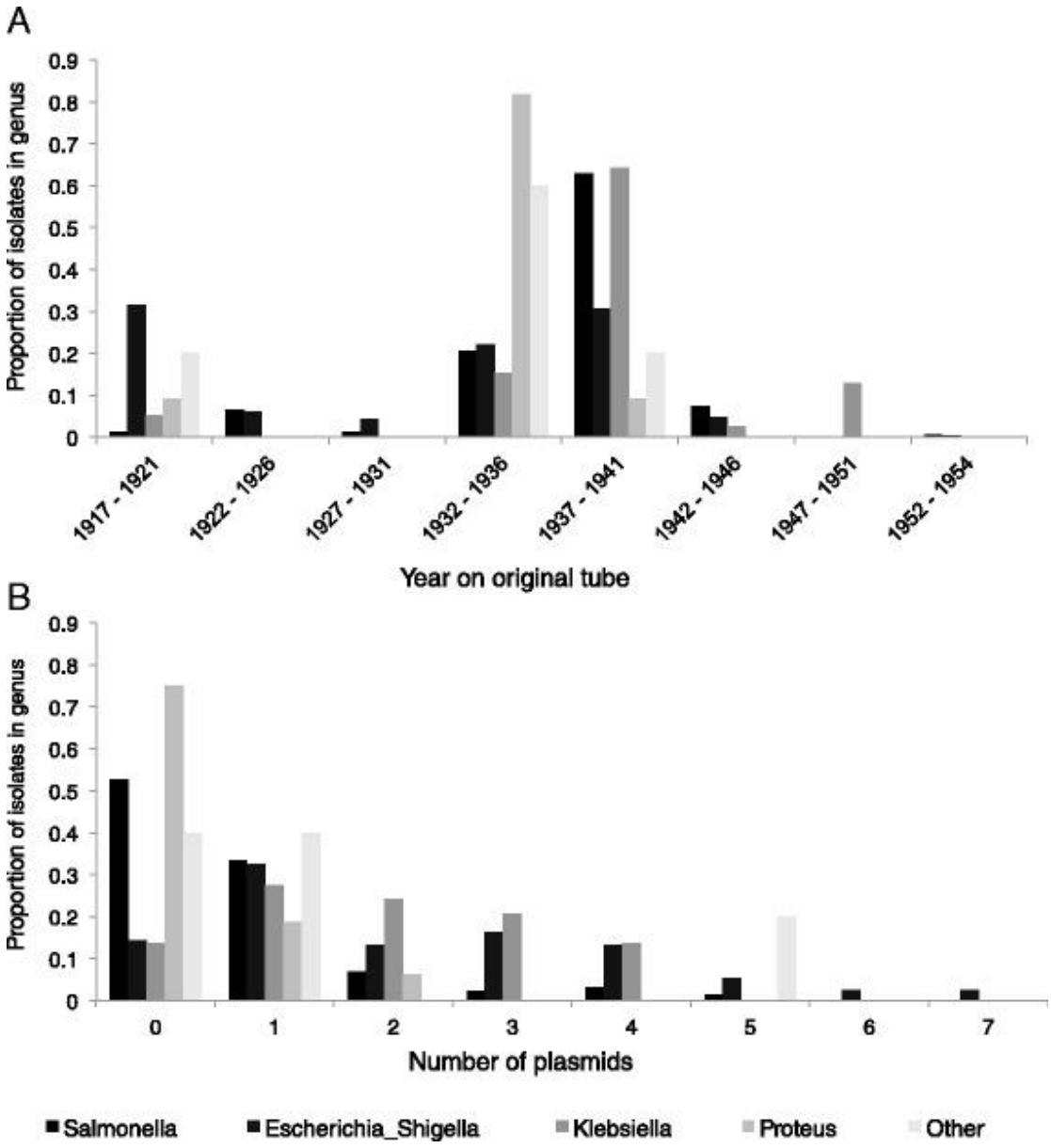


Figure 1. Metadata available for the Collection strains by genus, including year on original vial (A) and number of plasmids (B).

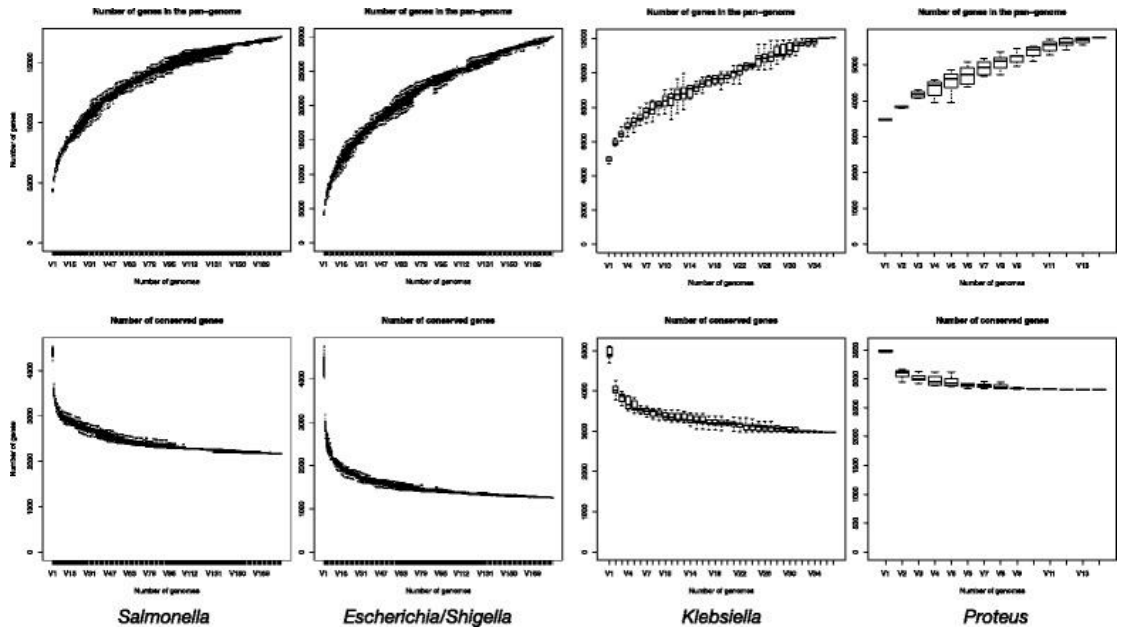


Figure 2. Rarefaction curves for pan- (above) and core- (below) genome sizes by genus.

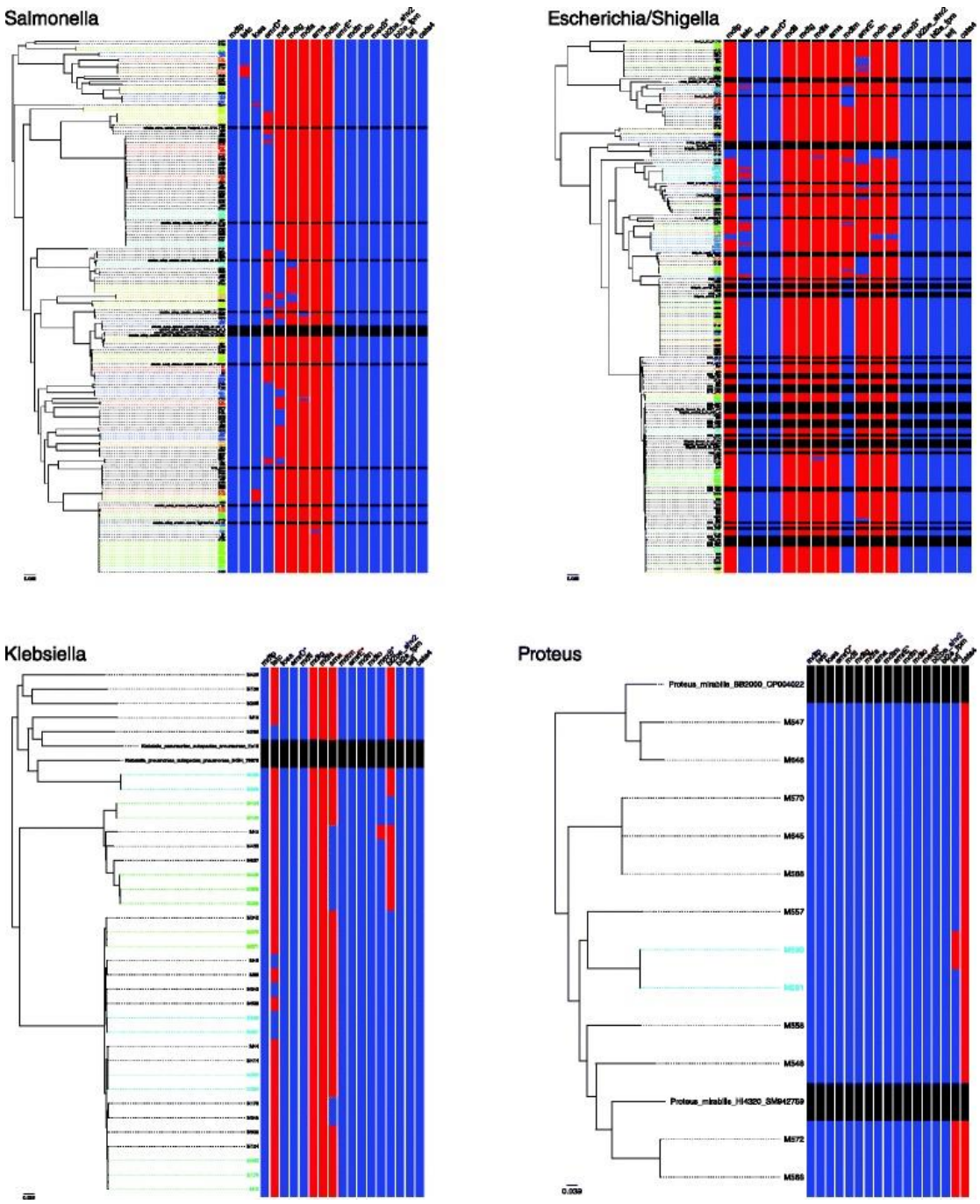
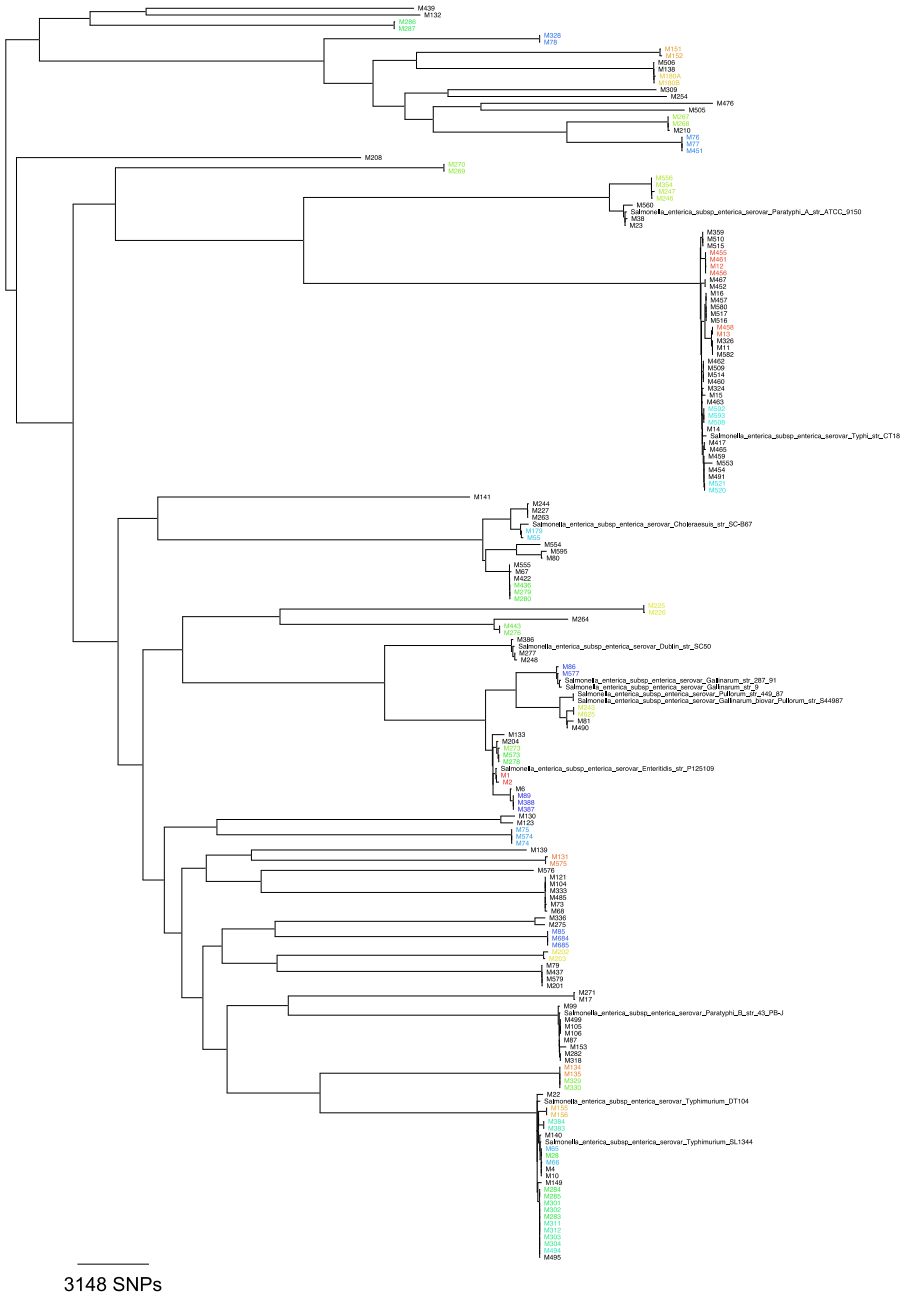


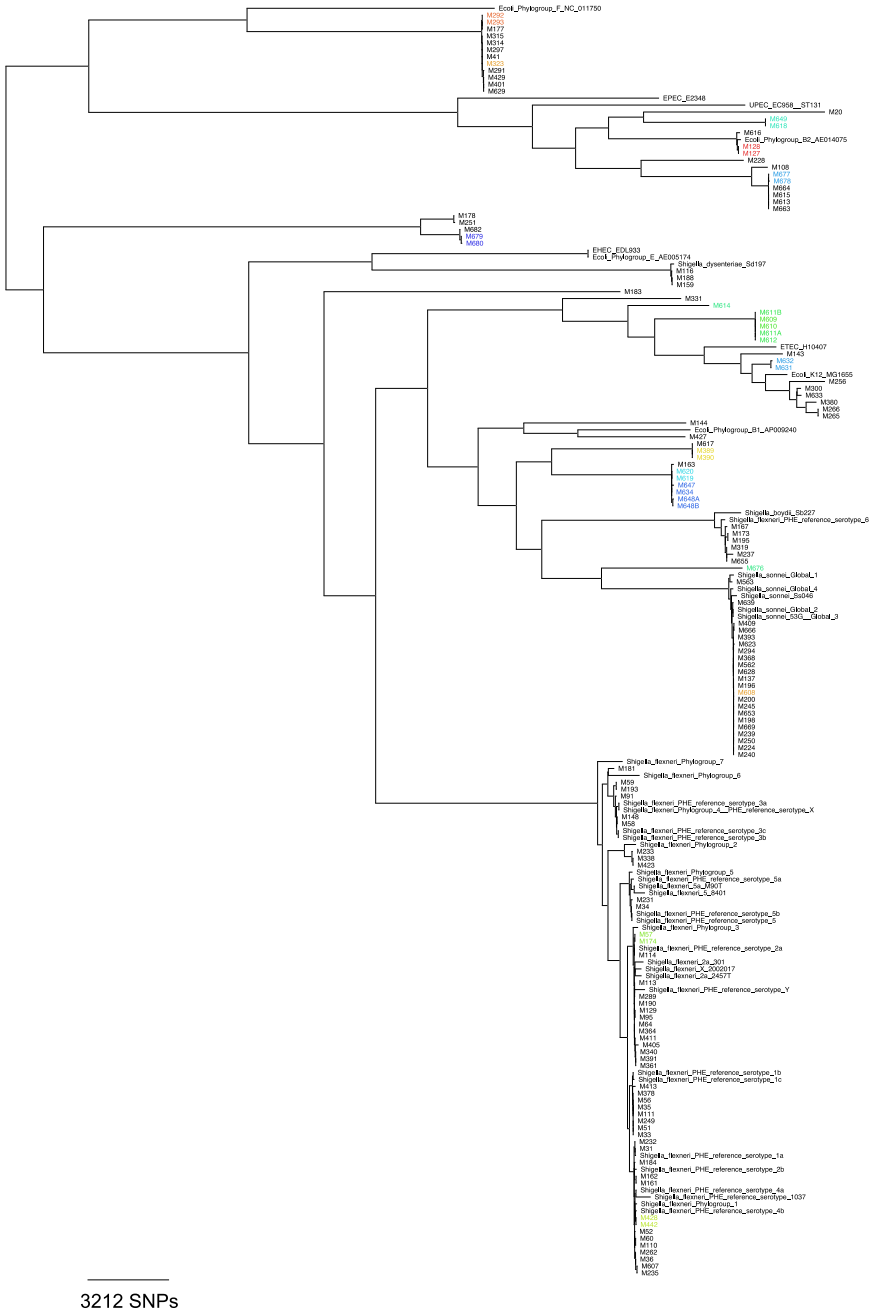
Figure 3. Presence (red) and absence (blue) of variably present antimicrobial resistance genes among the Collections strains overlaid adjacent to core genome phylogenies (as presented in Figures 2 – 5). The presence of genes in reference isolates was not determined (black).

Additional files

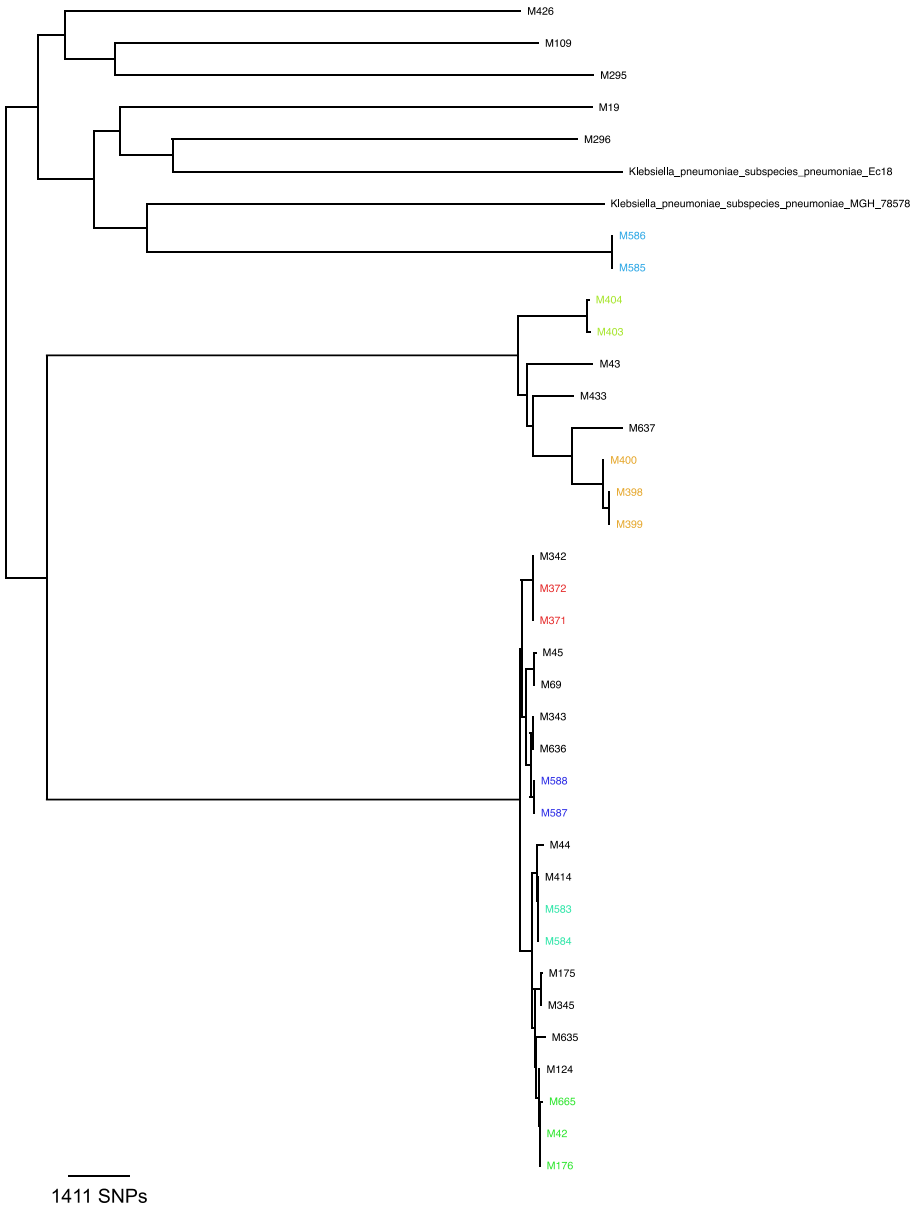
Additional file 1: Table S2. Original Collection metadata and laboratory determination of plasmid content and species.



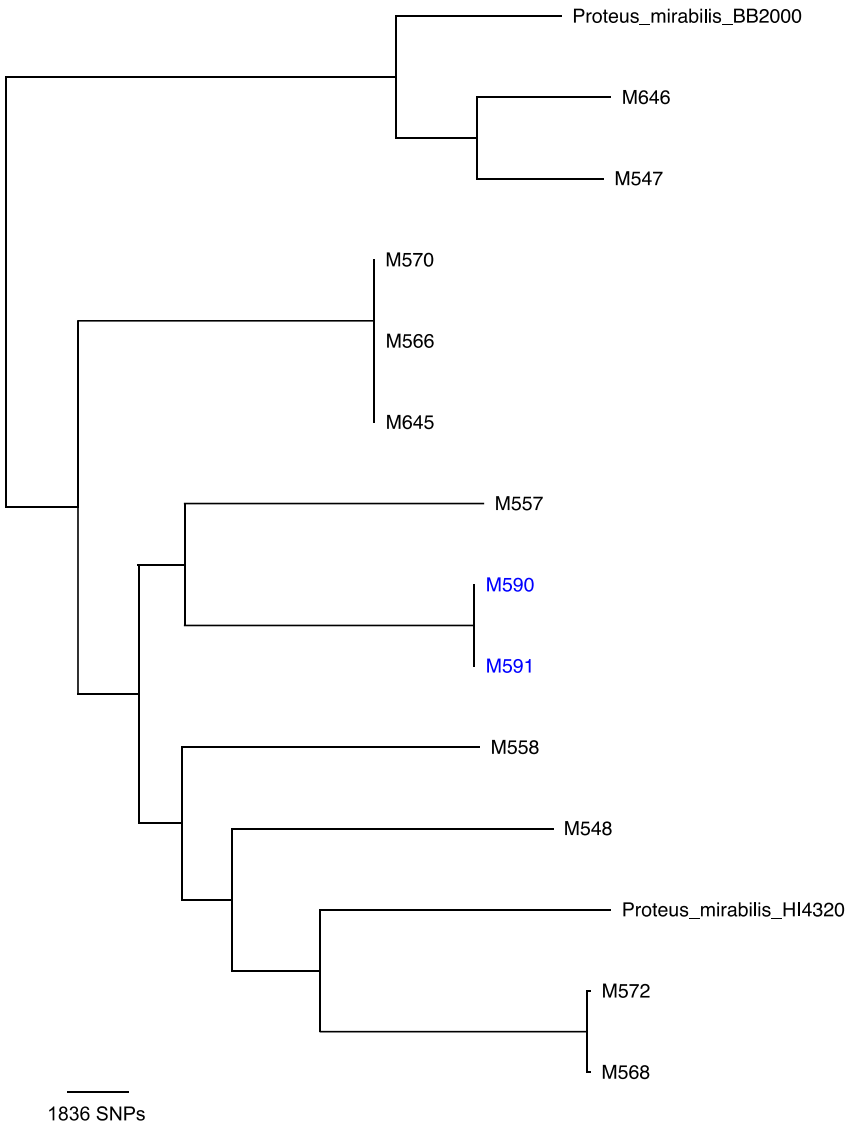
Additional file 2: Figure S1. Core genome phylogenetic tree for *Salmonella* sp. Strains noted to be in equivalence groups are similarly coloured.



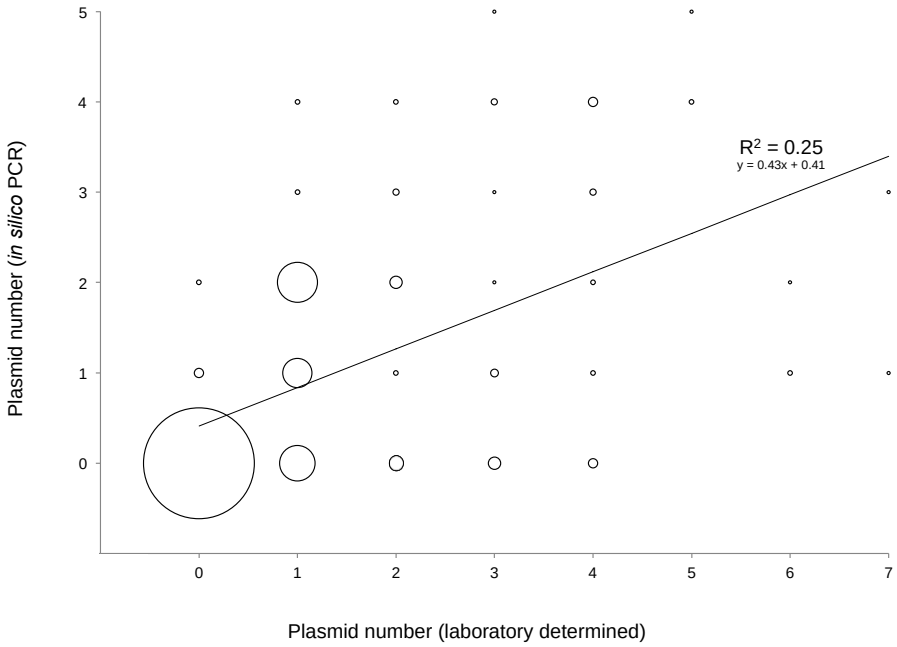
Additional file 3: Figure S2. Core genome phylogenetic tree for *Escherichia/Shigella* sp. Strains noted to be in equivalence groups are similarly coloured.



Additional file 4: Figure S3. Core genome phylogenetic tree for *Klebsiella* sp. Strains noted to be in equivalence groups are similarly coloured.



Additional file 5: Figure S4. Core genome phylogenetic tree for *Proteus* sp. Strains noted to be in equivalence groups are similarly coloured.



Additional file 6: Supplementary Material. Figure S5. Number of plasmids detected in 271 Collection strains by laboratory and *in silico* approaches. Marker size is scaled by the number of strains and the trendline represents the overall correlation.

Additional file 7: Table S3. Sequencing, assembly and gene content analyses for strains sequenced for this study.

Additional file 8: Table S1. Antimicrobial resistance genes in sequenced strains by genus

	Proportion of isolates containing gene			
Antibiotic Resistance Gene	*Salmonella*	*Escherichia/Shigella*	*Klebsiella*	*Proteus* (inc. *P. vulgaris*)
bl3_cpha	0.00	0.00	0.00	0.00
catA2	0.00	0.00	0.00	0.00
bl2e_fpm	0.00	0.00	0.00	0.14
tetJ	0.00	0.00	0.00	0.21
catA4	0.00	0.00	0.00	0.86
fosA	0.03	0.00	0.00	0.00
*ermD**	0.25	0.00	0.00	0.00
pbp2	1.00	0.00	0.00	0.00
*mexB**	0.00	0.00	0.03	0.00
bl2be_shv2	0.00	0.00	0.37	0.00
tetC	0.02	0.07	0.86	0.00
mdtM	1.00	0.44	0.00	0.00
*emrE**	0.00	0.87	0.00	0.00
mdtN	0.00	0.96	0.00	0.00
mdtO	0.00	0.96	0.00	0.00
mdtP	0.01	0.96	0.00	0.00
mdfA	0.99	0.99	1.00	0.00
bl1_ec	0.00	1.00	0.00	0.00
mdtE	0.00	1.00	0.00	0.00
mdtF	0.00	1.00	0.00	0.00
mdtL	0.83	1.00	0.00	0.00
arnA	0.99	1.00	0.77	0.00
mdtG	0.97	1.00	1.00	0.00
acrA	1.00	1.00	1.00	0.00
acrB	1.00	1.00	1.00	0.00
bacA	1.00	1.00	1.00	0.00
bcr	1.00	1.00	1.00	0.00
ksgA	1.00	1.00	1.00	0.00
macB	1.00	1.00	1.00	0.00
mdtH	1.00	1.00	1.00	0.00
mdtK	1.00	1.00	1.00	0.00
tolC	1.00	1.00	1.00	0.00
Total isolates (number)	174	140	35	14

* dependencies not met

Acknowledgements

The authors thank David Harris and the WTSI sequencing teams for coordination of sample sequencing, and Philippa Bracegirdle and Steven Brimble for assistance in designing initial work flows at PHE. WTSI authors were funded by grant number 980561. CB and AC are supported by MRC grant G1100100/1. KSB is in receipt of a Wellcome Trust Postdoctoral Training Fellowship for Clinicians (106690/Z/14/Z). The authors are also grateful to Vicki Hughes, Naomi Datta, Tegid Matthews, Peter Sneath, Laurence Rowland Hill, Rita Legros, and R.E.G. Murray who coordinated the transfer of the strains to the NCTC and performed the laboratory analysis of plasmid content.

Abbreviations

AMR	Antimicrobial resistant/resistance
NCTC	National Collection of Type Cultures
UK	United Kingdom
MLST	Multi-Locus Sequence Typing
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation - Time of Flight
rRNA	ribosomal Ribose Nucleic Acid
CG	Core Genome
GC	Guanine-Cytosine

Footnotes

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

KSB collated metadata, performed the whole genome sequence data analysis and drafted manuscript. EB and HMcG recovered strains, performed identification tests and prepared lysates for sequencing. ADG recovered and prepared lysates of HG3 isolates. CB and GL selected references and provided helpful discussions on the manuscript. AW and AC performed *in silico* plasmid analysis. NRT provided helpful discussions on the manuscript. JP and JER conceived study and facilitated and guided work performed at the WTSI and NCTC respectively. All co-authors contributed to manuscript writing and read and approved the final manuscript.

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Appendix E: Supplementary Conference Presentation related to Chapter 2

The following appendix, conference poster presentation, was published and presented during candidature.

The genetic analysis of various NDM-producing Gram-negatives to investigate the inter-species and inter-patient transmission of *bla*$_{NDM}$ within a clinical setting. **Wailan AM**, *Sartor AL*, *Paterson DL*, *Perry JD*, *Sidjabat HE*. 25th European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, Denmark, April 2015

Abstract

Objectives

First reported in 2008, *bla*$_{NDM}$ is a highly transmissible gene that provides Gram-negative bacteria resistance to virtually all beta-lactams including carbapenems. It is theorised to have originated from *Acinetobacter* species through the mobilisation of Tn*125*. This investigation analysed the genetic context of *bla*$_{NDM}$ within two sets of clinical isolates in order to describe and provide an insight into the interspecies and inter-patient clinical transmission of *bla*$_{NDM}$.

Methods

Clinical isolates were obtained from stool samples of three patients from two military hospitals in Pakistan. Plasmid Replicon typing was performed to identify plasmid Inc types. The first set (S1) included 4 NDM producing species isolated from one patient: *Enterobacter cloacae* (PN2 – IncA/C), *Acinetobacter baumannii* (PN3), *Klebsiella pneumoniae* (PN4 - IncN) *Citrobacter freundii* (PN5 - IncA/C). The second set (S2) included isolates from two different patients: First patient – *Pseudocitrobacter faecalis* (PN13 – IncN) and *Escherichia coli* (IncI1, IncN, Inc FIA, IncFIB) and second patient *P. faecalis* (IncN) and *E. coli* (IncHI1, IncA/C). Whole Genome Sequencing of these isolates was prepared by paired-end libraries and sequenced through the

Illumina MiSeq platform. CLC Genomics Workbench was used to *de novo* assembly the Illumina reads into contigs. PCR-gap closure was performed and contigs were analysed via BLAST and IS finder and manually annotated.

Results

Analysis of the genetic surroundings provided different contexts of *bla*$_{NDM}$ all of which contained a truncated Tn*125*. Within S1, PN2 had a NDM IncA/C genetic context similar to pNDM-US, PN3's NDM genetic context was the only structure with full IS*Aba125* and was similar to published Acinetobacter spp. sequences, pAbNDM-1 and pNDM-BJ01, PN4's NDM genetic context indicated a plasmid Class one integron capture and PN5's NDM genetic context had a 7, 288bp Tn*125* structure including Acinteobacter backbone (5'-IS*Aba14*|*aphA6*-3') similar to pNDM-BJ01. All S2 isolates (PN13, PN14, PN27, PN28) had identical genetic contexts involving a Class one integron/ IS*CR1* capture. This indicated a conjugation event amongst these isolates possibly with an IncN type plasmid. Further, all S2 isolates had genetic contexts identical to PN4 from S1, indicating an inter-species and inter-patient transmission of *bla*$_{NDM}$.

Conclusion

bla$_{NDM}$ is a highly transmissible gene that provides resistance to virtually all beta-lactams including carbapenems. The diversity of plasmid types and bacterial species harbouring *bla*$_{NDM}$ within one patient (S1) highlights the ability of *bla*$_{NDM}$ to be acquired and retained upon various plasmid backbones and chromosomes within different bacterial hosts. Furthermore, the ability for *bla*$_{NDM}$ to be acquired on various plasmids facilitates the potential for rapid transmission of carbapenem resistance to other species and patients, as observed with the IncN plasmid of this study.

ECCMID 1424

The genetic analysis of various NDM-producing Gram-negatives to investigate the inter-species and inter-patient transfer of *bla*$_{NDM}$

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Introduction

New Delhi Metallo-β-lactamase (*bla*$_{NDM}$)

- **Antimicrobial Resistance** to virtually all β-lactams including carbapenems[1,2,3]
- Highly transmissible gene via Enterobacteriaceae plasmids [2,3]
- Common in Gram-negative bacteria[1,2,3]
- Theorised origin: *Acinetobacter* spp.[4]
  - Mobilisation of all, or part of, Tn*125*[5]

Scenario

Two military hospitals in Rawalpindi, Pakistan

Four patients: Each multiple NDM-1 producers of different species

Investigation: Analyse the genome characteristics & genetic context of *bla*$_{NDM}$

Purpose: In order to describe and provide an insight into the interspecies and inter-patient clinical transmission of *bla*$_{NDM}$

Methods

11 NDM-producing species

- *Escherichia coli* (*n=3*)
- *Enterobacter cloacae* (*n=2*)
- *Citrobacter freundii* (*n=2*)
- *Pseudocitrobacter faecalis* (*n=2*)
- *Klebsiella pneumoniae* (*n=1*)
- *Acinteobacter baumannii* (*n=1*)

Plasmid characterisation

- Conjugation
- PCR-Based replicon typing[6]

Whole Genome Sequencing
Illumina Miseq – 150bp pair-end
Annotation and analysis

- CLC Genomics Workbench 7.5
- IS finder (www-is.biotoul.fr/)
- Center of Genomic Epidemiology
- MLST, Plasmid finder, Resfinder

Results – Genetic Contexts of *bla*$_{NDM}$

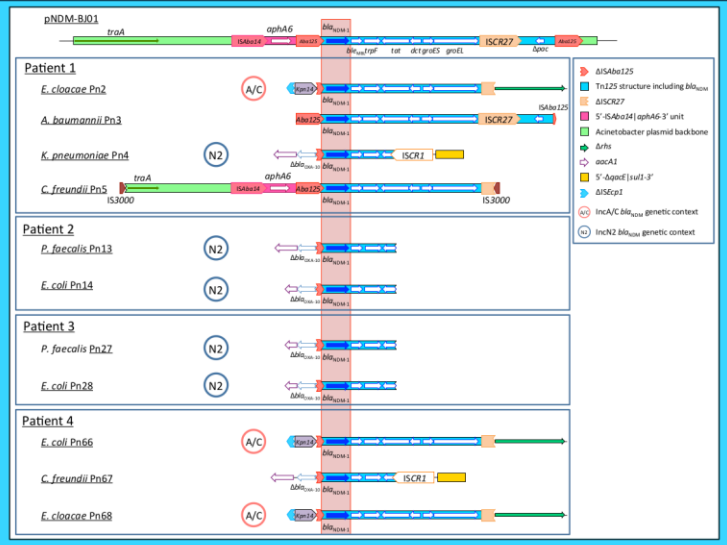

Figure 1. Schematic representation of the genetic context of *bla*$_{NDM}$ for each isolate, with two repeated *bla*$_{NDM}$ genetic contexts found on IncN2 and IncA/C plasmids in different species. Red box: Highlighting the *bla*$_{NDM-1}$ in each isolate. pNDM-BJ01 plasmid: Acinetobacter plasmid with full Tn*125* transposon (Genbank Accession no. JQ001791) .

Results – Genomic Characteristics

Table 1. Genomic characteristics of isolates within this study

Patient	Isolate number	Bacterial Species	MLST (ST)	Plasmid finder (Inc)		Antimicrobial resistance determinants			
						Beta-lactam (*bla*)		Aminoglycoside	Fluoroquinolone
1	Pn2	*Enterobacter cloacae*	171	**A/C(2)*** HI2 HI2A FIA(HI1)		NDM-1 CTX-M-15 OXA-1	ACT-7 CMY-6 TEM-1B	*rmtC* *aac(3)-IId* *aacA4* *aadA1*	*aac(6')Ib-cr* *qnrB1*
	Pn3	*Acinteobacter baumannii*	113	N/A		NDM-1 OXA-64		*aph(3')-VIa*	-
	Pn4	*Klebsiella pneumoniae*	101	**N2*** R FIB(K) FII		NDM-1 CTX-M-15 OXA-10	TEM-1B OXA-1 SHV-1	*aac(3)-IIa* *aadA5* *aadA16*	*aac(6')Ib-cr* *qnrB66* *oqxA* *oqxB*
	Pn5	*Citrobacter freundii*	20	**A/C(2)*** FIB(pHCM2)	HI2 HI2A	NDM-1 CTX-M-15 OXA-1		*aac(3)-IIa,* *aph(3')-VIa*	*aac(6')Ib-cr,* *qnrB34*
2	Pn13	*Pseudocitrobacter faecalis*	N/A	**N2*** FIB(K)	HI2 HI2A	NDM-1 CTX-M-15 OXA-10	TEM-1B OXA-1	*aadA16* *aadA1*	*aac(6')Ib-cr* *qnrB1* *qnrS1*
	Pn14	*Escherichia coli*	2598	**N2*** FIB*(AP001918) FIA* FIB(K)*	I1* HI2 HI2A Col156	NDM-1 CTX-M-15 OXA-10	TEM-1B OXA-1	*aadA16*	*qnrB1* *qnrS1*
3	Pn27	*Pseudocitrobacter faecalis*	N/A	**N2*** FIB(K)	HI2 HI2A	NDM-1 CTX-M-15 OXA-10	TEM-1B OXA-1	*aac(6')Ib-cr* *aadA16* *aadA1*	*aac(6')Ib-cr* *qnrB1* *qnrS1*
	Pn28	*Escherichia coli*	1431	HI2 HI2A HI1B(R27)* FII	HI1A* A/C2 Col(BS512)	NDM-1 CTX-M-15 CMY-4 OXA-9	OXA-10 TEM-1B OXA-1	*armA* *aadA16* *aadA1*	*aac(6')Ib-cr* *qnrB1* *qnrS1*
4	Pn66	*Escherichia coli*	10	**A/C2*** FIB(K)	Y*	NDM-1 CTX-M-15	CMY-6 TEM-1B	*rmtC* *aacA4*	*aac(6')Ib-cr qnrS1*
	Pn67	*Citrobacter freundii*	21	**FII(pMET)*** **FII(Yp)***		NDM-1 CMY-73	TEM-1B OXA-10	*aac(3)-IId* *aadA16* *aadA1*	*aac(6')Ib-cr qnrB54*
	Pn68	*Enterobacter cloacae*	171	**A/C(2)*** HI2 HI2A FIA(HI1)		NDM-1 CTX-M-15 CMY-6	OXA-1 ACT-7 TEM-1B	*rmtC* *aac(3)-IId* *aadA2*	*aac(3)-IIa* *aacA4* *aadA1* *armA*	*aac(6')Ib-cr* *qnrB1*

Inc – Plasmid Incompatibiltiy group N/A - No positive Plasmid Replicon Typing results for Acinetobacter species & no MLST scheme for *P. faecalis* Sequence Type (ST) * - Indicates the Replicon types identified via PBRT. Bold - Indicates Inc type of plasmid carrying *bla*$_{NDM}$

Genome characteristics differed between isolates (Table 1)

- ST, Plasmid replicon profiles, Antimicrobial resistance gene profile

All plasmids carrying *bla*$_{NDM}$ (except Pn3 and Pn5) were successfully conjugated

Two common plasmids were identified carrying *bla*$_{NDM}$ associated with specific genetic contexts (Figure 1)

- IncN2
- IncA/C

Conclusion

Here we describe the possibility of *bla*$_{NDM}$ dissemination via two plasmids (IncN2 and IncA/C), spreading carbapenem resistance below the level of nosocomial transmission i.e. between different bacterial species and genera.

Plasmid characterization and in-depth genetic assessment has provided an insight of *bla*$_{NDM}$-related spread, specifically associated with patients belonging to the same medical facility.

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Appendix F: Supplementary Conference Presentation related to Chapter 3

The following appendix, conference oral presentation, was published and presented during the candidature.

The prevalence plasmids types carrying *bla*$_{NDM}$ and genetic context of *bla*$_{NDM}$ providing carbapenem resistance to the *Enterobacteraceae* family in Australia. **Wailan AM**, *Paterson DL, Nimmo GR, Karina K, Ingram PR, Sidjabat HE*. Australian Society for Antimicrobials, 16th Annual Scientific Meeting – Antimicrobials 2015, Brisbane, Australia February 2015

Abstract

Background: New Delhi metallo-beta-lactamase gene (*bla*$_{NDM}$) is a worldwide concern as this gene provides carbapenem resistance to the *Enterobacteraiceae* family. While *bla*$_{NDM}$ is reported to transmit amongst *Acinetobacter* species within the transposon Tn*125*, *bla*$_{NDM}$ has been reported on different plasmid types within the Enterobacteracae with highly variable *bla*$_{NDM}$ genetic contexts, even on the same plasmid type. In recent years, there have been a number of reports of NDM producing Enterobacteraceae within Australia. This study aimed to characterise the *bla*$_{NDM}$ genetic context of NDM producing *Enterobacteraiceae* isolated within Australia to evaluate if there is a prevalent plasmid providing *Enterobacteraiceae* carbapenem resistance.

Methods:

10 NDM producing *Enterobacteriaceae* isolated between 2012 and 2014 were available for analysis. Each isolate was sequenced via the Illumina Hiseq2000 and *de novo* assembled via CLC Genomics Workbench. Isolates and their respective contig containing *bla*$_{NDM}$ were named as follows: pCR539-KP-NDM-1 (*Klebsiella pneumoniae* CR539), pCR77-ECL-NDM-1 (*Enterobacter cloacae* CR77), pCR774-EC-NDM-4 (*Escherichia coli* CR774), pCR121-EC-NDM-4 (*E. coli*

CR121), pCR473-ECL-NDM-7 (*E. cloacae* CR473), pSingWA-EC-NDM-4 (*E. coli* SingWA), pCR1113-KP-NDM-1 (*K. pneumoniae* CR1113), pCR1013-EC-NDM-1 (*E. coli* CR1013), pSDW-KP-NDM-7 (*K. pneumoniae* SDW), pKEC-EC-NDM-1 (*E. coli* KEC) and pCR1036-PR-NDM-1 (*Providencia rettgeri* CR1036).

Results: Each contig of the 10 isolates had a truncated Tn*125* structure carrying its *bla*$_{NDM}$. Two common plasmid types were identified, IncX types (3/10) and IncFII (5/10). The Tn*125* structure of each IncX type *bla*$_{NDM}$ context was flanked by IS*5* and IS*26*. There were three different *bla*$_{NDM}$ genetic contexts amongst the IncFII type plasmids, each with different IS elements flanking Tn*125*. pCR1036-PR-NDM-1 had an IncH1B-like *bla*$_{NDM}$ context and pCR539-KP-NDM-1 had an IncN2-like context.

Conclusion: Genetic characterisation of NDM producing *Enterobacterciaeae* has identified IncX type and IncFII as prevalent plasmids within Australia. Here we highlight the possibility for different plasmid types to acquire *bla*$_{NDM}$ and different *bla*$_{NDM}$ genetic context to exist in different *Enterobacteriaceae* species.

Appendix G: Supplementary Conference Presentation related to Chapter 4

The following appendix, conference poster presentation, was published and presented during candidature presented in this thesis.

The mechanisms of plasmid acquisition of *bla*$_{NDM}$ in *Enterobacteriaceae* ***Wailan AM,*** *Sidjabat HE* and *Paterson DL* Australasian Society for Infectious Diseases, Gram Negative 'Superbugs' Meeting, Gold Coast, Australia August 2013

Abstract

Genetic integrity of Tn*125* carrying *bla*$_{NDM}$ in *Enterobacteriaceae* from Australia and New Zealand

bla$_{NDM}$ is a gene encoding resistance to all β-lactam including the last line carbapenems and is known to be encoded on plasmids. It has been theorised that *bla*$_{NDM}$ originated in *Acinetobacter baumanni* and initially mobilised into plasmids by a transposon (Tn*125*) flanked by two insertion sequence (IS) elements, IS*Aba125*. The structure of Tn*125* comprises of multiple genes including *bla*$_{NDM}$ i.e. 5'-IS*Aba125*- *bla*$_{NDM}$-*ble*$_{MBL}$-*trpF-tat-dct-groES-groEL*-IS*CR27*-Δpac-IS*Aba125*-3'. This study aimed to observe the genetic structure of the Tn*125* structure within *Enterobacteriaceae* isolated in Australia and New Zealand.

NDM producing *Enterobacteriaceae* species included Australian *Klebsiella pneumoniae* (KP1), *Escherichia coli* (EC2) and *Enterobacter cloacae* (ECL3) and New Zealand *E. coli* (EC4). Analysis involved replicon typing, full genome sequencing, plasmid *de novo* assembly and annotation on CLC Genomics Workbench platform.

The plasmid backbones carrying *bla*$_{NDM}$ from were identified as IncA/C (KP1 and EC2) and IncFII (ECL3 and EC4). The Tn*125* structure of KP1, ECL3 and EC4 were similar 5'-ΔIS*Aba125-bla*$_{NDM}$-*ble*$_{MBL}$-*trpF-tat-dct-groES-groEL*-ΔIS*CR27*-3' with variation at the 3' end. EC2 had a variant

Tn*125* structure of 5'-ΔIS*Aba125*- *bla*$_{NDM}$-*ble*$_{MBL}$-Δ*trpF*-3'. All 5' end IS*Aba125* were partial, interrupted by an upstream IS element thus *bla*$_{NDM}$ was associated to this novel IS element. Associated IS elements were IS*Kpn14* (KP1), IS*903*-like (ECL3) while EC2 and EC4 had unknown IS elements. The similarities between the generalised structure of Tn*125* amongst these different isolates may indicate a mechanism *bla*$_{NDM}$ is able to traverse between different plasmid backbones, thus broadening the bacterial host range that can acquire *bla*$_{NDM}$ via plasmid transfer to become carbapenem resistant.

The mechanisms of plasmid acquisition of *bla*$_{NDM}$ in Enterobacteriaceae

Genetic Integrity of Tn*125* carrying *bla*$_{NDM}$ inEnterobacteriaceae from Australia and New Zealand

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2nd-3rd August 2013

Background

The recent emergence of New Delhi metallo-β-lactamase-1 (NDM-1), represents a significant public health threat providing pathogens with resistance to the last-line antibiotics, carbapenems, and leaving limited treatment options for such infections. *bla*$_{NDM}$ has been theorised to originate from *Acinetobacter baumannii* and initially mobilised from the chromosome onto plasmids via a transposon called Tn*125* [1]. This study investigated and determined the genetic environment of *bla*$_{NDM}$ in the context of Tn*125*, its surroundings and associated transposition mechanisms associated with *bla*$_{NDM}$ e.g. Insertion sequence (IS) elements, within NDM producing Enterobacteriaceae from Australia and New Zealand.

Methods

Four well-characterised strains of *bla*$_{NDM}$ harbouring Enterobacteriaceae were used in this study (Table 1). All four clinical isolates were genome sequenced using Illumina HiSeq 2000 and *de novo* assembled using CLC genomic workbench v 5.1. The *bla*$_{NDM-1}$ carrying plasmids were designated as follows: pKP1-NDM-1 (*Klebsiella pneumoniae* KP1), pEC2-NDM-3 (*Escherichia coli* EC2), pECL3-NDM-1 (*Enterobacter cloacae* ECL3) and pEC4-NDM-6 (*E. coli* EC4). The genetic surrounding of *bla*$_{NDM}$ in each plasmid (Table 1) was analysed and annotated according to available sequences from the GenBank, primarily using the Tn*125* sequence of *A. baumannii* 161/07 as a reference [2].

TABLE 1. NDM producing clinical isolates used in this study.

Donor isolate	Country of Isolation	Plasmid name	Plasmid Replicon Type	*bla*$_{NDM}$ variant
K. pneumoniae KP1[3]	Australia	pKP1-NDM-1	IncA/C	NDM-1
E. coli EC2 [4]	Australia	pEC2-NDM-3	IncA/C	NDM-3
E. cloacae ECL3 [4]	Australia	pECL3-NDM-1	IncFII	NDM-1
E. coli EC4 (ARL09/232) [5]	New Zealand	pEC4-NDM-6	IncFII	NDM-6

Results & Discussion

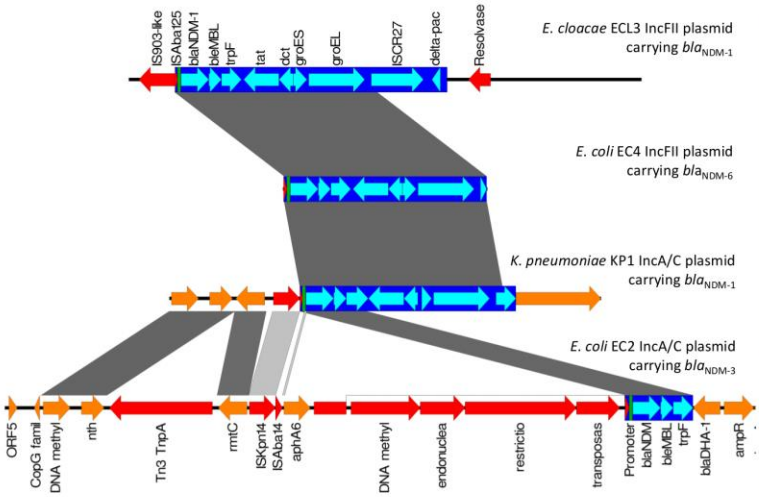

Figure 1. Contigs containing Tn*125* carrying *bla*$_{NDM}$ from Australian and New Zealand isolates
Red arrows – IS elements and transposases, Green box - Promoter, Blue arrows – Tn*125* associated genes, Blue rectangle – Remnant Tn*125* sequences, Orange arrows – other identified genes, Dark grey shade – 100% identical sequence, Light grey shade – 99% identical sequence, Inc – plasmid backbone *bla*$_{NDM}$ is located upon.

The genetic surroundings of each isolate's *bla*$_{NDM}$ is illustrated in Figure 1.

1. Tn*125* carrying *bla*$_{NDM}$ is a conserved region on all *bla*$_{NDM}$ harbouring plasmids

- *bla*$_{NDM-1}$ was located within Tn*125* in all isolates
  - Similar large Tn*125* structure: pKP1-NDM-1, pECL3-NDM-1 & pEC4-NDM-6
  - Smaller Tn*125* structure: pEC2-NDM-3

2. Common Tn*125* integration hotspot on IncA/C plasmids

Similarity between IncA/C plasmids harbouring *bla*$_{NDM-1}$

- pKP1-NDM-1 & pNDM-KN[6]:
  - Same Tn*125* structure, located in close proximity to *rmtC*
    - 1207bp vs. 1246bp i.e. IS*Kpn14*
- pEC2-NDM-3 & pKP1-NDM-1:
  - Upstream Tn*125* genetic surrounding = 100% similarity (disregarding 2 insertions)

Tn*125* insertion in the same vicinity to *rmtC* suggests a common integration hotspot for *bla*$_{NDM}$ transposition into IncA/C plasmid backbones.

3. Host IS element facilitate Tn*125* plasmids acquisition and structure depending on placement

- Each Tn*125* = ΔIS*Aba125*
- Novel IS elements associated with *bla*$_{NDM-1}$ & Tn*125*:
  - pKP1-NDM-1: IS*Kpn14*
  - pEC2-NDM-3: IS*Kpn14*-like element
    - IS*Kpn14*-like inserted further upstream from Tn*125*
      - Extended upstream gene mobilisation
      - Smaller Tn*125* structure
  - pECL3-NDM-1: IS*903*-like
  - pEC4-NDM-6: could not be determined

Tn*125* plasmid acquisition of is facilitated by native IS elements i.e. IS*Aba125* is not compatible for this transposition within Enterobacteriaceae hosts.

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Conclusion

Regardless of *bla*$_{NDM}$ variant, plasmid type and bacterial species:

1. Tn*125* is a conserved structure on *bla*$_{NDM}$ harbouring plasmids.
2. Transposition of Tn*125* is facilitated by native IS elements.
3. Tn*125* IncA/C plasmid acquisition may occur in a common hotspot.

This may infer that for this carbapenemase to rapidly spread to multiple species, native IS elements need to facilitate Tn*125* transposition in order for conjugative plasmids to acquire *bla*$_{NDM}$.

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Appendix H: Supplementary Conference Presentation related to Chapter 4

The following appendix, conference poster presentation, was published and presented during candidature presented in this thesis.

Complete Plasmid Sequence of IncA/C pKP1-NDM-1 from South East Queensland

Yam WK, **Wailan AW**, Alikhan N-F, Paterson DL, Petty N, Beatson SA, Schembri MA, Sidjabat HE Australasian Society for Infectious Diseases, Gram Negative 'Superbugs' Meeting, Gold Coast, Australia August 2013

Abstract

Background: New Delhi Metallo-β-lactamase (NDM-1) which confers resistance to carbapenems and third-generation cephalosporins has been reported to be mainly plasmid mediated with diverse plasmid replicon types, suggesting its mobility is due to mobile genetic elements (MGEs). The objective of this study was to characterise plasmid pKP1-NDM-1 recovered from *Klebsiella pneumoniae* sequence type (ST) 147 from South-East Queensland.

Methods: Complete DNA sequencing of pKP1-NDM-1 was obtained using HiSeq 2000 (Illumina). NDM-1 genetic environment was determined by plasmid cloning and sequenced by Sanger. Annotation was completed using Artemis and Pairwise alignment performed by a BLASTN and BLASTP homology search. PCR was performed to determine antimicrobial resistance genes and plasmid replicon type.

Results: pKP1-NDM-1 was ~170kb long and encoded ~151 predicted proteins. It harboured resistances genes encoding NDM-1 and RmtC within close proximity as well as CMY-6 and *ACC(6')-1b. Additionally several MGEs have been identified: class 1 integron, IS26 and IS1. It belongs to replicon type IncA/C* broad host range plasmid family with 9 out of 12 common region similarities. BLAST analysis showed 80% and 81% query with 100% identical with IncA/C pNDM-KN from *K. pneumoniae* from Kenya and IncA/C pNDM10469 from *Escherichia coli* from

Canada, respectively.

Conclusion: We described a broad host range multidrug resistance plasmid. pKP1-NDM-1 have close similarity to four other previously described IncA/C NDM-1 carrying plasmids with *rmtC* and $bla_{\text{CMY-6}}$ from other countries suggesting its affinity to this IncA/C type of plasmid. Whole plasmid sequencing provides information on the co-resistance carried by a broad host range plasmid.

Appendix I: Supplementary Conference Presentation related to Chapter 4

The following appendix, conference poster presentation, was published and presented during candidature.

Sequence comparison of plasmids carrying *bla*$_{NDM}$ from Australian and New Zealand *Enterobacteriaceae*, **Wailan AM**, *Petty NK, Sidjabat HE, Beatson SA, Schembri MA, Williamson DA and Paterson DL,* Australian Infectious Diseases, Lorne Infection & Immunity Conference 2014, Lorne, Australia February 2014.

Abstract

New Delhi metallo-β-lactamase-1 (NDM-1), a carbapenemase represents a significant global public health threat and theorised to have originated from *Acinetobacter* species through the mobilisation of a Tn*125*. This study investigated the genetic mechanisms of *bla*$_{NDM}$ spread from *Acinetobacter* species to the *Enterobacteriaceae* through the complete sequencing of plasmids carrying *bla*$_{NDM}$ from Australian clinical *Enterobacteriaceae* species, including *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacer cloacae* to theorise. The *bla*$_{NDM}$ genetic surrounding as well as plasmid structure was evaluated in order to describe *bla*$_{NDM}$ acquisition by IncA/C and IncFII plasmid as well as other genetic mobilisation of resistance mechanisms. This was achieved via next generation sequencing and bioinformatic analysis through both the Illumina platform and CLC Genomics workbench. The *bla*$_{NDM}$ genetic context in all isolates consisted of a truncated Tn*125* structure carrying *bla*$_{NDM}$ with a native IS element upstream e.g. IS*Kpn14*, regardless of *bla*$_{NDM}$ variant, plasmid backbone type and bacterial species. This may infer that plasmids capable of high conjugation rates have acquired *bla*$_{NDM}$ via transposition of a truncated Tn*125*, which could have provided the initial platform for *bla*$_{NDM}$'s rapid dissemination to multiple bacterial species within the *Enterobacteriaceae* family.

Sequence comparison of plasmids carrying *bla*$_{NDM}$ from Australian and New Zealand Enterobacteriaceae

Wailan AM[1,*], Petty NK[2,3,4], Sidjabat HE[1], Beatson SA[2,3], Schembri MA[2,3], Williamson DA[5] and Paterson DL[1,2,6,7,8]

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Introduction

New Delhi metallo-β-lactamase-1 (NDM-1), is a significant global public health threat providing resistance to the 'last-line' antibiotics, carbapenems[1]. This carbapenemase is commonly found on plasmids within Enterobacteriaceae[2]. These plasmids vary in their genetic backbone by the additional genes they have acquired. *bla*$_{NDM}$ is theorised to have originated from *Acinetobacter* species and has spread and acquired amongst these species via the mobilisation of a Tn*125* composed two flanking IS*Aba125*[3].

This study assembled the sequences of plasmids carrying *bla*$_{NDM}$ within Enterobacteriaceae from Australia and New Zealand, the *bla*$_{NDM}$ genetic environment in the context of Tn*125,* its surroundings for transposition mechanisms such as Insertion Sequence (IS) elements and plasmid comparison was also investigating.

Methods

Whole genome sequencing was performed on previously characterised NDM producing Australian & New Zealand Enterobacteriaceae (Table 1).

Table 1. NDM producing Enterobacteriaceae

Plasmid Name	Strain species & name	Country of Isolation	Plasmid Replicon	*bla*$_{NDM}$ variant
pEC2-NDM-3	*E. coli* EC2 [4]	Australia	IncA/C	NDM-3
pECL3-NDM-1	*E. cloacae* ECL3 [4]	Australia	IncFII	NDM-1
pEC4-NDM-6	*E. coli* EC4 (ARL09/232) [5]	New Zealand	IncFII	NDM-6

Sequences were assembled by:
- Sequencing: Illumina HiSeq 2000
- *De novo* assembly & Mapping
  - CLC Genomics Workbench 6.1
  - NCBI Genbank BLAST

- Annotation and Plasmid analysis
  - CLC Genomics Workbench 6.1
  - RAST (rast.nmpdr.org/)
  - IS finder (www-is.biotoul.fr/)
  - BRIG (sourceforge.net/projects/brig/) [6]

Results

A plasmid sequence carrying *bla*$_{NDM}$ from each NDM producing isolate was assembled

pEC2-NDM-3
- Size: 159, 834 bp
- Tn*125* size: 2, 946 bp
- IS element for *bla*$_{NDM}$ acquisition: IS*Kpn14*
- High similarity sequences:
  - pNDM102337,pNDM10469, pAR060302, pNDM10505
- Co-harbouring antimicrobial genes: *rmtC*, *aac3*, *bla*$_{CMY-6}$, *aac-6'-Ib*, *aphA6*, *bla*$_{DHA-1}$

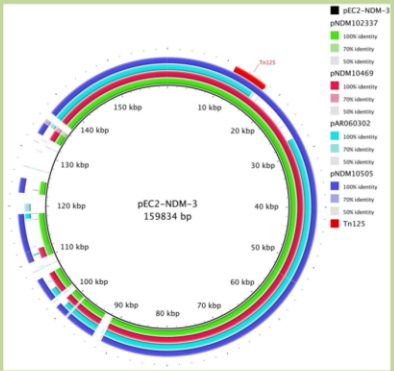

Figure 1. pEC2-NDM-3 BLAST comparison with pNDM102337, pNDM10469, pAR060302 and pNDM10505.

pECL3-NDM-1
- Size: 97, 252 bp
- Tn*125* size: 7, 977 bp
- IS element for *bla*$_{NDM}$ acquisition: IS*903*
- High similarity sequences:
  - pKOX_NDM1, plasmid MT, pG8786
- Co-harbouring antimicrobial genes: *rmtC*

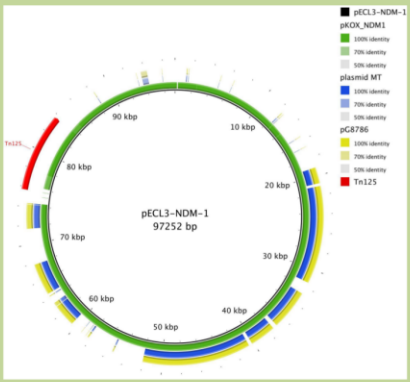

Figure 2. pEC2-NDM-3 BLAST comparison with pKOX_NDM1, plasmid MT and pG8786.

pEC4-NDM-6
- Size: 108, 970bp
- Tn*125* size: 5, 947 bp
- IS element for *bla*$_{NDM}$ acquisition: IS*CR27*
- High similarity sequences:
  - pKOX_NDM1, plasmid MT, pG8786
- Co-harbouring antimicrobial genes: *rmtC*

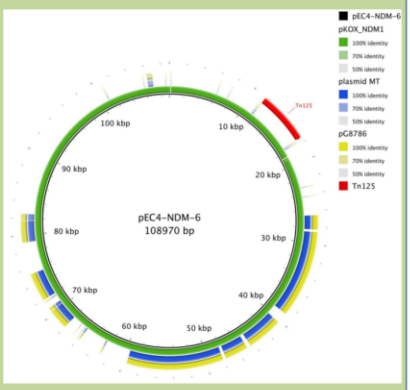

Figure 3. pEC4-NDM-6 BLAST comparison with pKOX_NDM1, plasmid MT and pG8786.

Discussion

pEC2-NDM-3
- The IncA/C plasmid is a common plasmid backbone[7] (~100 Kbp) to have acquired *bla*$_{NDM}$
- The variable region ~40kb contains various antimicrobial genes possibly acquired through IS elements

pECL3-NDM-1 (*E. cloacae)* & pEC4-NDM-6 (*E. coli*)
- Highest similarity to pKOX-NDM1 of *Klebsiella oxytoca*
- Plasmid MT and pG8786 are plamids both from *Yersinia pestis*
- The *Yersinia pestis* similarity may indicate the broad spectrum of these plasmid backbones and its ability to be retained by a broad-range of species

bla$_{NDM}$
- acquired within a Tn*125* structure
- mechanisms that facilitated acquisition include the IS elements founds in close proximity i.e. IS*Kpn14* , IS*903* and IS*CR27*

Conclusion

- **Plasmid backbones are able to acquire numerous antimicrobial genes leading to co-habouring of antimicrobial genes**
- ***bla*$_{NDM}$ can be acquired by IS elements onto plasmids in Enterobacteriaceae**

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Appendix J: Supplementary Conference Presentation related to Chapter 6

The following appendix, conference oral presentation, was presented and published during candidature. This presentation is provided to supplement discussion of this thesis and observed the increase in conjugation rate of plasmids harbouring bla_{NDM} genes in the presence of antimicrobials.

Conjugation Rates of the NDM Plasmid Conferring Carbapenem Resistance in *Enterobacteriaceae* ***Wailan A****, Paterson D, A Silvey, Williamson D, Sidjabat H* Australian Society for Antimicrobials, 13rd Annual Scientific Meeting – Antimicrobials 2012, Brisbane, Australia February 2012

Abstract

Objectives: The recent emergence of carbapenem resistance, New Delhi β-lactamase-1 (NDM-1), represents a significant threat and is found in numerous *Enterobacteriaceae* species, including nosocomial pathogens and human intestinal flora (*E. coli*). The bla_{NDM} gene can spread rapidly by residing in plasmids capable of a horizontal gene transfer process called conjugation. This study investigated the conjugation rate of NDM plasmids to *E. coli*, resembling bla_{NDM} gene acquisition by normal human flora in the gastrointestinal tract.

Methods: Five NDM producing *Enterobacteriaceae,* 2 *K. pneumoniae,* 2 *E. coli* and 1 E. cloacae, isolates were used as donor strains; and the recipient was sodium azide resistant *E. coli* J53. Conjugation was performed overnight on MacConkey agar with a 1:1 donor to recipient ratio. Transconjugants were analysed for plasmid acquisition through phenotypic and genotypic tests including S1 nuclease digestion. IncA/C plasmid conjugative machinery was determined pre- and post-conjugation.

Results: The replicon type of the NDM plasmid from each *K.pneumoniae* and *E.coli* was IncA/C, and the *E. cloacae* was IncFII. All but one (*K. pneumoniae*) of the NDM plasmids from the five

donor strains was transferable via conjugation. Conjugation rates of NDM plasmids from *K. pneumoniae* and *E. coli* were 9.0 x 10^{-4} and 1.4 x10^{-4} transconjugants per recipient, respectively. A synergistic effect of meropenem and ceftazidime in combination with sodium azide mitigated bla_{NDM} transconjugant growth. Sub-MIC ciprofloxacin inclusion into the environment *in vitro* enhanced the NDM plasmid conjugation rate by ~40 fold. Conjugation can alter the *tra* operon which may lead to defective conjugation machinery within transconjugants.

Conclusions: The spread of the NDM plasmid can occur at an astounding rate through conjugation. This process can be enhanced by ciprofloxacin introduction into the environment before conjugation, to either stimulate plasmid transfer or increase the frequency. However before a plasmid is able to transfer, it must encode genes known as the *tra* (transfer) genes to be able to initiate transfer from donor to recipient cells. Furthermore, the plasmid can become modified during the conjugation process to generate an alternative sized plasmid or multiple plasmids which can potentially enhance the spread of the bla_{NDM} gene.

Appendix K: Other Supplementary Manuscripts during Candidature

The following manuscripts were published and/or prepared during candidature, however do not form part of this thesis. The manuscripts that have been published are provided below.

Zowawi HM, Ibrahim E, Syrmis MW, **Wailan AM,** *AbdulWahab A, Paterson DL.* PME-1 Producing *Pseudomonas aeruginosa* in Qatar. Antimicrob. Agents Chemother. 2015 Jun;59(6):3692-3. doi: 10.1128/AAC.00424-15.

Toh B, Paterson D, Witchuda Kamolvit W, Zowawi H, Kvaskoff D, Sidjabat H, **Wailan AM**, *Peleg A and Huber C*. Species identification within the *Acinetobacter calcoaceticus - baumannii* complex using MALDI - TOF MS. J. Microbiol. Methods. 2015 Nov;118:128-32. doi: 10.1016/j.mimet.2015.09.006.

Toh B, Paterson DL, Pfluege V, Kvaskoff D, **Wailan AM**, *Riley T, Harper J, Flohr G, Huber C*. Relationships between whole genome sequencing, PCR ribotyping and MALDI - TOF MS in the subtyping of *Clostridium difficile*. Submitted to Diagn. Microbiol. Infect. Dis, 2015.

Zowawi HM, Ibrahim E, Syrmis MW, **Wailan AM,** *AbdulWahab A, Paterson DL.* PME-1 Producing *Pseudomonas aeruginosa* in Qatar. Antimicrob. Agents Chemother. 2015 Jun;59(6):3692-3. doi: 10.1128/AAC.00424-15.

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PME-1 Producing *Pseudomonas aeruginosa* in Qatar

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Running Title: PME-1 producing *P. aeruginosa* in Qatar

Key words: ESBL, PME-1, *Pseudomonas aeruginosa*, Qatar, Gulf.

Abstract

The newly discovered extended-spectrum β-lactamase, PME-1 was identified in a *Pseudomonas aeruginosa* isolated from pulmonary secretions of a 3 year old Qatari child, receiving prolonged ventilation in Doha. The patient had past hospitalizations in Saudi Arabia and the United Kingdom. Using whole genome sequencing, the isolate was found as sequence type (ST 654) and carrying multiple antibiotic resistance determinates beside $bla_{PME\text{-}1}$ such as $bla_{\text{GES-5,}}$ *strA*, *aph(3')-Via*, *aph(3')-IIb*, *strB*, *fosA*, *catB7*, *sul1*, *tet(A)* and *tet(G).*

Main body

The novel extended-spectrum beta-lactamase (ESBL), PME-1 was first described in 2010 from a Pseudomonas aeruginosa strain obtained from blood, surgical wound, and urine specimens from a single patient admitted to University of Pittsburgh Medical Center in 2008. The patient had prolonged hospitalization (6-months) in Dubai, United Arab Emirates, immediately before being transferred to the United States (1). We describe here the first case of P. aeruginosa carrying blaPME-1 isolated from Qatar and the second report to the date of this enzyme.

A 3 year old Qatari child developed pneumonia due to P. aeruginosa and Serratia marcescens. Her past history included tetralogy of Fallot for which she had undergone cardiac surgery in Saudi Arabia at the age of four months. Post-operatively she had a cardiac arrest and developed hypoxic ischemic encephalopathy. After seeking further medical care in the United Kingdom she became ventilator dependent. She was subsequently accommodated in a Paediatric Long Term Ventilation unit in Qatar, where she underwent mechanical ventilation via a tracheostomy. During this period she developed purulent pulmonary secretions and new infiltrate on her chest radiographs. These pulmonary secretions grew P. aeruginosa and S. marcescens. Treatment with piperacillin/tazobactam was successfully administered for ten days, but she remained chronically ventilated.

The P. aeruginosa HZ-QTR-51 isolate was sent to the reference laboratory at The University of Queensland, Centre for Clinical Research (UQCCR) as part of a region-wide collaborative study on multidrug resistant Gram-negative bacilli (2, 3). P. aeruginosa HZ-QTR-51 isolate underwent antibiotic susceptibility testing using E-test to measure the minimal inhibitory concentration of several antimicrobial compounds as listed in table 1.

The bacterial genomic DNA was extracted using the UltraClean Microbial DNA Isolation kit (MO BIO Laboratories) as recommended by the manufacturer. Species identification was performed using PAduplex assay that targets ecfX and gyrB genes, as previously described (4). Paired-end

libraries of whole genomic DNA of HZ-QTR-51 was prepared via Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, USA) and sequenced by Illumina HiSeq platform (Illumina, San Diego, USA). The 100bp pair-end reads were de novo assembled using CLC Genomic Workbench v.7.5.1 (CLC Bio, Aarhus, Denmark) with a minimum contig length of 200bp. 167 contigs were assembled with a depth coverage of ca. 100x.

The identities of the P. aeruginosa HZ-QTR-51 sequence type (ST) strain was confirmed by in silico multilocus sequence typing (MLST) (https://cge.cbs.dtu.dk/services/MLST/) using the P. aeruginosa MLST 1.7 database targeting seven potential loci (acs, aro, gua, mut, nuo, pps, and trp) (5) and defined as sequence type 654 (ST 654). ResFinder 2.1 platform (http://cge.cbs.dtu.dk/services/ResFinder/) (6) was also used to characterize acquired antimicrobial resistance mechanism genes among draft genomes. We found that the isolate encoded blaPME-1 besides blaOXA-50, blaGES-5 and blaPAO contributing to β-lactam resistance; and strA, aph(3')-Via, aph(3')-IIb, and strB for aminoglycoside resistance. The isolate also carried fosA for fosfomycin resistance, catB7 for phenicol resistance, sul1 for sulphonamide resistance; and tet(A) and tet(G) for tetracycline resistance.

For further confirmation, blaPME specific primers were designed and PCR was carried out using GoTaq® Green Master Mix and the following primers; PME-F (5'-CGCATTGCTGCTGTTTATGC-3') and PME-R (5'-GTGGGCATCGGATTCGTA-3'), yielding an 849-bp product. The run conditions used for this reaction started with denaturation at 95oC for 3 min; followed by 34 cycles at 95oC for 30 s, 55oC for 30 s, 72oC for 60 s; and a final extension at 72oC for 5 min. The sample was also PCR screened for other major groups that confer clinically relevant resistance to carbapenems, blaVIM-type (7), blaIMP-types (8), and blaNDM-type, blaKPC-type in a duplex reaction (2), but no positive PCR result was observed.

P. aeruginosa HZ-QTR-51 was phenotypically resistant to all tested antibiotics and was on the breakpoint border for amikacin (Table 1). The initial PME-1 producing P. aeuginosa GB771

identified in Pittsburgh was resistant to all β-lactams, all aminoglycosides except amikacin, and all fluoroquinolones. Although PME is not a carbapenemase, this might be due to the other antibiotic resistance mechanism that P. aeruginosa known to carry such as chromosomally encoded AmpC cephalosporins, modified outer membrane porin OprD, and multidrug efflux pumps.(9)

P. aeruginosa ST 654 is noteworthy for several reasons. VIM-2 producing P. aeruginosa ST 654 has been isolated from a patient in Sweden following hospitalization in Tunisia (10). ST 654 was also associated with KPC producing P. aeruginosa from Argentina (11). More recently, VIM-2 producing P. aeruginosa ST 654 was identified among the international 'high-risk clones' in the United Kingdom (12).

In conclusion, this study shows the first description of PME-1 producing P. aeruginosa in Qatar and the second in the world (13). Although our patient has a history of medical travel to United Kingdom and Saudi Arabia, she has not received any medical management in Dubai, where the first blaPME-1 producing P. aeruginosa is believed to have originated from. This might highlight the possibility that PME-1 producing P. aeruginosa are disseminated in the Gulf region. The currently described P. aeruginosa isolate belongs to the successful international clone ST 654, which might contribute to the global spread of blaPME-1. Hence, we suggest active surveillance for multidrug resistant P. aeruginosa to assess the dissemination and prevalence of PME mediated antibiotic resistance.

Acknowledgement

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Table 1: MICs of PME-1 producing *P. aeruginosa* HZ-QTR-51.

Antimicrobial Category	**Antimicrobial Agent**	**MIC (mg/L)**	**EUCAST Interpretation**
Aminoglycosides	Gentamicin	>256	R
	Amikacin	16	NS
	Netilmicin	>8	R
Antipseudomonal Penicillins and Beta-lactamase Inhibitors	Ticarcillin/Clavulanate	>256	R
	Piperacillin/Tazobactam	>32	R
Carbapenems	Imipenem	>32	R
	Meropenem	>32	R
	Doripenem	>32	R
Extended Spectrum Cephalosporins	Ceftazidime	32	R
	Cefepime	16	R
Fluoroquinolones	Ciprofloxacin	>32	R
Monobactams	Aztreonam	128	R

MIC. Minimum inhibitory concentration; R, resistant, NS, non susceptible

Toh B, Paterson D, Witchuda Kamolvit W, Zowawi H, Kvaskoff D, Sidjabat H, **Wailan AM**, *Peleg A and Huber C*. Species identification within the *Acinetobacter calcoaceticus - baumannii* complex using MALDI - TOF MS. J. Microbiol. Methods. 2015 Nov;118:128-32. doi: 10.1016/j.mimet.2015.09.006.

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Species identification within *Acinetobacter calcoaceticus - baumannii* complex using MALDI – TOF MS

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Running Title: Species identification of Acb complex using MALDI - TOF MS

Key words: *A. baumannii*, Mass spectra, Rapid diagnostics, *Acb* complex

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Abstract

Acinetobacter baumannii, one of the more clinically relevant species in the *Acinetobacter* genus is well known to be multi-drug resistant and associated with bacteremia, urinary tract infection,

pneumonia, wound infection and meningitis. However, it cannot be differentiated from closely related species such as *A. calcoaceticus, A. pittii* and *A. nosocomialis* by most phenotypic tests and can only be differentiated by specific, time consuming genotypic tests with very limited use in clinical microbiological laboratories. As a result, these species are grouped into the *A. calcoaceticus – A. baumannii* (*Acb*) complex. Herein we investigated the mass spectra of 73 *Acinetobacter* spp., representing ten different species, using an AB SCIEX 5800 MALDI – TOF MS to differentiate members of the *Acinetobacter* genus, including the species of the *Acb* complex. *RpoB* gene sequencing, 16S rRNA sequencing, and *gyrB* multiplex PCR were also evaluated as orthogonal methods to identify the organisms used in this study. We found that whilst 16S rRNA and *rpoB* gene sequencing could not differentiate *A. pittii* or *A. calcoaceticus*, they can be differentiated using *gyrB* multiplex PCR and MALDI – TOF MS. All ten *Acinetobacter* species investigated could be differentiated by their MALDI – TOF mass spectra.

1. Introduction

Acinetobacter baumannii is an opportunistic, aerobic, Gram-negative nosocomial pathogen that has become one of the six most important multi-drug resistant microorganisms worldwide (Antunes et al., 2014). A. baumannii is commonly known to cause difficult to treat pneumonia, bacteremia, urinary tract infection, wound infection and meningitis (Maragakis and Perl, 2008).

Accurate identification of A. baumannii and its closely related species A. pittii, A. nosocomialis and A. calcoaceticus is important as each of these species may display different characteristics in regards to antimicrobial susceptibilities, pathogenicity and clinical outcomes (Chuang et al., 2011; Sedo et al., 2013). The environmental strain A. calcoaceticus has not been reported to cause infection in humans whilst the other organisms are all of clinical significance (Peleg et al., 2008; Peleg et al., 2012). A recent paper has described increased severity and mortality in bacteraemia patients infected with A. baumannii compared with those infected with A. pitii and A. nosocomialis, emphasising the need for accurate differentiation (Fitzpatrick et al., 2015).

However, these four species which together make up the A. calcoaceticus – A. baumannii (Acb) complex are indistinguishable by phenotypic based tests (Peleg et al., 2008; Wang et al., 2013; Lee et al., 2014).

Molecular methods such as 16S rRNA and rpoB gene sequencing and have shown to be useful in differentiating members of the Acinetobacter genus (La Scola et al., 2006; Zarrilli et al., 2009; Wang et al., 2014). However, neither method is sufficient to differentiate species such as A. pittii and A. calcoaceticus (Higgins et al., 2010), and other molecular methods such as PCR amplification of intrinsic resistance genes or gyrB are used. However, these methods are mainly applied in research settings and have very limited use in clinical laboratories (La Scola et al., 2006; Higgins et al., 2010; Kamolvit et al., 2014).

Matrix assisted laser desorption ionisation – time of flight mass spectrometry (MALDI – TOF MS) has been shown to be a rapid and highly discriminatory method for the identification of bacteria (Kliem, 2010; Welker et al., 2011; Sedo et al., 2013). MALDI – TOF instruments that are

commonly used in clinical settings for bacterial identification rely on the use of proprietary identification software and spectral databases (Martiny et al., 2012).

In this study, we evaluated the use of a research-use-only (RUO) 5800 MALDI – TOF MS (AB SCIEX, Concord, ON, Canada) to differentiate and identify various species in the Acb complex, as well as other Acinetobacter spp. using only the standard instrument software and freely available open-source software for the acquisition, processing and interpretation of spectra.

2. Materials and Methods

2. 1. Bacterial isolates and reference strains

The strains investigated in our study included thirty two multilocus sequence typed (MLST typed) A. baumannii isolates, six Acinetobacter reference strains and thirty five additional Acinetobacter isolates as described below:

The MLST typed A. baumanni isolates included sixteen isolates of sequence type (ST)110, four isolates of ST92 and three isolates of ST109 (Huber et al., 2014). In addition to this, four isolates of from the Arabian peninsula were added to the study, including one isolate from Saudi Arabia of the ST195 and one of ST436, respectively, plus one isolate of ST208 from Kuwait and one of ST229 from Qatar (Zowawi et al., 2015).

Two additional isolates of ST208 were of Japanese origin, and one isolate of ST208 was from Thailand, and two Singaporian strains were of ST491 (Kamolvit et al., unpublished data). The isolates from Japan, Thailand and Singapore were collected between 2008 and 2010, and the MLST typing was performed in silico. The Kenyan strains and the strains from the Arabian peninsula were collected and MLST typed as previously described (Huber et al., 2014; Zowawi et al., 2015). MLST typing has been performed according to the Oxford scheme for all isolates (http://pubmlst.org/abaumannii/).

The following reference strains and previously published isolates and were added to the study; A. baumannii ATCC 19606, A. calcoaceticus ATCC 14987, A. lwoffi ATCC 15309 and ATCC 17986, A. johnsonii ATCC 17909, A. junii ATCC 17908, A. baylyi (n=1), A. calcoaceticus (n=1), A. pitii (n=1) (Peleg et al., 2012) and A. nosocomialis (n=2) (Peleg et al., 2012; Carruthers et al., 2013).

Various additional Acinetobacter spp. from Kenya (n=4, provided by the AGA KHAN University hospital in Nairobi, Kenya and collected between 2010-2011), Japan (n=12, provided by the Toho University in Tokyo, Japan and collected in 2010), Australia (n=2, collected at the Royal Brisbane and Women's hospital in Brisbane, Australia in 2004 and 2006 respectively), Thailand (n=7, provided by the Siriaj Hospital in Bangkok), Singapore (n=5, collected in 2008 and provided by the National University of Singapore). All isolates were grown on Mueller Hinton agar and incubated for 24 hours in a 37oC incubator, and identification and confirmation of species was performed as described in chapter 2. 2.

2. 2. 16S rRNA identification

All Acinetobacter spp. isolates were initially identified by the sequencing of the 16S rRNA gene as previously described (Misbah et al., 2005). Sequencing was performed by Macrogen Inc, Seoul, Korea, and sequences were blasted on NCBI using the megablast function against the 16S ribosomal RNA sequences database with maximum target sequences being set at 100. If 16S rRNA sequencing was unable to identify an isolate using the highest percentage identity, score and an E-value of 0 resulting in a sequence that matches two species with identical lengths, rpoB gene sequencing of zone 1 and 2 was performed as previously described (La Scola et al., 2006). A previously described gyrB multiplex PCR (Higgins et al., 2010) was used to differentiate A. calcoaceticus and A. pittii.

2. 3. MALDI - TOF MS

MALDI – TOF MS analyses were conducted on a 5800 TOF/TOF set in linear positive mode running the TOF/TOF Series Explorer acquisition software (AB SCIEX, Framingham, Massachusetts) at a laser frequency of 100 Hz with a set mass range of 3,000 to 20,000 Da. A continuous stage motion set in a random pattern at 600 μm/sec was used for sampling.

An in-house sinapinic acid matrix consisting of 10mg of sinapinic acid (>99.0% for MALDI-MS, Fluka 85429) in 500 μL acetonitrile, 475 μL distilled water and 25 μL 80% trifluoroacetic acid (TFA, LC-MS grade, Fluka 40967) was adapted from a previously published protocol (Freiwald and Sauer, 2009). Calibration was performed using calibration mixture 2 (AB SCIEX, Framingham, Massachusetts) which contained Angiotensin I, ACTH (1-17 clip), ACTH (18-39 clip), ACTH (7-38 clip) and insulin (bovine) to ensure mass accuracy within 5 ppm.

2. 4. Acquisition of mass spectra

A small amount of bacteria (approximately 106 – 108 cfu) was transferred from a 24 hour culture by spreading a thin layer onto a sample spot on an Opti-TOF 384 MALDI plate insert (AB SCIEX, Framingham, Massachusetts) and overlaid with 1 μL of sinapinic acid matrix. Each isolate was spotted in quadruplicate and each replicate scanned once. Laser intensity was set at 4322 units and at a pulse rate of 100 Hz with a total of 1000 spectra accumulated for each sample. A mass range of m/z 3,000 to m/z 20,000 and a continuous stage motion set in a random pattern at 600 μm/sec was used for sampling. The TOF/TOF Series Explorer acquisition software (AB SCIEX, Framingham, Massachusetts) was used to acquire mass spectra.

2. 5. Processing of spectra

Mass spectra files were non-manipulatively converted from t2d files to mzXML files using a t2d converter (http://www.pepchem.org) and processed using mMass version 5.50 (Strohalm et al., 2008) (http://www.mmass.org/). Processing of raw spectra was conducted in mMass 5.5 (Martin Strolham) with a peak picking algorithm that used baseline correction, Savitzky-Golay smoothing and a signal to noise ratio of 3. Replicates of the same isolates were averaged to form a consensus spectrum.

3. Results

3. 1. Bacterial identification

Using 16S rRNA identification, we confirmed the following Acinetobacter spp. in our collection; A. baumannii (n=37), A. nosocomialis (n=15), Acinetobacter junii (n=3), Acinetobacter lwoffii (n=2), Acinetobacter johnsonii (n=1), Acinetobacter baylyi (n=1), Acinetobacter soli (n=1) and Acinetobacter bereziniae (n=1).

Using rpoB gene sequencing and gyrB multiplex PCR we determined the remaining fourteen strains to be A. pittii (n=12) and A. calcoaceticus (n=2). The results are as summarized in Table 1.

3. 2. MALDI – TOF MS based characterisation

All ten Acinetobacter species investigated in our study had sufficient differences in their mass spectra to be characterised and differentiated using MALDI – TOF MS. In all of the A. baumannii strains investigated (n = 35) we observed the presence of a characteristic, high intensity mass of m/z 5743.05, as well as two other specific masses of m/z 8583.00 and m/z 8715.00 that could be used to distinguish A. baumannii from other Acinetobacter spp. (Figure 1). Additionally, all the species that were not part of the Acb complex could also be characterised and differentiated (Summarised in Table 1).

Nine of the twelve A. pittii isolates studied had a representative high intensity mass of m/z 5780.03 and two other specific masses of m/z 8620.00 and m/z 8822.00. In addition, three of the twelve isolates were also characterized by a mass at m/z 5743.05, with similar intensity to that of the one in A. baumannii. However, the mass at m/z 5780.03 was absent (Figure 1).

In A. nosocomialis we observed a representative mass at m/z 8134.00 in all isolates, and another mass at m/z 8542.00 in fourteen of fifteen isolates (Figure 2). In A. calcoaceticus, we observed a representative prominent peak at m/z 5829.00 that occurred in both isolates of this species (Figure 2).

4. Discussion

Retrospective studies found that A. baumannii was associated with higher mortality rates, greater antimicrobial resistance and different clinical characteristics than the ones of A. nosocomialis and A. pittii (Chuang et al., 2011; Lee et al., 2011; Yang et al., 2013). This raises the importance of being able to differentiate the organisms within the Acb complex to allow for treatment to be optimised for specific organisms.

A. calcoaceticus – A. baumannii complex organisms are hard to differentiate because of high phenotypic similarities (Gerner-Smidt et al., 1991). Molecular methods such as 16S rDNA and rpoB sequencing as well as gyrB PCR amplification are possible methods to discriminate between these strains. We observed that 16S rRNA sequencing or rpoB gene sequencing were not sufficient to differentiate A. pittii and A. calcoaceticus, and that the identification of these two organisms required gyrB multiplex PCR. However, in our investigation these species showed MALDI-TOF mass spectra that are clearly distinct from each other.

MALDI is a soft desorption ionization method which may result in the formation of ions without significantly breaking chemical bonds, which is particularly useful in protein analysis. MALDI instruments commonly used for bacterial identification use licenced software packages that are incompatible with instruments of other brands. Such instruments are usually single TOF mass analysers that do not allow for efficient structure and sequence investigation of analytes. The ABSciex 5800 instrument in our laboratory is a TOF/TOF instrument which can overcome the limitations of a single TOF mass analyser by linking two TOF mass analysers in series, making it a much more powerful tool in protein research (Ng et al., 2014). However, due to the lack of a database, bacterial identification is not readily performed with this instrument.

The use of freeware to augment the abilities of RUO instruments such as the 5800 TOF/ TOF greatly increase the functionality and availability of these instruments for bacterial identification. The ability to differentiate species without using automated systems may be a step back from the

automated systems that the VITEK – MS and the Biotyper offer, but it validates the ability of MALDI – TOF instruments to visualise spectral differences for greater discrimination between closely related species.

As we have seen in our study, MALDI – TOF was able to differentiate closely related species such as A. pittii and A. calcoaceticus. It appears that MALDI – TOF may also differentiate various Acinetobacter spp. from each other, as well as organisms that fall within the Acb complex. Automated processes would be required if this method is to be used as part of a diagnostic procedure.

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