The mobility and dissemination of *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM}}$ carbapenemase genes within clinical *Escherichia coli* isolates

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Chapter 1: Introduction

1.1 ANTIBIOTIC DISCOVERY

Antibiotics are fundamentally chemicals that possess a bactericidal or bacteriostatic effect. There are a wide range of different classes some discovered from natural sources, such as fungi and bacteria, and others that are synthetically produced modified versions of their naturally occuring counterparts. In nature, antibiotics are produced by microbes as an evolutionary advantage, allowing them to kill or inhibit the growth of competing microbes in their environment both distantly and closely related. One of the most well-known examples of this is the bacterial genus *Streptomyces,* who produce a range of antivirals, antifungals and especially antibiotics (Lima Procópio et al., 2012). The antibiotics produced varies between specific species of *Streptomyces* allowing them to maintain their ecological permanence by eliminating a sleugh of other microbial competitors. *Streptomyces'* prolific antibiotic production has resulted in them being instrumental in antibiotic drug discovery and thus responsible for two thirds of all known antibiotics (Manteca and Yagüe, 2019). However, as humans have introduced themselves into this evolutionary arms race of antibiotic production against new resistance mechanisms, we have discovered that with our excessive use of antibiotics that many of the naturally occuring antibiotic compunds have reached the limit of their efficacy.

The first natural antibiotic discovery is attributed to Alexander Fleming in 1928. While many times had antibacterial properties been associated with the presence of certain microbes (Dougherty TJ, Pucci MJ, 2011), no solid conclusions were drawn, this changed with Fleming's discovery of the production of a compound by *Penicillium rubens* that bared notable antibacterial properties, this compound later became known as the widely known drug "Penicillin" (Landau R, Achilladelis B, Scriabine A, 1999). The discovery of penicillin was not immediately lauded as revolutionary as the potential it possessed was not truly realised due to the struggles experienced in the identifying the active component in the drug and therefore difficulties purifying it.

The advent of The Second World War and the mass causalities it produced provided the perfect global situation to showcase the lifesaving abilities of penicillin and antibiotics as a whole. It pushed two individuals, Florey and Chain, who pioneered a new system of producing and purifying penicillin and even proved its effectiveness by testing it on mice (Ligon, 2004).

Penicillin was instrumental in the war effort, saving countless lives and enabling the continuation of a strong and effective war effort by the Allies (Quinn, 2013). In return the war had established the immense value of antibiotics still observable to this day by the alternative methods of treatment for bacterial infections pioneered in countries not privy to the discovery of antibiotics, for example the use of Bacteriophage (phage) as a therapeutic in the former Soviet Union and the establishment of the Eliava Institute in Georgia, 1923, (Chanishvili, 2012) who to this day devote themselves to the sustainable development of phage-based therapeutics.

While being a revolutionary discovery of the time, even then the limitations of penicillin and other potential drugs like it was truly realised very quickly by Fleming himself. Flemming had discovered alongside the wide-bactericidal effect of penicillin that the isolate "*Bacillus coli*", now known as *Escherichia coli*, and other bacteria from the "colityphoid" group were actually resistant to penicillin and their growth was not inhibited by its presence (Abraham and Chain, 1940). They discovered the production of penicillinases by the *E. coli*, enzymes that were capable of breaking down penicillin, nullifying its bactericidal effects. This acted as a prelude to the critical global health issue we face in the modern day, antibiotic resistance.

The discovery of naturally-occuring antibiotics was groundbreaking due to their potential for lost cost, mass production, but it wasn't the first time humans had discovered and applied molecules with antibacterial properties in therapeutics. The true dawn of the antibiotic era could arguably be attributed to Paul Ehrlich and his concept of the "Magic Bullet", a concept of a drug that could target specific pathogens and cure disease.

In the 19th century, the dye industry had begun utilising synthetic chemistry to attempt to find more economical ways to procure certain compounds that they used as key precursors to a range of dyes, which previously would've required extraction from natural sources which was expensive and time-consuming. The production of aniline is a prime examples of this.

Aniline is the precursor molecule to aniline dye that was previously extracted from indigo plants. In 1854, Antoine Béchamp discovered an economic synthetic pathway to produce aniline by reducing nitrobenzene with hydrochloric acid, using iron as a catalyst. This set off the synthetic dye industry, but unknowing would have an even greater impact on medicinal

chemistry. Béchamp's continued experimentation with producing chemical derivates of aniline led to produce another dye molecule named atoxyl.

Atoxyl later became the focus of Ehrlich, who was investigating the antimicrobial potential of synthetic dyes due to their affinity for living cells. In 1891, alongside Paul Guttman, he already reported the successful treatment of malaria using a fully synthetic dye, methylene blue. After reading about atoxyl's apparent activity against *Trypanosoma*, he focused his research on the compound and came to discover it's chemical structure had been mischaracterised and was in fact much easier to synthesise but also to modify to produce derivatives.

Subsequently, hundreds of structurally similar derivatives were produced with varying degrees of activity against the *Trypanosoma*. Upon suggestion, these derivatives were also tested against the causative agent of syphilis, *Treponema pallidum*, and this summated in the discovery of a molecule named salvarsan in 1909. Salvarsan successfully treated syphilis and is considered the first antibacterial drug, becoming a global success and Ehrlich's long sought-after "Magic Bullet".

The role that the dye industry would play in synthetic antibacterial drug discovery was not yet finished however as in subsequent years the addition of sulphonamide groups to dye molecules was found to further increase their affinity for living cells and leading to the discovery of "sulfa drugs", another class of synthetic antibacterial molecules, some of which are still used today such as sulfamethoxazole.

These synthetically produced antibiotics were a great success but generally had higher risk of side effects and increased cost to produce when compared to the naturally sourced antibiotics that replaced them. However, this ability to chemically modify the structure of the drug molecules was invaluable in increasing their effectiveness and circumventing total resistance to them and so many antibiotics in use now are semisynthetic, combining the cheapness of mass-producing natural antibiotic molecules as a precursor and chemically modifying them to increase their range of effectiveness, making distinct new antibiotics.

1.2 CLASSES OF ANTIBIOTICS

Although penicillin was realised not to be a universal solution to bacterial infections, the discovery of a range of more antibiotics, made up for its shortcomings.

Due to the huge range and variety of the molecules that come under the term antibiotics, they are being distinguished and regrouped constantly, with the nomenclature and grouping of them constantly changing and then varying from person to person. Penicillin, for example, resides within a group of antibiotics called the penicillins but also belongs to a family of antibiotics called Beta-lactams due to their shared structural features. There are numerous antibiotic families with the each having their own sub-groups and scopes of bacterial targets (Calhoun and Hall, 2023). Even NHS England has stated that antibiotics can be loosely split into 6 "groups", these being Penicillins, Cephalosporins, Aminoglycosides, Tetracyclines, Macrolides and Fluroquinolones (NHS, 2022).

There are many different antibiotics belonging to these families with a range of different targets within a bacterium's metabolism, but they can be split into two classifications of "bacteriostatic" and "bactericidal" (Calhoun and Hall, 2023). Bacteriostatic drugs are generally classified as such for their ability to inhibit bacterial growth rather than cause immediate cell death, while bactericidal have a more direct lethal action. This rigid binary is not completely accurate in reality as both types of antibiotic can have lethal effects on bacteria but to different degrees, enabling their distinction. Clinically, "bacteriostatic" antibiotics have been observed to have strong lethal effects to bacterial populations, within 18-24 hours, after their administration due to the disruption of vital metabolic functions in the bacterium, but do not kill sufficient proportions to be classified as "bactericidal". Similarly, some "bactericidal" antibiotic can be ineffective at killing every bacterium present in a population. The range conditions within live patients can vary drastically, affecting the ability of the antibiotics to fit neatly into their distinct classifications. This leads to different clinical applications with the antibiotics considered "bactericidal" being administered for strongly established infections with an insufficient host immune responses and immediate threat to an individual's health, such as meningitis and sepsis. Conversely, "bacteriostatic" antibiotics would be administered for infections where the infections are less established and there is a wider treatment widow available (Pankey and Sabath, 2004). In practice, a combination of the two has generally been considered an optimal treatment option.

Classes of antibiotics that are bacteriostatic include Amphenicols, Macrolides, Sulfonamides and Tetracyclines, with notable specific examples being chloramphenicol and trimethoprim/sulfamethoxazole combination. The most common mechanism of action in these bacteriostatic antibiotics is the inhibition of aspects of protein synthesis in bacteria.

Tetracyclines target the 30s subunit of the bacterial ribosomes. The drug molecule aminoacyl site (A-site) which prevents the entrance of tRNA molecules and their bound amino acids into the ribosome during protein translation or even blocking initial entry preventing protein production totally (Vicente and Pérez-Trallero, 2010). Macrolides target the 50s subunit and target the exit site (E-site) of the ribosome, preventing the exit of spent tRNA molecules, which inhibits the entrance of new tRNAs carrying amino acids for the protein and blocks the translation process (Vázquez-Laslop and Mankin, 2018). Amphenicols target the peptidyl-transferase site (P site) on the 50s subunit of the ribosome. This P site is the site between the A and E site and is responsible for the catalysis of peptide bond formation between the amino acid units that are to make up the finished protein (Tereshchenkov et al., 2018). The binding of the amphenicol molecules halts the translation of proteins in the ribosomes.

Some examples of bactericidal antibiotics include β-lactams, Aminoglycosides and Quinolones. Bactericidal antibiotics have more of a range to their targets than bacteriostatic antibiotics and thus more mechanims of killing bacteria, which include inhibition of cell wall syntheisis, DNA replication and synthesis, and causing protein mistranslation (Kohanski, Dwyer and Collins, 2010). Aminoglycosides affect protein translation by binding to bacterial ribosomes, however unlike some bacteriostatic antibiotics, they do not cause the halting and inhibition of protein translation but instead cause the incorporation of incorrect amino acids into the proteins synthesised by the bacterium. Some of these proteins include membrane proteins and the modification of these proteins can increase the uptake of the aminoglycosides and other antibiotics by the bacterium (Krause et al., 2016). This increase uptake can result in bactericidal levels of the antibiotic within the cell, through the severity of the translational disruption. Quinolones target the enzymes DNA gyrase and Topoisomerase IV, both essential enzymes for controlling the supercoiling of DNA for DNA replication. The complex that DNA gyrase and topoisomerase IV produce relaxes supercoiling of bacterial DNA by controlled cleaving and rejoining of the DNA strands, allowing DNA polymerases to bind and replicate the DNA (Aldred, Kerns and Osheroff, 2014). The quinolones target this complex and inhibits the rejoining of the DNA strands, this results in the inhibition of DNA replication and causes damage to the bacterial chromosome, leading to cell death. β-lactams inhibit cell wall synthesis within the bacteria by binding to specific proteins that catalyses critical bonds within the N-acetylglucosamine and N-acetylmuramic acid molecules that make up a stable peptidoglycan cell wall (Oelschlaeger, 2021). This mechanims will be expanded on later.

1.3 ANTIBIOTIC RESISTANCE

With each varied way that antibiotics can inhibit and eliminate bacterial growth, there are mechanisms that the bacteria have evolved to resist their action. Due to the presence of antibiotics as a selective pressure within nature, many bacteria have evolved mechanisms that allow them to survive in their presence, and this means that from the initial coopting of antibiotics in therapeutics there were specific bacteria within their range of activity that were resistant to them. The main methods that bacteria can resist antibiotics includes enzymatic degradation or modification, overproduction of efflux pumps, mutation of porin proteins to prevent their entry, production of repair mechanims to limit the damage of their presence or even mutations producing an alteration in the target site of the antibiotics effecting their efficacy (Reygaert, 2018).

Some bacteria employ multiple of these resistance mechanims to provide complete resistance to specific broad classes of antibiotics, but clinically other antibiotic classes would be implemented to treat infections caused by these bacteria. However, there has been rapid and vast spread of strains that possess a sleugh of resistance mechanisms that confer resistance to many groups of these antibiotic classes. Severe multi-drug resistance has proved to be a serious threat and triggered a global health crisis (Chinemerem Nwobodo et al., 2022).

Bacteria reproduce through binary fission, which is an asexual form of reproduction, allowing for no production of new genetic combination to be produced and all progeny being clonal copies. Aside from standard mutations of their genome, asexual reproduction does not provide much genetic variation to bacteria, but some bacteria also contain loops of DNA far smaller than their genomes called plasmids that are packed with a variety of genes that provide them selective advantages, including virulence factors that help them produce persistent infections, nutrient scavenging mechanism and resistance to toxic molecules (O'Donnell, Langston and Stillman, 2013) (Finks and Martiny, 2023). The key advantage of possessing these plasmids is their penchant for mobilising themselves and being able to transfer themselves between bacterial strains and species. Bacterial conjugation is the process by which two bacteria can horizontally transfer genetic material in the form of plasmids through pili which produce a temporary fusion of two bacterial cells. The plasmid DNA shared through this union can provide the same advantages the genes conferred to the donor to the recipient (Virolle et al., 2020) (Guzmán-Herrador and Llosa, 2019). This process can be rapid and repeated multiple times allowing for the swift dissemination of many genes through plasmids within an environment, especially antibiotic resistance genes. A small number of resistance genes

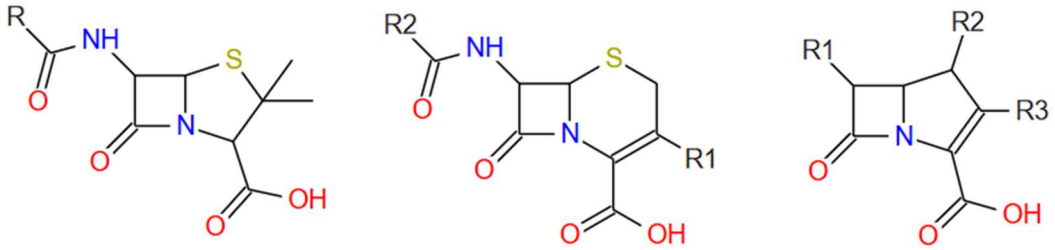
within a mobile plasmid may not provide an extreme resistance, but plasmids are also hosts to a range mobile genetic elements, such as transposons and integrons. These mobile genetic elements can integrate themselves into plasmids from the genome and vice versa; this can result in highly mobile plasmids that contain a variety of different resistance genes present within a range of strains within a bacterial population.

The presence of multiple antibiotic resistance genes within bacteria that are also pathogens, or opportunistic pathogens produce the immense challenge of treating the infections they cause. As multi-drug resistance increases in occurrence and severity, fewer and fewer antibiotics are available to be used as an effective therapeutic. Certain antibiotics are considered critical/last resort antibiotics due to their strength, range of their activity and most importantly the lack of resistance against them in the bacterial populations (Miteu et al., 2023). Their use is typically avoided so as to prevent the production of new resistant strains or due to their toxicity to the patient. As more severe antibiotic resistances increase in number, the reliance on these last resort antibiotics is increasing exponentially and as a result so is the prevalence of isolates resistant to their function. The carbapenem antibiotic sub-class are prime example of this situation.

1.4 CARBAPENEMS

Carbapenems are antibiotics within the beta-lactam family of antibiotics. The beta-lactams are antibiotics that all share the same core “beta-lactam ring” structure in their molecule. Different groups of beta-lactams will have their own additional structures alongside their beta-lactam ring. Figure 1 shows the core structures of the Penicillins, Cephalosporins and Carbapenems, the R-groups are where the different structures within these groups varies further between the specific antibiotics.

Figure 1.



Penicillins **Cephalosporins** **Carbapenems**

Structures of the three beta-lactam antibiotic backbones for Penicillins, Cephalosporins and Carbapenemases.

They target the enzymatic penicillin-binding proteins (PBPs) in bacteria which are responsible for producing the bonds that adjoin the chains of N-acetylglucosamine and N-acetylmuramic acid that make up the layers of the peptidoglycan that most bacterial walls are made up of (Papp-Wallace et al., 2011). They do this by structurally mimicking the d-ala-d-ala dipeptide that the enzyme binds to produce the peptidoglycan chains. The beta-lactams covalently bond to the active sites of the PBPs preventing them from producing stable bacterial cells walls and this means that during binary fission the bacterial cell will lyse, and this results in cell death. As the active region, the beta-lactam ring is the target of degradation by beta-lactamases which hydrolyse the beta-lactam ring. The enzymes target the carbonyl carbon of the ring as it is bound to an oxygen and nitrogen group, which are two highly electronegative groups and result in this carbonyl carbon being very vulnerable to nucleophilic attack (Majiduddin, Materon and Palzkill, 2002). Once hydrolysed the resemblance to the d-ala-d-ala dipeptide is destroyed and the molecule is rendered inactive.

Carbapenems were previously valued for their strength and broad-range of activity against aerobic and anaerobic, Gram-positive and Gram-negative bacteria due to the molecule's high affinity to a wide range of different subtypes of PBPs. These features had them conserved as a last-resort antibiotic treatment, especially against extended-spectrum beta-lactamase (ESBL) producing bacterial isolates (Papp-Wallace et al., 2011). The chemical structure of carbapenems also helps them resist hydrolysis as the hydroxy-ethyl side chain produce a steric affect shielding the beta-lactam ring and reducing the

chance of beta-lactamases being able to successfully bind, this is called steric hindrance.

Due to the increase in ESBL producing bacteria, the use of carbapenems has increased and as a result there has been an increase in the number of bacterial strains that are becoming resistant to carbapenems and particularly a number of those who produce beta-lactamases capable of hydrolysing carbapenems, named carbapenemases (Codjoe and Donkor, 2017).

1.5 CARBAPENEM RESISTANCE

Carbapenem resistance has multiple mechanims, lower levels can be provided through the modification of the outer membrane porins (OMPs), in particular OmpF and OmpC proteins which prevent the movement of carbapenems into the cell to target the PBPs or by the excess production of efflux pump proteins which expel the carbapenems within the cell back out before it can target the PBPs (Codjoe and Donkor, 2017) (Meletis, 2016). Higher levels can be provided through the presence of carbapenemases, especially in conjunction with porin mutation or efflux pump overproduction.

Carbapenemases are the resistance mechanism of most interest due to their wide variety, different origins and high activity against carbapenems. Some of the main carbapenemases include KPC, NDM, OXA-48-like, IMP, IMI, GES, VIM (Queenan and Bush, 2007).

In the UK an analysis of carbapenemases between 2003 and 2017 was done with found the majority of isolates belonging to Enterobacterales (84.4%) and the top three carbapenemases were VIM (30.9%), NDM (23.0%) and KPC (17.7%) =with others of note being OXA-48 (17.7%), IMP (7.4%), NDM+IMP (0.8%) and NDM+OXA-48 (0.8%). Within all these isolates *Escherichia coli* made up 24.5%, being the third most common species (Zhao et al., 2021).

Worldwide most countries are dominated by *Klebsiella pneumoniae,* particularly in countries where the KPC carbapenemase are the most common, for example the United States where van Duin et al. found that within across 49 hospitals in the US that 57% of CRE isolates were *K. pneumoniae*, followed by 11% *E. coli* and 18% *Enterobacter spp.* and of these CRE isolates 54% were made up of KPC, with 30% being KPC-2 and 24% being KPC-3 (van Duin et al., 2020).

Although, there are outliers. A study in Japan has found that the primary CRE in from their hospitals is *E. cloacae*, making up 43% of the CRE isolates examined

and Klebsiella aerogenes following it up at 31%. Continuing the divergence from the trend, the isolates were to all be solely IMP producers.

A study conducted in Japan included 179 CRE strains from 2014 to 2016 reported that *E. cloacae complex* (43%) and *Klebsiella aerogenes* (31%) exceeded *K. pneumoniae* (7%) and *E. coli* (5%) (Oka et al., 2022).

CRE strains from urine, sputum and blood were collected in Thailand from 2016 to 2018 which were composed of 72% *K. pneumoniae* and 22% *E. coli*, 80% of the CRE strains produced carbapenemases, 17% (629/4296) produced more than one carbapenemases, and the most common type of carbapenemase was NDM, accounting for 65% (2392/4296) ((Paveenkittiporn et al., 2021)

In 2020, the UK government acted towards more stringent monitoring of carbapenem resistance. They required that all diagnostic laboratories in the UK were required to report any carbapenem resistant Gram-negative bacteria derived from human samples, their antimicrobial sensitivity profiles and the resistance mechanism of these isolates.

Recent data has shown a shift in the prevalence of different carbapenem genes and their isolates. Between June 2023 and June 2024, *E. coli* accounted for the most common carbapenemase producing organism (CPO) at 33.8%. Within all Enterobacterales OXA-48 and NDM have become the most prevalent carbapenemase genes, finding 44.4% of *E. coli* isolates containing OXA-48 and 42.4% containing NDM. Between 2020 to 2024, all carbapenemases apart from OXA-48-like, NDM, KPC, VIM and IMP made up less than 0.5% of reports. This shows a clear domination within the dissemination of the carbapenemase families, with OXA-48 and NDM showing to have succeeded others in becoming the most dominant, not due to a decrease in others but an increase in their numbers. The fluctuation between OXA-48 and NDM being the most dominant carbapenemase in isolates has been observed over the four years, with an alarming observation of the most common dual carbapenemase producers being OXA-48 and NDM. Through the past four years of carbapenem-resistance surveillance, no changes were reported between different demographics were reported between age, sex and ethnic group, which indicates that the isolates are spreading strongly endemically throughout the entire UK (GOV.UK, 2024).

While the UK has implemented significant measures in place to monitor and control it's spread of carbapenem resistance, it is not the only country struggling with the rise of CPOs. Although globally there is notable variations in

the predominance of carbapenemase genes within each country's native bacterial populations.

For example, in contrast to the prevalence of OXA-48-like and NDM strains, in the United States, Greece and Israel KPC has become endemic and is the dominant carbapenemase in the CRE population (Arnold et al., 2011) (Logan and Weinstein, 2017).

Similar to the UK, NDM remains endemic in India, where it was first identified. A study on carbapenemase producing isolates from Indian hospitals found that 67% of the producers were NDM producers and the second most common being 18.4% with VIM (Garg et al., 2019), this shows a clear lead NDM dissemination has taken over the others. OXA-48-like carbapenemases remain endemic throughout the middle-east, especially in Turkey, where OXA-48 was initially discovered (Poirel et al., 2003).

1.6 CARBAPENEMASES

Carbapenemase encoding genes are frequently found on very large plasmids that are commonly filled with many other resistance genes (Yuan et al., 2024), often alongside aminoglycoside-modifying enzymes, sulfonamide resistance genes *sul1* and *sul2*, and tetracycline resistance genes such as *tetA* and *tetB* (Carattoli, 2013). While plasmids of such sizes have a high fitness cost for the bacteria carrying them, carbapenemases generally have a wide spectrum of activity and the variety of other antibiotic resistance genes they contain make retaining them a viable effort. Carbapenemase genes are found associated with different transpositional sequences such as IS*Aba125* which frequently flanks bla_{NDM-1} and allow for the segement of DNA containing the gene to excise itself from one and insert itself into other multi-drug resistance plasmids (Fortunato et al., 2023). This means that multidrug resistant bacteria can contain fewer plasmids as all their resistances can be localised on one plasmid. This is also useful when considering the role that incompatibility groups within plasmids play, meaning that multiple plasmids of the sample incompatibility groups cannot exist with the same cell, due to competition for the replicative and transcriptional systems for their specific replicons (Wang et al., 2019).

1.6.1 Serine vs Metallo beta-lactamases

Serine beta-lactamases have a serine residue as an active site. They break down the beta-lactam ring by acylating the carbonyl carbon within the ring and

binding to it which then enables a water molecule to attack this bound intermediate, and this then releases a hydrolysed form of the molecule with an opened beta-lactam ring, leaving the molecule inactivated (Mora-Ochomogo and Lohans, 2021). Ambler classes A and D are both serine based.

Metallo-beta-lactamases have a zinc ion as their active site, rather than a serine residue. This zinc ion activates water molecules at the site of the antibiotic, forming a hydroxide ion which will attack they carbonyl carbon, opening up the ring and leaving an inactive hydrolysed product (Page and Badarau, 2008). Ambler class B consists of the metallo beta-lactamases.

1.6.1.1 NDM

New-Delhi Metallo-B-lactamase (NDM) is a carbapenemase that possesses two positively zinc ions within its protein structure, which enable it to hydrolyse the beta-lactam ring in beta-lactam antibiotics. Due to the presence of zinc ions in its structure it is a metallo-β-lactamase (MBL) belonging to the Ambler class B (H. Halat, A. Moubareck, 2020). NDM has a broad range of hydrolytic activity towards beta-lactam antibiotics, being able to breakdown almost all the beta-lactam class of antibiotics. NDM was first identified in patient in New-Delhi, 2008. The patient was receiving treatment for a urinary-tract infection caused by a carbapenem-resistant *Klebsiella pneumoniae* strain. The isolate was identified as producing MBL but contained no known MBL genes. Upon amplification of they identified a new MBL gene associated with an IS*26* sequence, later designated as $bla_{\text{NDM-1}}$ (Yong et al 2009).
NDM is unaffected by β-lactamase inhibitors and is able to efficiently hydrolyse even the later generations of cephalosporin, which gives it a wide range of activity without the need to co-produce other β-lactamases. However, NDM is notably unable to hydrolyse the monobactam aztreonam (Ioana Miriana Cismaru et al., 2024).

1.6.1.2 OXA-48

OXA-48 and OXA-48-like enzymes are subtypes of the Oxacillinase (OXA) group of penicillinases. They have a high efficiency at hydrolysing penicillins especially oxacillin, that being its name-sake. The OXA-48 enzyme is serine based, differentiating it from other metallo-β-lactamases, and belongs to the ambler class D (Poirel et al., 2003). The OXA-48 subtype is characterised by its low-level ability to hydrolyse carbapenem antibiotics (Boyd et al., 2022). It also possess a lack of inhibition by β-lactamase inhibitors, but it is unable to break down later

generation of cephalosporins, such as cefoxitin or cefotaxime. The ability to hydrolyse carbapenemases is what distinguishes it from the other OXA sub types, whilst this hydrolysis of carbapenems is low level, it can be increased with the presence of other beta-lactamases such as ESBL and overproduction of AmpC. The OXA-48-like group is a sub-group itself of the OXA-48 subgroup, it covers a range of carbapenem hydrolysing OXA enzymes that have extremely close relation to OXA-48 with a range of 1 to 5 amino acid substitutions, the most common of these are OXA-162, OXA-163, OXA-181, OXA-232 and OXA-204 (Pitout et al., 2019). Some of these variants for example OXA-181 actually have a much higher hydrolytic activity towards carbapenems, resulting in it being one of the more common OXA-48-like enzymes in carbapenemase producing bacteria in certain global regions (V. Bakthavatchalam and S. Anandan, 2016). The original discovery of OXA-48 was in Turkey, 2001, where it was discovered in an *E. coli* isolate. It was found to only have a remote relation to other OXA enzymes, with around 46% similarity in their amino acid sequence and it was also lacking an associated integron which is common for the other OXAs. It was found immediately downstream from an IS*1999* insertion sequence.

1.7 NOSOCOMIAL *E. COLI* INFECTIONS

Escherichia coli have been observed to be an especially problematic strain of bacteria in the dissemination of carbapenem resistance. *Escherichia coli* is a species of Gram-negative bacteria that are routinely associated with the bacteria flora of the lower intestines of humans and are considered to generally be part of a healthy microbiome. *E. coli* strains are mostly non-pathogenic; however, horizontal gene transfer has resulted in the production of highly virulent and pathogenic strains of *E. coli*. While *E. coli* colonise the intestines and as a result a lot of the pathogenic strains cause infections centred in that region, there are also extra-intestinal pathogenic *E*. coli (ExPEC) strains which cause infections at other sites throughout the body (Mueller and Tainter, 2023). ExPEC strains are some of the major causes of nosocomial infections within hospitals.

Nosocomial infections are infections that are acquired or closely associated with healthcare settings. They commonly caused by bacteria that are part of the normal human microbiome, such as *Enterococcus, Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus*, and typically become pathogenic due to the weakened or immunocompromised state of the individual's receiving treatment in the healthcare settings (R. McFee, 2009). Nosocomial infections are concerning due to the extra burden they can add onto the health of individuals who are already requiring intervention to sustain it. An aspect that

makes them especially concerning is their association with the healthcare environment; the proportion of antibiotic and multidrug resistant bacteria is exceptionally high and can often result in the nosocomial infections being extremely resistant to conventional treatment techniques (Abban et al., 2023). Nosocomial infections tend to be chronic or frequently reoccurring in nature, which is increasingly problematic if the bacteria causing the infection is already antibiotic resistant as repetitive treatment may result in the induction of further antibiotic resistance.

The most common nosocomial infections include ventilator-associated pneumonia (VAP), urinary tract infections (UTIs), venous catheter infections and surgical site infections. ExPEC strains are some of the bacteria that can be the cause of these infections, especially UTIs. UTIs are one of the most more common nosocomial infections, particularly due to their tendency to be recurrent. Uropathogenic *E. coli* (UPEC) are one the most common ExPEC to cause UTIs. UPEC strains (Terlizzi, Gribaudo and Maffei, 2017).

E. coli's prolific uptake and accumulation of plasmids and other mobile genetic elements through conjugation and transfection, means many strains gain significant pathogenicity islands from other bacterial species allowing more virulent and pathogenic strains causing more resilient and dangerous infections (Feng and Wang, 2024). It also means that as a species they are a hub of multidrug resistance plasmid passage and this has resulted in *E. coli* becoming a species of concern, making it onto the ESKAPE pathogen list (Mulani et al., 2019), due to its tendency to carry and disseminate many newer and dangerous antibiotic resistance genes, in particular OXA-48s and NDMs. This penchant for acquiring a variety of virulence genes, antibiotic resistance genes and the predominance in nosocomial infection make the state of antibiotic resistance in *E. coli* a critical topic to investigate.

1.8 PRIMARY AIM

The main aim of this thesis is to assess the genetic mobility of bla_{NDM} and bla_{OXA-48} within a clinically significant bacterium such as *E. coli* and to gain an insight into what characteristics in OXA-48 and NDM producing bacteria possess that aids their swift and vast dissemination within clinical settings. The main focuses would be the location of these genes on either the chromosome or on plasmids, which genetic features may be commonly associated with the genes that may provide them an advantage at spreading throughout a population, such as co-carriage of other antibiotic resistance genes or mobile genetic elements and the specific sub-types of OXA-48 and NDM present currently within the isolates and

if this reflects what has been observed currently in clinical environments in the UK. These aims work to get an insight what is facilitating this carbapenem resistance spread within *E. coli* specifically, as the role they play within clinical settings through nosocomial infections is both lethal and expensive. The focus will be UK centric and thus will use samples derived from carbapenem resistant infection being treated in a UK-based hospital and will focus on the most predominant carbapenemase being reported in hospitals, NDM and OXA-48.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Bacterial strains

The bacterial strains used as positive and negative controls for phenotypic testing and the strain used in the conjugation assay are listed in Table 1.

The hospital isolates bearing carbapenem resistance and the relevant information pertaining to them are listed in Table 2. These isolates were collected by the hospital as part of routine carbapenem-resistance screening and were selected for this research due to their carbapenem resistance mechanisms.

In Table 2 all MALDI-TOF species identifications scored values between 2.09 – 2.55. Identification scores ≥ 2.0 are accepted for a reliable identification at the species level, as specified by the manufacturer. The Presumptive identifications were generated via micro-scan and disc diffusion assays being performed on the isolates and their resistance patterns being matched to resistance mechanisms.

Table 1.

Strain	Description	Use	Reference
ATTC 25922	*E. coli* strain with no antibiotic resistances	Negative control in experiments including antibiotic selectivity	American Type Culture Collection (ATCC)
NCTC 13476	*E. coli* producing the IMP carbapenemase	Positive control in experiment with imipenem as a selective agent	National Collection of Type Cultures (NCTC)
Modified GFP J53 Azi^{R}	*E. coli K-12 derivative resistant to sodium azide,* with the insertion of a modified GFP encoding plasmid producing purple colouration to the isolate.	As the recipient strain in conjugation assays	(Yi et al., 2012)

N.B. When “control strains” are mentioned this refers to ATTC 25922 and NCTC 13476.

Table 2.

Isolate number	Species identity via MALDI-TOF	Presumed resistance mechanism	Sample type
1	*Escherichia coli*	NDM	Tip from wound drain site
2	*Escherichia coli*	NDM	Faeces
3	*Escherichia coli*	OXA-48	Faeces
4	*Escherichia coli*	NDM	Rectum swab
5	*Escherichia coli*	OXA-48	Blood culture white waste
6	*Escherichia coli*	OXA-48	Urine
7	*Escherichia coli*	NDM	Swab
8	*Escherichia coli*	OXA-48	Faeces
9	*Escherichia coli*	OXA-48	Mid Stream Urine
10	*Escherichia coli*	NDM	Rectum swab
11	*Escherichia coli*	CRE mechanism unknown	Sputum
12	*Escherichia coli*	OXA-48	Rectum swab
13	*Escherichia coli*	NDM	Rectum swab
14	*Escherichia coli*	CRE mechanism unknown	Blood Culture
15	*Escherichia coli*	OXA-48	Clean Catch Urine

2.1.2 Media

All media were prepared using Milli-Q purified water (Millipore) and sterilised by autoclaving at 121°C for 15 minutes at 15psi, unless specified otherwise. Where appropriate, supplements were added to the media once it had cooled to 50°C. All growth media were obtained from Sigma-Aldrich, unless specified otherwise.

2.1.2.1 Lysogeny broth (Lennox) and Lysogeny broth (Lennox) with agar

Lysogeny broth (LB) was prepared to a final concentration of 10g L^{-1} tryptone, 5g L^{-1} sodium chloride and 5g L^{-1} yeast extract. LB with agar used the same concentrations with the addition of 15g L^{-1} agar.

2.1.2.2 Mueller-Hinton broth and agar Mueller-Hinton

Mueller-Hinton (MH) broth was prepared to a final concentration of 2g L^{-1} beef infusion solids, 17.5g L^{-1} casein digest and 1.5g L^{-1} starch. MH agar used the same concentrations with the addition of 17g L^{-1} agar.

2.1.2.3 Tryptone Soya Broth (Thermo Fisher Scientific)

Tryptone Soya Broth (TSB) was prepared to a final concentration of 17g L^{-1} casein digest, 3g L^{-1} soya digest, 5g L^{-1} sodium chloride, 2.5g L^{-1} dipotassium hydrogen phosphate and 2.5g L^{-1} glucose.

2.1.2.4 Tryptone Bile X-glucuronide agar

Tryptone Bile X-glucuronide agar (TBX) was prepared to a final concentration of 15g L^{-1} agar, 1.5g L^{-1} bile salts, 0.075g L^{-1} X-β-D-glucuronide and 20g L^{-1} peptone.

2.1.2.5 CHROMagar mSuperCARBA agar

CHROMagar mSuperCARBA agar (CHROMagar) was prepared to a final concentration of 1g L^{-1} agar, 20g L^{-1} peptones, 5g L^{-1} sodium chloride, 0.8g L^{-1} chromogenic and selective mix, 1.7g L^{-1} growth factors and 2mL L^{-1} growth factors mix supplement. RO water was used to make the desired volume before bringing to the boil on a hotplate. This was followed by the addition of 0.25g L^{-1} 'selective mix supplement' once the agar had cooled to 50°C

2.1.3 Buffers and Solutions

All buffers and solutions were prepared using Milli-Q purified water (Millipore) and sterilised by autoclaving at 121°C for 15 minutes at 15 psi, unless specified otherwise. All were obtained from Sigma-Aldrich, Gillingham, UK, unless specified otherwise

2.1.3.1 Maximum Recovery Diluent

Maximum Recovery Diluent (MRD) was prepared to a final concentration of 8.5g L^{-1} sodium chloride and 1g L^{-1} peptone.

2.1.3.2 Phosphate Buffered Saline

Phosphate Buffered Saline (PBS) was prepared to a final concentration of 8.5g L^{-1} sodium chloride, 2.16g L^{-1} sodium phosphate dibasic, 0.2g L^{-1} potassium chloride and 0.2g L^{-1} potassium phosphate monobasic.

2.1.3.3 Ethylenediaminetetraacetic acid

Ethylenediaminetetraacetic acid (EDTA) was prepared as a 0.5M solution by dissolving 186.1g of EDTA in 800mL of RO water, adjusted to pH 8.0 with 1M sodium hydroxide.

2.1.3.4 Phenol Red

Phenol red indicator solutions were prepared with phenol red powder to a concentration of 5g L^{-1}, adjusted to pH 8.0 with either 1M hydrochloric acid or 1M sodium hydroxide.

2.1.3.5 Tris-EDTA buffer

Tris-EDTA (TE) buffer was prepared to a final concentration of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

2.1.3.6 Tris-acetate-EDTA buffer

Tris-acetate-EDTA (TAE) buffer was prepared to a final concentration of 40mM Tris-HCl, 1mM EDTA and 20mM glacial acetic acid, adjusted to pH 8.0 with either 1M hydrochloric acid or 1M sodium hydroxide.

2.1.3.7 Glycerol

Glycerol solution was prepared to a final concentration of 50% or 10% (v/v) as required.

2.1.3.8 Ethanol

Ethanol solution was mixed with sterile RO water to a final concentration of 80% or 95% (v/v) as required. It was not autoclaved

2.1.3.9 Isopropanol

Isopropanol was used undiluted at ~99% so no modification was necessary, and neither was autoclave sterilisation.

2.1.3.10 Zinc Sulphate

240mM zinc sulphate was dissolved in sterile Milli-Q purified water and filter sterilised using a 0.22µm filter (Sartorius) and not autoclaving.

2.1.4 Antibiotic stock solutions

All antibiotic powers used were obtained from Sigma-Aldrich, stocks were prepared using Milli-Q purified water (Millipore), filter sterilised using a 0.22 µm filter (Sartorius, Epsom) and stored in sterile microcentrifuge tubes at -20°C.

2.1.4.1 Imipenem

10mg mL^{-1} of imipenem (APExBIO) was dissolved in sterile water and used at a working concentration of 2µg mL^{-1} unless specified otherwise.

2.1.4.2 Sodium Azide

100mg mL^{-1} of sodium azide was dissolved in sterile water and used at a working concentration of 100µg mL^{-1} .

2.1.5 Routine Equiptment

2.1.5.1 Centrifugation of samples ≤1.5mL

All samples up to 1.5mL were centrifuged using the Sigma 1-16k, Sigma Laboratory Centrifuges.

2.1.5.2 Centrifugation of samples ≤15mL

All samples ranging between 1.5 – 50mL were centrifuged using the EBA 12R, Hettich.

2.1.5.3 Centrifugation of samples ≤50mL

All samples ranging between 15 - 50mL were centrifuged using the Heraeus Megafuge 16, Thermo Scientific.

2.1.5.4 Heat block

All samples requiring heating in a heat block were placed in the Thermomixer R, Eppendorf.

2.1 Storage of isolates

2.1.1 Production of long-term stocks

The isolates provided from the QMC were on agar slopes, and they were then sub-streaked onto LB agar to confirm the purity of the isolate. Then a broth culture was produced with 5mL of inoculated LB broth and incubated as standard. This culture was then used to produce glycerol stocks.

0.5 mL of the culture was combined with 0.5mL of a 50% glycerol solution and was transferred to a cryotube and stored at -80°C for future use.

Revival of these stock cultures was done by scrapping the stock with a 1µL sterile loop and streaking onto a fresh LB agar plate for incubation.

2.1.2 Short term storage

Short term storage of bacterial cultures or nucleic acids were stored at -2°C and for no longer than the span of a week.

Methodology

2.2 Confirmation of *Escherichia coli* identity

2.2.1 Oxidase testing

A fresh plate of each isolate and ATTC 25922 was produced for testing. A plastic loop was used to transfer part of a single colony to the testing site on an Oxidase testing strip (MilliporeSigma) and observed for any colour changes.

2.2.2 Indole testing

Another colony of each isolate and ATTC 25922 was tested for the capacity for tryptophane degradation. A colony was collected with a loop and mixed with a drop of the RapID Spot Indole Reagent (Remel, ThermoFisher). The reagent contains dimethylaminocinnamaldehyde, which reacts with indole producing rapid blue-green colour change. The drop was then observed for the appearance of a blue-green colour change.

2.2.3 Growth on TBX agar

The isolates and ATTC 25922 were all streaked onto TBX selective agar. The main constituents of the media were casein digest, bile salts and x-glucuronide. The presence of bile salts as a selective agent against Gram-positive bacteria and only casein digest as a nutrient source to select for tryptophan metabolising isolates. The presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) in the media means that bacteria producing β-glucuronidases produce visible blue/blue-green colonies. These constituents allow the presumptive identification of *E. coli* from blue colonies.

2.3 Measures of carbapenemase activity

2.3.1 CHROMagar mSuperCARBA agar

All the isolates and control strains were streaked onto CHROMagar mSuperCARBA agar so as to identify carbapenemase production and further confirmation of *E. coli* identification. The agar has specific selective and chromogenic agents that allow for the identification of carbapenem-resistant genera of bacteria and species in the case

of *E. coli*. These plates were then incubated at 37°C for 18 hours. The growth level and colour appearance was recorded.

2.3.2 Agar dilution MIC

MH agar plates containing varying concentrations of imipenem were produced. There were three concentrations of imipenem, which were 1µg/mL, 2µg/mL, and 4µg/mL, due to the variation of hydrolytic capacity amongst carbapenemases.

Each isolate and the control strains were streaked onto these plates and incubated at 37°C for 18 hours.

2.3.3 Broth dilution MIC

Further minimum inhibitory concentration were calculated using the broth microdilution method. A culture of each isolate and the control strains were grown in non-selective Muller-Hinton (MH) broth, for 18 hours at 37°C.

MH broth and imipenem solutions were produced to 5 concentrations of imipenem, which were 16µg mL^{-1}, 8µg mL^{-1}, 4µg/mL^{-1}, 2µg/mL^{-1} and 1µg/mL^{-1}. Into row of wells of a Corning 96 Well Clear Microplate (SigmaAldrich) 199µl of each concentration of the MH broth was pipetted. Into each row of these wells, 1µl of the cultures were inoculated. They were then incubated at 37°C for 24 hours.

They were then removed and examined by eye. Each of the well contents was then spotted out onto fresh non-selective LB agar to confirm viable cell presence.

2.3.4 Zinc-supplemented Carbapenem Inactivation Method (zCIM)

All isolates and control strains were screened with zCIM as another measure of their carbapenem resistance.

Firstly, 400µL of TSB, supplemented with $ZnSO_4$ to a concentration of 0.3mM, was added to a sterile microcentrifuge tube. A 10µL loopful of each isolate was added to the broth to inoculate it. A 10µg imipenem antibiotic disc (SIRscan, i2a diagnostics) was added to each tube and incubated at 37°C for 2 hours. This was also repeated with the positive and negative control strains, NCTC 13476 and ATTC 25922.

After the incubation time elapsed, a plate of Muller-Hinton agar (MHA) was streaked entirely as in 2.3.1 with the *E. coli* strain ATTC 25922, which bears no antibiotic resistance. Then from each of the incubated cultures the imipenem disc was removed with sterile tweezers and placed evenly onto the inoculated MHA plates. An additional uncontaminated imipenem disc was placed also as a control. The plates were then incubated at 37°C for 18 hours.

After incubation the plates were examined. The zones of inhibition around the discs were measured, and isolates with zone diameters of ≥20mm were recorded as positive for carbapenemase production.

2.3.5 EDTA-modified Carbapenem inactivation method (eCIM)

To get a better insight into the Ambler classification of the carbapenemase producers, the isolates testing positive in the zCIM were subject to the eCIM and the results of the zCIM was used as the standard to produce the results of the eCIM. The eCIM allows the identification of class B beta-lactamases as the chelating agent EDTA binds to the zinc ion that makes up their active site of these metallo beta-lactamases.

Firstly, 400µL of TSB, supplemented with EDTA to a concentration of 0.1mM, was added to a sterile microcentrifuge tube. A 10µL loopful of each isolate and control strains were added to the broth to inoculate it. A 10µg imipenem antibiotic disc (Sirscan) was added to each tube and incubated at 37°C for 2 hours. This was also repeated with the positive and negative control strains, NCTC 13476 and ATTC 25922.

After the incubation time elapsed, a plate of MHA was streaked entirely as in 2.3.1 with the *E. coli* strain ATTC 25922, which bears no antibiotic resistance. Then from each of the incubated cultures the imipenem disc was removed with sterile tweezers and placed evenly onto the inoculated MHA plates. An additional uncontaminated imipenem disc was placed also as a control. The plates were then incubated at 37°C for 18 hours.

After the incubation, the zones of clearing were measured. If the zone size is ≥5 mm compared to the zone size on the zCIM, the isolate is considered a metallo beta-lactamase and belongs to the Ambler classification B. If the zone is ≤4 mm it belongs to another class.

2.3.6 Modified Carba NP test

The modified carba NP test (mCNP) (Dortet et al., 2012) is another method used to distinguish between different classes of carbapenemases. The mCNP provides further confirmation of enzymatic breakdown of carbapenems due to the addition of phenol red pH indicator. The hydrolysis of carbapenems produces a derivative which bears another carboxylic acid group, causing a pH change.

The isolates that were screened in the zCIM were subjected to the mCNP.

Three test cultures were produced. In the first, the isolates and control strains were inoculated in TS broth with imipenem and $ZnSO_4$ at a concentration of 2µg/mL and 0.3mM, respectively. The second set were in cultures of the same specification, but with the addition of EDTA to a concentration of 0.1mM. The third set of these cultures were produced but excluding imipenem and ETDA. These cultures were incubated for 24 hours at 37°C. After 24 hours their colour changes were observed.

2.3.7 ESBL and AmpC differentiation

The isolates that presented as carbapenem-resistant but not carbapenemase-producing and ATCC 25922 were subjected to the ESBL + AmpC Screen Kit (ROSCO DIAGNOSTICA). To CLSI standard MH agar square plates were produced and were MH broth cultures produced to a 0.5 MacFarland standard and streaked entirely over the surface of the agar plates. Then the four discs from the kit were placed equally interspaced on the plate. The discs were impregnated with cefotaxime (50µg), cefotaxime + clavulanic acid (50µg), cefotaxime + cloxacillin (50µg), cefotaxime + clavulanic acid + cloxacillin (50µg). The plates were then incubated at 37°C for 18 hours and the zones of clearing were measured afterwards. The zones of clearing determined the presence of ESBL, AmpC or neither. Table 3 shows the criteria for each.

Table 3.

Antibiotic disc	Difference in zone diameters when compared			β-lactamase
	Cefotaxime	Cefotaxime+Clavulanic acid	Cefotaxime+Clavulanic acid+Cloxacillin	
Cefotaxime+Clavulanic acid	≥5 mm	-	-	ESBL
Cefotaxime+Clavulanic acid+Cloxacillin	-	<5 mm	≥5 mm	
Cefotaxime+Cloxacillin	≥5 mm	-	-	AmpC
Cefotaxime+Clavulanic acid+Cloxacillin	-	≥5 mm	<5 mm	
Cefotaxime+Clavulanic acid	<5 mm	-	-	ESBL + AmpC
Cefotaxime+Clavulanic acid+Cloxacillin	-	≥5 mm (AmpC)	≥5 mm (ESBL)	

2.4 Multi drug antibiotic sensitivity

2.4.1 Disc diffusion assay

A Kirby-Bauer disc diffusion assay was carried out on the isolates, to gauge their antibiotic susceptibilities. The assays were carried out to the performance standards outlined in the CLSI M100: Performance Standards for Antimicrobial Susceptibility Testing, 34th Edition (2024).

The isolates and the control strains were suspended in Mueller-Hinton broth to a 0.5 MacFarland standard of turbidity. These suspensions were then inoculated onto Mueller-Hinton agar plates with a sterile swab. The entirety of the agar surface was swabbed twice from opposing corners of the plate to ensure equal isolate coverage.

Then 16 antibiotic discs were dispensed onto the surface of the agar using a 16-disc antibiotic disc dispenser (i2a diagnostics). The antibiotic discs were SIRscan discs, also a product of i2a diagnostics. The discs applied were Ciprofloxacin 5ug (CIP), Colistin 10ug (CT), Nitrofurantoin 300ug (F), Meropenem 10ug (MEM), Streptomycin 10ug (S10), Imipenem 10ug (IPM), Tetracycline 30ug (TE), Cefpodoxime 10ug (CPD), Cefotaxime 30ug (CTX), Nalidixic acid 30ug (NA), Cefoxitin 30ug (FOX), Ampicillin 10ug (AM), Amoxicillin-Clavulanic acid 30ug (AMC) Ceftazidime 30ug (CAZ), Chloramphenicol 30ug (C), and Aztreonam 30ug (ATM). Once the discs were dispensed the plates were incubated at 37°C for 18 hours. The zones of inhibition were measured and recorded.

The breakpoints to classify isolates as resistant, intermediate and suceptible were also derived from the CLSI M100: Performance Standards for Antimicrobial Susceptibility Testing, 34th Edition (2024).

2.5 DNA and Plasmid preparation

2.5.1 Crude DNA extraction

A fresh colony of each isolate was resuspended in 100µL of Milli-Q purified water in a sterile microcentrifuge tube. The sample was then placed in a heat block at 100°C for 30 minutes.

The sample was then centrifuged at 16,000 x *g* for 10 minutes. The supernatant was then pipetted into a fresh tube, leaving the pellet undisturbed. These extracts were stored at -20°C.

2.5.2 Monarch® Plasmid mini-prep

For gene amplification on the plasmids, the plasmids within the isolates were extracted using the Monarch® Plasmid Miniprep Kit.

All centrifugation steps within this process took place at 16,000 x *g*.

Each isolate was inoculated into 5 mL of LB broth and incubated at 37°C for 18 hours. These cultures were then centrifuged for 30 seconds to pellet the bacteria. The supernatant was discarded, and the pellet was resuspended in 200µL of “Plasmid Resuspension Buffer” and vortexed to ensure complete and even resuspension.

200µL of “Plasmid Lysis Buffer” was then added to the resuspension and the tubes were inverted gently 5 times until the solution turned dark pink, from which it was incubated at room temperature for one minute.

400µL of "Plasmid Neutralisation Buffer" was added and the tube was gently inverted again 5 further times or until the solution was a uniform yellow colour with a precipitate formed. It was incubated at room temperature for 2 minutes.

This lysate was then spun for 5 minutes to pellet the cell debris, and the supernatant was transferred to the provided spin columns in collection tubes. The spin columns were then spun for 1 minute.

The flow through was discarded from the collection tubes, the spin column was re-inserted and 200µL of "Plasmid Wash Buffer 1" was added and the column was spun for 1 minute. Then 400µL of "Plasmid Wash Buffer 2" was added and the column was spun for 2 minutes. The spin column was then removed and transferred to a fresh collection tube, without coming into contact with the flow-through.

50µL of "DNA Elution Buffer", warmed to 50°C, was then added to the column. The column was allowed to rest for one minute and was then spun for another minute. The elute collected in the collection tube was then transferred to a sterile microcentrifuge tube and stored at -20°C for further application.

2.5.3 QIAGEN® Large construct kit

For the extraction of the large carbapenemase-producing plasmids for sequencing, the QIAGEN® Large-Construct Kit was used.

A 500mL culture of each isolate was produced. Each consisted of LB broth, imipenem at 4µg/mL and 1mL of freshly grown starter culture and it was then incubated at 37°C for 16 hours. The culture was expected to contain 3-4 x 10^9 CFU mL^{-1}.

This culture was pelleted by centrifugation at 6000 x *g* for 15 minutes. 20mL of "Buffer P1" was then added and the solution was vortexed to produce an even resuspension.

20mL of "Buffer P2" was added and the solution was mixed by 5 inversions and allowed to incubate at room temperature for 5 minutes. After 5 minutes exactly, 20mL of "Buffer P3", chilled to 4°C, was added and the tube was inverted 5 times. The solution was then incubated on ice for 10 minutes.

This solution was then centrifuged at 20,000 x *g* for 30 minutes, after which the supernatant was decanted into a fresh vessel, through a pre-moistened filter paper.

The DNA in this supernatant was precipitated out with 0.6 volumes of isopropanol and centrifuged at 15,000 x *g* for 30 minutes. The supernatant was removed, and the pellet was washed in 5mL of 70% ethanol and spun again at 15,000 x *g* for 15 minutes.

The ethanol was then decanted, and the DNA pellet was allowed to air-dry for 3 minutes.

The DNA was then completely redissolved in 9.5mL of "Buffer EX". 200µL of ATP-dependent exonuclease was added alongside 300µL of a 100mM ATP solution. This solution was mixed gently and incubated at 37°C for 60 minutes, in a water bath.

A “QIAGEN® -tip 500” tip was equilibrated by the addition of 10mL of “Buffer QBT” which was allowed to empty through by gravity flow.

10 mL of “Buffer QS was added to the digest and the whole sample was added to the equilibrated tip and allowed to flow through by gravity flow.

Once the flow halts the tip was washed through twice by 30mL of “Buffer QC”. After which the DNA was eluted with 15mL of “Buffer QF” prewarmed to 65°C and the elute was collected in a fresh tube.

The DNA precipitation step was then repeated again, and the pellet was allowed to airdry for 5 minutes. It was then redissolved in 200µL of TE buffer.

The samples were then stored at -20°C for later use.

2.5.4 GenElute gel extraction kit

For extraction of DNA bands from agarose gel, the GenElute (Millipore Sigma) kit was used.

All centrifugation steps took place at 16,000 x *g*.

Excised gel samples were weighed and combined in a sterile microcentrifuge tube with 3 gel volumes of the “Gel Solubilisation Solution”. The sample was then placed in a heat block, incubated at 60°C for 5 minutes, gently vortexed and then returned to the block until the gel had completely dissolved into solution. One gel volume of 100% isopropanol was then added and mixed.

A spin column was added to a collection tube. To prepare it for the sample, 0.5mL of “Column Preparation Solution” was added and spun for 1 minute, with the flow-through discarded.

Then the gel solution was added to the spin column and spun again for 1 minute, with the flow-through discarded. 700µL of “Wash Solution” was added to the spin column and spun for 1 minute and the flow-through was discarded. The column was then spun again for another minute, and the spin column was transferred to a fresh collection column.

50µL of “Elution Solution” was pre-heated to 65°C and then added to the spin column and allowed to incubate for 1 minute. The column was then spun for 1 minute and the spin column discarded.

The eluate was then stored at -20°C.

2.5.5 DNA quantification

DNA quantification was done with the BioDrop uLITE spectrophotometer. Using 1µL of the sample the absorbance of UV light was measured and absorbance value ratios at

260/280 nm and 260/230nm were calculated, providing measures of the purity and DNA concentration in ng/µL.

2.6 Polymerase Chain Reaction (PCR) Amplification

2.6.1 Primers

All primers were synthesised by Sigma-Aldrich, using desalt purification to a concentration of 100 µM in nuclease-free water. Working stocks were prepared at 10 µM by dilution in nuclease-free water and stored at -20°C.

2.6.1.1 Carbapenemase gene primer

The primers of the carbapenemase genes used are listed in Table 4.

Table 4.

GENE	PRIMER SEQUENCE	PRODUCT SIZE (BASE PAIRS)	REFERENCE
NDM (FW)	GGTTTGGCGATCTGGTTTTC	621	Poirel et al., 2011.
NDM (RV)	CGGAATGGCTCATCACGATC		
OXA-48 (FW)	GCGTGGTTAAGGATGAACAC	438	Poirel et al., 2011.
OXA-48 (RV)	CATCAAGTTCAACCCAACCG		

2.6.1.2 Incompatibility group primer

The primers used to identify incompatibility groups are listed in Table 5.

Table 5.

PRIMER	PRIMER SEQUENCE	PRODUCT SIZE (BASE PAIRS)	REFERENCE
HI1 (FW)	GGAGCGATGGATTACTTCAGTAC	471	Carattoli et al., 2005. Villa et al., 2010.
HI1 (RV)	TGCCGTTTCACCTCGTGAGTA		
HI2 (FW)	TTTCTCCTGAGTCACCTGTTAACAC	644	
HI2 (RV)	GGCTCACTACCGTTGTCATCCT		
I1-1γ (FW)	CGAAAGCCGGACGGCAGAA	139	
I1-Iγ (RV)	TCGTCGTTCCGCCAAGTTCGT		
X (FW)	AACCTTAGAGGCTATTTAAGTTGCTGAT	376	
X (RV)	TGAGAGTCAATTTTTATCTCATGTTTTAGC		
L/M (FW)	GGATGAAAACTATCAGCATCTGAAG	785	
L/M (RV)	CTGCAGGGGCGATTCTTTAGG		
N (FW)	GTCTAACGAGCTTACCGAAG	559	
N (RV)	GTTTCAACTCTGCCAAGTTC		
FIA (FW)	CCATGCTGGTTCTAGAGAAGGTG	462	
FIA (RV)	GTATATCCTTACTGGCTTCCGCAG		
FIB (FW)	TCTGTTTATTCTTTTACTGTCCAC	683	
FIB (RV)	CTCCCGTCGCTTCAGGGCATT		
W (FW)	CCTAAGAACAACAAAGCCCCCG	242	
W (RV)	GGTGCGCGGCATAGAACCGT		
Y (FW)	AATTCAAACAACACTGTGCAGCCTG	765	
Y (RV)	GCGAGAATGGACGATTACAAAACTTT		
P (FW)	CTATGGCCCTGCAAACGCGCCAGAAA	534	
P (RV)	TCACGCGCCAGGGCGCAGCC		
FIC (FW)	GTGAACTGGCAGATGAGGAAGG	262	
FIC (RV)	TTCTCCTCGTCGCCAAACTAGAT		
A/C (FW)	GAGAACCAAAGACAAAGACCTGGA	465	
A/C (RV)	ACGACAAACCTGAATTGCCTCCTT		
T (FW)	TTGGCCTGTTTGTGCCTAAACCAT	750	
T (RV)	CGTTGATTACACTTAGCTTTGGAC		
FIIS (FW)	CTGTCGTAAGCTGATGGC	270	
FIIS (RV)	CTCTGCCACAAACTTCAGC		

FII (FW)	CTGATCGTTTAAGGAATTTT	258-262	
FII (RV)	CACACCATCCTGCACTTA		
X3 (FW)	GTTTTCTCCACGCCCTTGTTCA	351	Johnson et al., 2012.
X3 (RV)	CTTTGTGCTTGGCTATCATAA		

2.6.2 Amplification of Carbapenem genes

2.6.2.1 NDM

All isolates were screened with the NDM primers.

20µL of the total reaction mixture was combined in a 200µL PCR tube (Thermo Scientific). This reaction mixture consisted of 16µL of Dream Taq, 1µL of the forward (FW) primer, 1µL of the reverse (RV) primer, 2µL of crude DNA extract with the final volume being made up with 6µL of nuclease-free water.

The reaction mixture was placed into a PCR thermocycler and subject to the conditions listed in Table 6.

Table 6.

Number of cycles	Stage	Temperature (°C)	Time (seconds)
1	Initial denaturation	94	600
35	Denaturation	94	30
	Annealing	52	40
	Extension	72	50
1	Final extension	72	300

The reaction mixture was then removed from the thermocycler and stored short term at 2-4°C until ready for visualisation.

2.6.2.2 OXA-48

All isolates were screened with the OXA-48 primers

20µL of the total reaction mixture was combined in a 200µL PCR tube (Thermo Scientific). This reaction mixture consisted of 16µL of Dream Taq, 1µL of the forward (FW) primer, 1µL of the reverse (RV) primer, 2µL of crude DNA extract with the final volume being made up with 6µL of nuclease-free water.

The reaction mixture was placed into a PCR thermocycler and the cycle conditions were the same as those used in 2.6.2.1, listed in Table 5.

The reaction mixture was then removed from the thermocycler and stored short term at 2-4°C until ready for visualisation.

2.6.2.3 Plasmid carriage

All excised purified plasmids were screened again for the carbapenemase gene(s) that were previously found in their crude extracts.

20µL of the total reaction mixture included 16µL of Dream Taq, 1µL of the FW primer, 1µL of the RV primer, 2µL of purified plasmid extract with the final volume being made up with 6µL of nuclease-free water. In the case that an isolate contained both OXA-48 and NDM, the volume of the nuclease-free water was reduced to 4µL, to accommodate for the additional 1µL of both FW and RV primers added.

The reaction mixture was placed into a PCR thermocycler and the cycle conditions was the same as those used in 2.6.2.1 and 2.6.2.2, shown in Table 6. Their storage conditions also remained the same.

2.6.3 Incompatibility group typing

The plasmids identified to contain the carbapenemase gene(s) were then subject to incompatibility group typing. The purified plasmids were subject to 5 multiplex and 1 simplex replicon sequence amplification, using 17 of the most common replicon types identified on plasmids with *Enterobacteriaceae* as suggested by Carattoli et al., 2005.

The primer types used in each multiplex were as follows, Multiplex 1: HI1, HI2, I1-Iγ, Multiplex 2: X, L/M, N, Multiplex 3: FIA, FIB, W, Multiplex 4: Y, P, FIC, Multiplex 5: A/C, T, FIIS. The lone simplex was done with the X3 primers. All primers can be located in Table 5.

The contents of the multiplex reaction mixtures consisted of 10µL of DreamTaq, 0.5 µL of each of the FW and RV primers, 2µL of the purified plasmid extract and then 6.5µL of nuclease-free water to make the volume up to 20µL.

The makeup of the X3 simplex reaction mixture was the same as 2.6.2.1, with the primers substituted for X3.

The conditions of the multiplex reaction thermocycling are listed in Table 7.

Table 7.

Number of cycles	Stage	Temperature (°C)	Time (seconds)
1	Initial denaturation	94	300
35	Denaturation	94	60
	Annealing	60	30
	Extension	72	60
1	Final extension	72	300

The conditions for the X3 simplex reaction thermocycling differed only in the annealing temperature which was modified to 52°C.

After thermocycling, the reaction mixtures were stored short-term at 2-4°C until ready they were ready for visualisation.

2.6.4 Visualisation of DNA

The amplicons from the PCR reactions and the purified plasmids from the large-construct kit were visualised using agarose gel electrophoresis. In all agarose gel visualisations, a negative control was also run using sterile MilliQ water.

2.6.4.1 Running of amplicons

Amplicons were visualised on 1% agarose gels, consisting of 0.5g of agarose powder dissolved in 50mL of 1xTAE buffer for smaller gels or 2g of agarose powder dissolved in 200mL of 1xTAE buffer for the larger gels.

The agarose powder was completely dissolved in the 1xTAE buffer by implementing microwave heating. Once cooled to ~50°C, before the agarose solidified, Ethidium Bromide (EtBr) was added to the gels to a concentration of 0.5µg mL-1, depending on the gel volume. Once the EtBr was incorporated into the solution, it was poured into a casting tray of an appropriate size with a well comb of an appropriate number of wells and allowed to set at room temperature.

Once set, the gel was then transferred to a gel tank filled with 1xTAE buffer and the samples were loaded alongside a 100bp DNA ladder (NEB). Each sample was loaded at a volume of 10µL and a volume of 5µL of the DNA ladder.

The gel tank was then powered and allowed to run at 100V for 30 minutes.

2.6.4.2 Running of plasmid extracts

The gel for visualising extracted plasmids was made to the same specifications as the larger gels previously made. The gels made were consistent and were 1% agarose gels made to a volume of 200 mL, so consisted of 2 g of agarose added to 200mL of 1xTAE buffer. The gel was prepared in the same manner as in 2.6.4.2 up to the loading stage.

Purple loading dye was added to the plasmid samples prior to loading at a 1:5 ratio, in this instance 4µL of dye to 16µL of sample. The DNA ladder used for these visualisations was the 1kb plus ladder. The ladder was loaded at 5µL, and the DNA samples were loaded at 20µL.

The samples were then run at the lower voltage of 80V for 90 minutes to account for their much larger size.

2.6.4.3 Visualisation of gels

The gels of the amplicons were visualised using UV transilluminator (Gel Doc XR+, Bio-rad) and their images were saved for further analysis.

The gels of the purified plasmids were visualised on a UV transilluminator (UPV TM-20, now Analytik Jena, Jena, Germany) to allow for the excision of the bands for further purification using the GenElute kit.

2.7 Conjugation of resistance plasmids

The isolates identified as carbapenem resistant and containing the carbapenemase gene on their plasmids were subject to conjugation assays using a modified strain of $J53_{AziR}$ as a recipient.

2.7.1 Conjugation into the recipient strain

From a fresh plate culture of the resistant isolate (donor), a single colony was inoculated into 20mL of LB broth with imipenem at 2µg/mL and incubated statically at 37°C for 18 hours. A separate culture was made of the modified J53 strain (recipient). A single fresh colony from the modified J53 strain was inoculated into 20mL of LB broth without a selective agent and was incubated shaking at 37°C for 18 hours.

After the incubation time elapsed, the donor isolates were removed from the incubator and stored at 2-4°C. The overnight cultures of the recipient strain were refreshed by transferring 500µL of the overnight cultures into 4.5mL of fresh LB broth and returning to the shaking incubator, at 37°C, for a further 2.5 hours.

After the 2.5 hours of incubation, 5mL of both the donor and refreshed recipient cultures were centrifuged at 1487 x *g* for 10 minutes to form a pellet. The supernatant was discarded, and the pellet was resuspended completely in 5mL of phosphate buffered saline (PBS). The centrifugation step was then repeated again with the supernatant discarded again. The pellet was then resuspended in 500µL of PBS and transferred to a fresh sterile microcentrifuge tube.

100µL of both the recipient and donor suspensions were pipetted into a single microcentrifuge tube and mixed by pipetting the mixture up and down. This mixture was then spotted onto the centre of a fresh LB agar plate and incubated at 37°C for 18 hours, with an additional 200µL of the pure donor and recipient cultures spotted on separate plates as controls.

To determine the CFU mL^{-1} of both the donor and recipient cultures were then serially diluted down to 10^{-8} with maximum recovery diluent (MRD). 100µL of the

concentrations 10^{-6}, 10^{-7}, and 10^{-8} were spread onto individual fresh LB agar plates with a sterile L-spreader and incubated at 37°C for 18 hours.

After 18 hours the plate count was determined for the pure donor and recipient cultures. The plates with the spot of the mixed culture had 1.5mL of PBS added to them and using an L-spreader, the colony was resuspended in the PBS and pipetted to a sterile microcentrifuge. These suspensions were then diluted with MRD to 10^{-4}; 100ul of each dilution was then spread on fresh LB agar containing 2µg/mL of imipenem and 100µg/mL of sodium azide. These plates were incubated at 37°C for 18 hours.

The plates were then removed from incubation and stored at room temperature for an hour. The plates were then observed and those containing discreet purple colonies were considered suspected transconjugants, the dilution within a countable range was counted and recorded.

Three colonies from each of these suspended transconjugants were then streaked into fresh LB agar plates, containing both imipenem and sodium azide to previously specified concentrations, and incubated at 37°C for 18 hours.

Long-term stocks of these suspected transconjugants were then produced, as described in 2.1.1.

2.7.2 Conjugational efficiencies

Conjugational efficiencies were calculated for each suspected transconjugant, using the CFU mL^{-1} of the donor and the suspected transconjugant in the following calculation:

$$\textit{Conjugational efficiency} = \frac{CFU\ mL^{-1}\ of\ suspected\ transconjugant}{CFU\ mL^{-1}\ of\ donor}$$

2.7.3 Confirmation of carbapenemase gene transference

In suspected transconjugants, the plasmids were extracted using Monarch® plasmid miniprep kit (2.5.2). The extracted plasmids were then subject to PCR reactions to identify the presence of the relevant carbapenemase gene(s), this process followed those listed in 2.6.2.1 and 2.6.2.2. Those containing these genes and also producing purple colonies that were resistant to sodium azide, were considered confirmed transconjugants.

2.7.3 Identifying the carbapenemase-producing plasmid

A fresh culture of the confirmed transconjugants were produced in LB broth with 2μg/mL of imipenem and 100μg/mL of sodium azide to maximise plasmid concentration and they were incubated for 37°C for 18 hours. This culture was then used in the Monarch® plasmid miniprep kit (2.5.2). Once the plasmids were purified, they were separated on an agarose gel (2.6.4.2) and then excised and purified (2.6.4.3).

Once the plasmids were purified, they were subject to PCR reactions to detect the presence of their carbapenemase gene (2.6.2.1 and 2.6.2.2). These amplicons were then visualised on an agarose gel (2.6.4.2 and 2.6.4.3). to identify which plasmid band contained the resistance gene.

2.8 Sequencing of transmissible plasmids

To understand the mechanisms enabling such swift and widespread dissemination of the OXA-48 and NDM genes through plasmids, they must be sequenced.

2.8.1 Purification of large plasmids from transconjugants

The carbapenem resistance plasmids were purified from their transconjugant isolates using the QIAGEN® Large-Construct Kit (). The extracts were then run on agarose gels (2.6.4.2) to separate the plasmid bands. From there the bands previously identified as the relevant plasmids were excised using a sterile scalpel and purified using the GenElute kit (2.5.4).

The plasmids had their purity and concentration confirmed using the BioDrop uLITE. The samples were then either diluted with more TE buffer to bring them within the required volume and concentration parameters for sequencing.

2.8.2 Sequencing of carbapenemase-producing plasmids

The sequencing was done with Plasmidsaurus, who carried out long-read whole plasmid sequencing. Due to their size, the plasmids were within the “Big” category (25-125kb) and required the samples to be a minimum volume of 20μL and a concentration between 50-400ng/μL. Dilutions to this concentration were made with TE buffer.

Chapter 3: Results

3.1 Confirmation of *Escherichia coli* identity

All isolates (1-15) were all expected to be *Escherichia coli* with some degree of carbapenem resistance. To confirm this they were subject to biochemical testing to confirm their identity as *E. coli*. All isolates and the control ATCC 25922, were found to be oxidase negative (2.2.1) and indole positive (2.2.2). All isolates streaked onto TBX agar (2.2.3) produced blue/green colonies, indicating the production of β-glucuronidase and therefore identifying them as *E. coli*. The isolates, except isolates 6, 11 and 14, streaked into mSuperCARBA agar produced colonies with the colouration of dark pink to reddish. This identified that as both *E. coli* and carbapenem-resistant.

3.2 Measures of carbapenemase activity

All isolates were then subjected to assays that measured and roughly identified their carbapenem resistance mechanisms. The isolates 6, 11, and 14 were still included in these assays so as to completely confirm their lack of clinically-relevant carbapenem resistance.

3.2.1 Minimum inhibitory concentration of imipenem

The minimum inhibitory concentrations (MICS) of imipenem in agar dilution assays (2.3.2) and broth dilution assays (2.3.3) were used as a way to classify the strength of carbapenem resistance within the isolates. As Imipenem was the antibiotic of choice, the CLSI breakpoints used were ≥4μg mL^{-1} resistant and ≥2 μg mL^{-1} for intermediate.

The agar dilution provided a visual of the strength of carbapenemase activity that each isolate possessed by the portion of the agar the colonies covered. Strong growth and therefore stronger carbapenemase activity was attributed if the isolate took over more than ~50% of the agars surface, whereas weak growth was less than ~50% of the plate covered. They judgements were able to be drawn as all isolates could colonise the entire agar plate in the absence of any antibiotics. The growth strength between the two concentrations of imipenem and the conclusion drawn is shown in Table 8.

Table 8.

	Isolate number								
	1	2	4	7	10	13	8	9	15
Growth at 2µg mL^{-1}	Strong	Strong	Strong	Strong	Strong	Strong	Strong	Strong	Strong
Growth at 4µg mL^{-1}	Strong	Strong	Strong	Strong	Strong	Strong	Weak	Weak	Weak
Final judgement	Strong activity	Strong activity	Strong activity	Strong activity	Strong activity	Strong activity	Intermediate activity	Intermediate activity	Intermediate activity

	Isolate number					
	3	5	12	6	11	14
Growth at 2µg mL^{-1}	Weak	Weak	Weak	None	None	None
Growth at 4µg mL^{-1}	Weak	Weak	Weak	None	None	None
Final judgement	Weak activity	Weak activity	Weak activity	No activity	No activity	No activity

Broth dilution (2.3.3) with a wider range of dilutions was then carried out to more clearly divide the isolates imipenem MICs. Out of the 15 isolates 12/15 (80%) were within the breakpoints to be considered carbapenem resistant with MIC proportions of >10μg mL^{-1} = 8 (53.3%), 4μg mL^{-1} = 1 (6.7%), 2μg mL^{-1} = 2 (13.3%), and 3/15 (20%) considered susceptible with the remaining MIC being <1μg mL^{-1} = 3 (20%). None of isolates MICs were 6μg mL^{-1} or 8μg mL^{-1}. These values are laid out in Table 9.

Table 9.

Isolate	*Minimum inhibitory concentration (μg mL^{-1})*
1	>10
2	>10
3	2
4	>10
5	4
6	<1
7	>10
8	>10
9	>10
10	>10
11	<1
12	2
13	>10
14	<1
15	>10

Table 9 showing the minimum inhibitory concentration of imipenem for all the different isolates

3.2.2 Identification of carbapenemase-activity

The isolates were used in zCIM (2.3.4) and eCIM (2.3.5) assays, the results of these could provide an inference into the Ambler classification of the isolates. The results of this assay is laid out in Table 10.

Table 10.

Isolate	zCIM zone size (mm)	eCIM zone size (mm)	Size difference (mm)
1	0	13	13
2	0	15	15
3	0	0	0
4	0	16	16
5	0	0	0
6	14	15	1
7	0	17	17
8	0	0	0
9	0	0	0
10	0	14	14
11	14	14	0
12	0	0	0
13	0	14	14
14	13	15	2
15	0	0	0
+	0	13	13
-	15	16	1

Table 10 showing the difference in the sizes of the zones of inhibition produced in the zCIM and eCIM assays.

The isolates with zones of 0mm in assays were taking to have functioning carbapenem resistance mechanims. The isolate zones that increase by 5mm or more from the zCIM to the eCIM were interpreted as having carbapenemases that are inhibited by the chelating effect of EDTA, meaning they are Class B metallo-β-lactamases. The isolates classified as such were isolate 1, 2, 4, 7, 10, 13, and NCTC 13476, which is confirmed to produce the class B IMP carbapenemase.

The mCNP test (2.3.6) was carried out to confirm if all isolates with no zones of clearing in the zCIM were carbapenemase-producing *E. coli* (CPE). The colour changes observed in both the standard and EDTA supplemented assays and they were compared as a confirmation in the conclusions made between the zCIM and eCIM. In the standard assay, all isolates exhibited the colour change to orange-yellow, except for 6, 11, and 14. In the EDTA-supplemented series, all the isolate identified as metallo-β-lactamases from the eCIM displayed no colour change, confirming this classification. This data is laid out in Table 11.

Table 11.

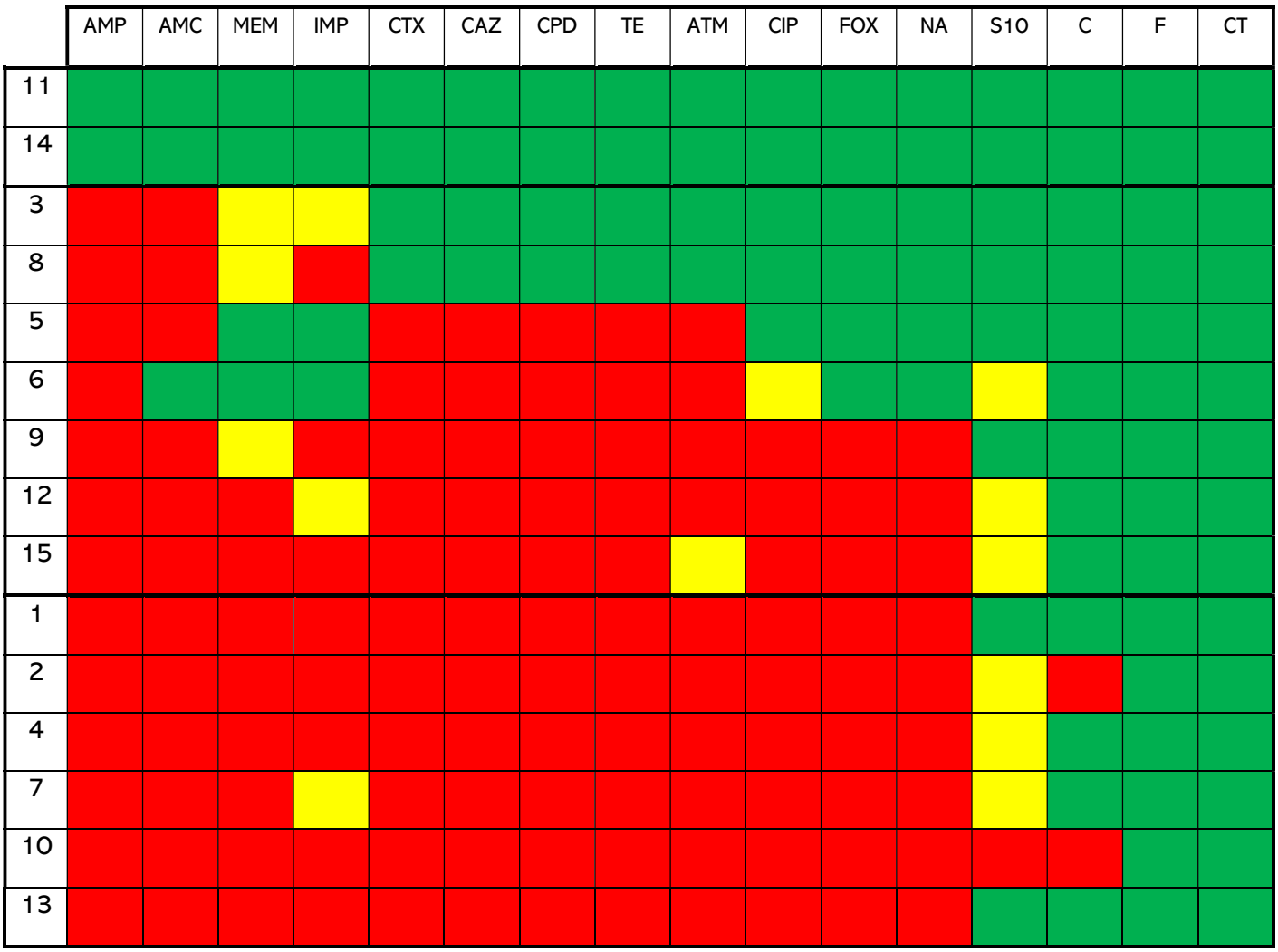
ISOLATE	COLOUR		EDTA INHIBITED CARBAPENEMASE ACTION
	Standard	With EDTA	
1	Yellow	Red	Yes
2	Yellow	Red	Yes
3	Yellow	Yellow	No
4	Yellow	Red	Yes
5	Yellow	Yellow	No
6	Red	Red	No
7	Yellow	Red	Yes
8	Yellow	Yellow	No
9	Yellow	Yellow	No
10	Yellow	Red	Yes
11	Red	Red	No
12	Yellow	Yellow	No
13	Yellow	Red	Yes
14	Red	Red	No
15	Yellow	Yellow	No
NCTC 13476	Yellow	Red	Yes
ATCC 25922	Red	Red	No

Table 11 showing the colour of the cultures after incubation. Isolates all started red and the change to yellow indicates carbapenem hydrolysis. It also contains the interpretation of the colour changes, or lack thereof.

3.3 Identification of other resistances

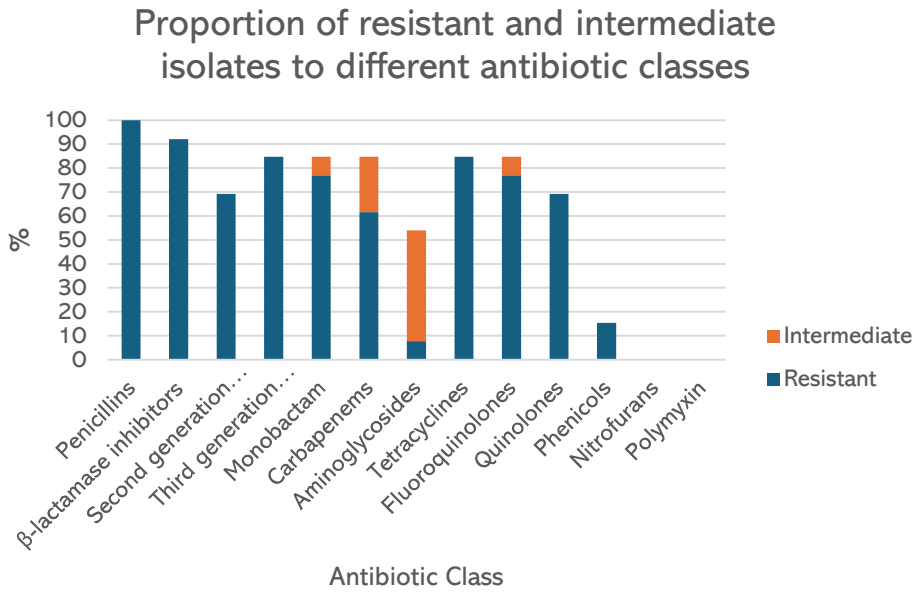
The isolates were subjected to further screening to identify multidrug resistance phenotype with disc diffusion antibiotic sensitivity assays (2.4.1) and their resistance patterns are shown in Figure 2 and a visualisation of the proportion of the antibiotic class resisted is shown in Figure 3.

Figure 2.



Resistance heatmap showing the distribution of resistance to the tested antibiotics. The isolates are blocked off into the three different groups derived from the zCIM & eCIM. Red=resistant, yellow=intermediate and green=susceptible. Antibiotic abbreviations: Ampicillin (AMP), Amoxicillin-clavulanic acid (AMC), Cefoxitin (FOX), Cefotaxime (CTX), Cefpodoxime (CPD), Aztreonam (ATM), Meropenem (MEM), Imipenem (IMP) Streptomycin (S10), Oxytetracycline (TET), Ciprofloxacin (CIP), Nalidixic Acid (NA), Colistin (CT), Chloramphenicol (C), Nitrofurantoin (F).

Figure 3.



The variety of resistance towards the different antibiotic classes, only within the carbapenem resistant isolates.

The isolates 6, 11, and 14 were removed from the subsequent experiments carried out, content that they did not present as carbapenemase producers. However, one final attempt to understand their phenotypes was carried out using an AmpC/ESBL screening kit. The results of this screening is shown in Table 12, Table 13 and Table 14.

Table 12.

Isolate 6	
Antibiotic combination	**Zone of inhibition (mm)**
CTX	9
CTX+Clav	27
CTX+Clox	12
CTX+Clav+Clox	22

Table 13.

Isolate 11	
Antibiotic combination	**Zone of inhibition (mm)**
CTX	37
CTX+Clav	39
CTX+Clox	38
CTX+Clav+Clox	39

Table 14.

Isolate 14	
Antibiotic combination	**Zone of inhibition (mm)**
CTX	37
CTX+Clav	39
CTX+Clox	38
CTX+Clav+Clox	38

Table 12, 13, and 14 showing the size of the zones of inhibition for isolates 6, 11 and 14 with the different antibiotic discs. Antibiotic abbreviations: Cefotaxime (CTX), Clavulanic acid (Clav), and Cloxacillin (Clox).

The zones of inhibition for isolate 6 indicate its beta-lactamase activity is inhibited by clavulanic acid and not by cloxacillin. This indicates that it has no AmpC overproduction and is an ESBL producer. This aligns with the AST result for the isolate being resistant to 3rd generation cephalosporins and aztreonam. It also had tetracycline resistance which is commonly associated with ESBL-producing organisms due to co-carriage of the two resistance genes on the same plasmid. Certain ESBLs have been observed to hydrolyse carbapenems at a very low level and this may be why the isolate was picked up in the hospitals carbapenem-resistance screening.

The zones of inhibition for isolates 11 and 14 show the two isolates possess neither AmpC over production nor ESBL production. This is corroborated by the AST results which found the isolates unable to hydrolyse even ampicillin. The lack of beta-lactam resistance and the inconclusive identification of the resistance mechanism by the hospital leads me to believe that, excluding an error in categorisation, that the isolates may possess some nonenzymatic carbapenem resistance mechanisms such as efflux pump overproduction or porin modification. These mechanisms may by themselves produce a slightly higher inhibitory concentration to carbapenems when compared to a completely susceptible isolate and thus may have been picked up in the carbapenem-resistance screening.

3.4 Genotypic profiles of isolates

The PCR amplification found the isolates consisted of OXA-48 and NDM. The NDM positive isolates were 1, 2, 4, 7, 10, and 13. The OXA-48 positive isolates were 3, 5, 8, 9, 12, 13, 15. These results are shown in Table 15.

Table 15.

Genotype	Isolate
NDM	1
	2
	4
	7
	10
OXA-48	3
	5
	8
	9
	12
	15
Both	13

3.5 Conjugation assays

The isolates that were able to successfully conjugate during the assay were isolate 1, 2, 4, 7, 8, 9, 10, and 12. Their calculated conjugational efficiencies are listed in Table 16.

A Paired T-test was performed on the conjugational efficiencies of NDM vs OXA-48 to see if there was a significant difference between the two and the calculated P-value was 0.3163 and therefore there was not a statistically significant difference between the two.

Table 16.

DONOR ISOLATE	CONJUGATIONAL EFFICIENCY	CARBAPENEMASE GENE
1	1.39×10^{-6}	NDM
2	4.00×10^{-7}	NDM
4	7.88×10^{-7}	NDM
7	5.52×10^{-6}	NDM
8	1.38×10^{-5}	OXA-48
9	9.81×10^{-5}	OXA-48
10	4.20×10^{-5}	NDM
12	7.59×10^{-6}	OXA-48

Table 16 showing the calculated conjugational efficiencies of each isolate, calculated from the donor and transconjugant counts recorded in the conjugation assay, alongside the carbapenemase gene transferred.

The antibiotic phenotypes of the transconjugants were then determined using the disc diffusion antibiotic sensitivity test results and compared to the results of the donors listed in Figure 1. The resistances maintained and lost are listed in Table 17.

Table 17.

Isolate	Carbapenemase gene	Maintained resistances	Lost resistances
1.1	NDM	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, MEM, IMP	None
1.2	NDM	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, MEM, IMP	None
2.1	NDM	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, S10, C, MEM, IMP	None
4.1	NDM	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, S10, MEM, IMP	None
4.2	NDM	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, S10, MEM, IMP	None
7.1	NDM	AMP, AMC, NA	FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP
7.2	NDM	AMP, AMC, NA	FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP
8.1	OXA-48	AMP, AMC, MEM, IMP	None
8.2	OXA-48	AMP, AMC, MEM, IMP	None
8.3	OXA-48	AMP, AMC, MEM, IMP	None
9.1	OXA-48	AMP, AMC, FOX, CAZ, CTX, CPD, TE, MEM, IMP	ATM, CIP, NA
9.2	OXA-48	AMP, AMC, MEM(i), IMP	FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA
10.1	NDM	AMP, AMC, FOX, CAZ, CTX, CPD, S10, TE, MEM, IMP	ATM, CIP, NA, C
10.2	NDM	AMP, AMC, FOX, CAZ, CTX, CPD, S10, TE, MEM, IMP	ATM, CIP, NA, C
12.1	OXA-48	AMP, AMC, MEM, IMP (i)	FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP
12.2	OXA-48	AMP, AMC, MEM, IMP (i)	FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP
12.3	OXA-48	AMP, AMC, MEM, IMP (i)	FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP

Table 17 showing the carbapenem genes within the transconjugants and the class resistances they conserved and lost. "(i)" indicates that the resistance is within the intermediate breakpoints.

Antibiotic abbreviations: Ampicillin (AMP), Amoxicillin-clavulanic acid (AMC), Cefoxitin (FOX), Cefotaxime (CTX), Cefpodoxime (CPD), Aztreonam (ATM), Meropenem (MEM), Imipenem (IMP) Streptomycin (S10), Oxytetracycline (TET), Ciprofloxacin (CIP), Nalidixic Acid (NA), Colistin (CT), Chloramphenicol (C), Nitrofurantoin (F).

The plasmids extracted from the gel of the transconjugant plasmid extracts that tested positive for the carbapenemase gene were all over 48kb

3.6 Sequencing data

3.6.1 Sequencing outcome

Whole plasmid sequencing was attempted on the large transmissible plasmids to investigate the genomic context of the bla_{OXA-48} and bla_{NDM} genes. This would have produced a visual of any mobile genetic elements and the antibioitic resistance genes they could have been associated with and gave an insight into what enables the successful dissemination of the plasmid. This was done with Plasmidsaurus who performed whole plasmid sequencing using continuous long reads, however due to the size of the plasmids and the likely low copy numbers they had, we were unable to meet their requirements for a successful outcome.

The plasmids as of writing this thesis are currently undergoing short read sequencing. Due to unforeseen delays in sequencing, the data from this reading will not be available intime for the inclusion in this thesis. Once the data is returned the findings may be used to build upon the finding reported in this thesis.

While this study successfully characterised the phenotypic and genotypic properties of the carbapenemase-producing *E. coli* isolates, the lack of whole plasmid sequencing data limits the ability to analyse the genetic context of the carbapenemase dissemination within the clinical isolates through the plasmid structures and potentially infer future horizontal gene transfer events.

3.6.2 Analysing sequencing data

While I was unable to retrieve the sequencing data from these conjugative plasmids, the main features within the plasmids I would have analysed to find would have been commonly associated antibiotic resistance genes, insertion sequences/transposons specific to both OXA-48 and NDM, and the incompatibility groups of the plasmids.

In the literature there is a strong association between IS*Aba125* and NDM, this insertion sequence sits upstream from the bla_{NDM} gene and acts as a strong promoter, increasing the expression of the gene. The insertion sequence and bla_{NDM} gene would likely accompany other genes and make up the transposon Tn*125* which enables the mobilisation of the bla_{NDM} gene, it would also have been interesting to have seen if any other antimicrobial resistance genes were carried within this transposon (Kikuchi et al., 2022) (Poirel et al., 2012).

For OXA-48 the literature has highlighted the main insertion sequence associated with OXA-48 and its spread, IS*1999*. IS*1999* flanks OXA-48 and together make up Tn*1999*, of which that are many variants which can differ in the associated insertion sequences but also with their contents, which leads to increased transmissibility and to some becoming the dominant variant in specific populations and overall facilitates OXA-48 in its dissemination within the bacterial populations (Giani et al., 2012) (Sattler et al., 2022).

I would also use it to conclusively assign my plasmids to their incompatibility groups, which would then be compared to the common carbapenemase associated incompatibility groups reported in the literature. For examples the primary incompatibility group seen to be associated with $bla_{OXA\text{-}48\text{-}like}$ genes are IncL plasmids. IncL are a highly stable and conjugative group of plasmids which when carrying $bla_{OXA\text{-}48}$ will result in higher retention rates of the gene within the population, especially in periods with a lower selective pressure towards it, and increased change of horizontal gene transfer throughout the population (Carattoli, 2013) (Brehony et al., 2019).

Bla_{NDM} has larger selection of incompatibility groups that it is found alongside, attesting to its high genetic mobility. These include IncA/C, IncFII, IncHI1 and IncL, but the main group associated with NDM is IncX3 which is concerning due to its observed rapid global dissemination (Rozwandowicz et al., 2018) (Guo et al., 2022). The incompatibility groups and the general plasmid backbone would also provide an interesting insight into if the dominant strains of either carbapenemase are a result of clonal dissemination or if the plasmid carrying the carbapenemase gene was itself extremely mobile.

3.7 Discussion

The key points of data produced from the isolates in this study are combined and presented in Table 18 to provide ease of comparison between them.

Table 18.

Isolate	Carbapenemase gene	Carbapenemase strength	Antibiotic resistances	Conjugatable?	Conjugational efficiency	Resistances gained	Resistance changes
1.1, 1.2	NDM	Strong	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, MEM, IMP	Yes	1.39×10^{-6}	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, MEM, IMP	None
2.1	NDM	Strong	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, S10, C, MEM, IMP	Yes	4.00×10^{-7}	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, S10, C, MEM, IMP	None
3	OXA-48	Weak	AMP, AMC, MEM, IMP	No	N/A	N/A	N/A
4.1, 4.2	NDM	Strong	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, S10, MEM, IMP	Yes	7.88×10^{-7}	AMP, AMC, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA, S10, MEM, IMP	None
5	OXA-48	Weak	AMP, AMC, CAZ, CTX, CPD, ATM, TE	No	N/A	N/A	N/A
6	N/A	None	None	N/A	N/A	None	N/A
7.1, 7.2	NDM	Strong	AMP, AMC, NA, FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP	Yes	5.52×10^{-6}	AMP, AMC, NA	FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP
8.1, 8.2, 8.3	OXA-48	Moderate	AMP, AMC, MEM, IMP	Yes	1.38×10^{-5}	AMP, AMC, MEM, IMP	None
9.1	OXA-48	Moderate	AMP, AMC, FOX, CAZ, CTX, CPD, TE, MEM, IMP, ATM, CIP, NA	Yes	9.81×10^{-5}	AMP, AMC, FOX, CAZ, CTX, CPD, TE, MEM, IMP	ATM, CIP, NA
9.2	OXA-48	Moderate	AMP, AMC, MEM, IMP, FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA	Yes	9.81×10^{-5}	AMP, AMC, MEM(i), IMP	FOX, CAZ, CTX, CPD, ATM, TE, CIP, NA
10.1, 10.2	NDM	Strong	AMP, AMC, FOX, CAZ, CTX, CPD, S10, TE, MEM, IMP, ATM, CIP, NA, C	Yes	4.20×10^{-5}	AMP, AMC, FOX, CAZ, CTX, CPD, S10, TE, MEM, IMP	ATM, CIP, NA, C
11	N/A	None	None	N/A	N/A	None	N/A
12.1, 12.2	OXA-48	Weak	AMP, AMC, MEM, IMP , FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP	Yes	7.59×10^{-6}	AMP, AMC, MEM, IMP (i)	FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP
13	NDM	Strong	AMP, AMC, FOX, CAZ, CTX, CPD, TE, MEM, IMP, ATM, CIP, NA	No	N/A	N/A	N/A
14	N/A	None	None	N/A	N/A	None	N/A
15	OXA-48	Moderate	AMP, AMC, MEM, IMP , FOX, CAZ, CTX, CPD, ATM, S10, TE, CIP, MEM, IMP	No	N/A	N/A	N/A

Table 18 showing the gathered genotypic and phenotypic data for isolates 1-15

The isolates that were identified as NDM producers all displayed strong hydrolysis of imipenem, having MICs of over 10μg mL^{-1}. They produced the difference in results that would be expected of a metallo-β-lactamase, in the zCIM and eCIM. The EDTA chelated with the Zn^{2+} ions that make up the NDM enzymes active site. This was further confirmed with the lack of colour change in the EDTA supplemented mCNP test, indicating that no imipenem was able to be hydrolysed in the presence of EDTA.
In the ASTs the NDM producers all presented with resistance to penicillins, cephalosporins, and carbapenems, which is the expected range of their hydrolytic activity. The non-beta-lactam resistances observed however should not be accredited to the NDM, especially aztreonam as NDM cannot hydrolyse aztreonam. The consistent resistance to certain other antibiotic classes by the NDM isolates may indicate the presence of another shared resistance plasmid amongst them, chromosomally encoded resistances shared amongst closely related isolates or even a shared plasmid backbone that their NDM genes are a part of, that contains other resistance genes.

The two transconjugants of isolate 10 lost their aztreonam resistance which indicates the original isolate could have been carrying another plasmid encoding an ESBL or the isolate was over producing AmpC, which would not be transferred through conjugation if it was chromosomal-mediated-*ampC*.The last idea seems less likely with the AST results of the NDM transconjugants which indicate the consistent loss of some resistance genes, which indicates they are not sharing a plasmid with the NDM gene. The lack of any of the isolates retaining these genes further indicates that the genes are either chromosomal and cannot be transconjugated or on non-conjugable plasmids, but these assumptions cannot be confirmed without more conjugation assays.

The isolates that were identified as OXA-48 has a more mixed range of hydrolytic capabilities towards imipenem. Some of the isolates showed very weak breakdown of carbapenems, some only just being classified as non-susceptible. This may be due to a variety in OXA-48-like genes, with some inherently having higher carbapenemase activity, whilst other have a slightly wider range of β-lactamase activity at lower levels. These isolates showed no alteration to their carbapenemase activity in the presence of EDTA in the zCIM/eCIM or the mCNP tests, which is expected as OXA-48 enzymes are serine-based and belong to the ambler class D, so EDTA would have no inhibitory effect. The ASTs of the isolates did find a range of resistance profiles, all including the expected patterns for OXA-48, that being strong penicillin hydrolysis, resistance to β-lactamase inhibitors and weak or strong hydrolysis of carbapenems. The additional resistances included second and third generation cephalosporins and tetracyclines, which I believe is likely due to additional ESBL genes on the OXA-48 plasmid or even additional plasmids carrying ESBL within the bacteria. The conjugation assays produced transconjugants that after subjecting to ASTs appear to show some losing their extend spectrum beta-lactamase activity along with losing some activity against carbapenems, while others maintained it, likely due to the conjugation of multiple plasmids simultaneously.

Both OXA-48 and NDM were located in isolates that had the ability to conjugate and transfer their carbapenemase-producing plasmids. This demonstrates on a smaller scale of what is happening in clinical setting to help disseminate these carbapenem-resistance genes and the transference of a wide range of resistances to different antibiotic classes explains how frequently multidrug resistant isolates are identified. The conjugational efficiency of NDM was observed to be lower than that of OXA-48, this may help to explain the fluctuations of which strain is the most common in clinical isolates, while OXA-48 alone provides a smaller spectrum of resistances than NDM alone, it may retain its position as one of the most common carbapenemases produced by isolates due to the higher rate of dissemination of its plasmid into the bacterial populations, potentially alongside other highly transmissible drug resistant plasmids.

Of the three isolates that displayed no notable phenotypic carbapenem resistance, only isolate 6 produced a positive result in the ESBL/AmpC kit for ESBL production, which may explain why it was identified initially as potentially carbapenem resistant. The other two displayed no presence of AmpC or ESBL, on top of lacking any notable phenotypic carbapenem resistance. These isolates may potentially have mutations causing the overexpression of efflux pumps or modification of porin structure which may have explained its initial identification as carbapenem resistant.

Chapter 4: Conclusion

4.1 Conclusion

This study investigated the dissemination and resistance patterns associated with bla_{OXA-48} and bla_{NDM} carbapenemase genes in clinical *Escherichia coli* isolates. Through phenotypic and genotypic characterization, both genes were found to align with the general resistance patterns report in the literature, with the NDM-producers showing a broad spectrum of activity for all tested β-lactam, with the exception of aztreonam in some transconjugated isolates. OXA-48 producers initially presented to have a larger range of antibiotic resistance than expected, but once the OXA-48 plasmid was transconjugated the resistance profile was high activity against penicillins and lower but notable activity against the carbapenems.

The conjugation assays verified the high transmissibility of both genes, which appeared to show higher conjugational efficiency than NDM, however there was found to be no statistically significant difference, likely in part to the variety of efficiencies within both the OXA-48 group and NDM group, this variation is exactly how these genes maintain their oppressive presence in clinical settings. Transconjugating the NDM plasmids also displayed the retention of resistance against non- β-lactam antibiotics such as tetracyclines, fluoroquinolone/quinolones and in some cases aminoglycosides, indicating continued presence of bla_{NDM} on multi-drug plasmids. The transconjugation of the OXA-48 plasmids indicated the co-existence of multiple antibiotic resistance plasmids or other non-enzymatic resistance mechanisms. This highlights the difference in the ways that NDM and OXA-48 maintain their presence in the CRE landscape in the UK, with NDM-encoding plasmids accruing a range of other resistance genes and being more self-sufficient and OXA-48-producing plasmids relying on preexisting antibiotic resistance genes in their recipients to increase their survivability and in the case of other β-lactamases increase their carbapenemase activity.

Globally, carbapenem resistance poses a severe and expanding threat, with NDM being most prevalent carbapenemase in South Asia and OXA-48 carbapenemases dominating in the Middle East and parts of Europe. While this study focused on UK-derived isolates and the carbapenem resistance landscape appears to vary immensely between countries worldwide, its findings reflect broader global concerns regarding the rapid and increasing plasmid-mediated dissemination of resistance mechanism towards critical antibiotics.

The findings from this study should serve to stress the importance and urgency of antibiotic resistance surveillance, especially in healthcare environments which provide the perfect environment for the multidrug resistant isolates detailed in this study to be produced, as even in the UK where there is already established surveillance and controls being implemented the dissemination of these resistance is dire. Further

work, including plasmid sequencing and incompatibility group typing (once the sequencing data is available), will be necessary to confirm the genomic characteristics and co-resistance potential of these mobile genetic elements.

Chapter 5: Bibliography

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