The Effect of Multi-drug Resistance on the Fitness and Pathogenicity of Carbapenem Resistant Organisms (CROs) in the Absence of Selective Pressure

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The effect of multi-drug resistance on the fitness and pathogenicity of carbapenem resistant organisms (CROs) in the absence of selective pressure

A thesis submitted for the degree of Doctor of Philosophy

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Some of the content has been removed (Figures 1.4 and 1.6)
Abstract

Antimicrobial resistance (AMR) is a global threat estimated to cause millions of deaths and trillions of dollars in economic loss if not dealt with urgently. Carbapenems are one of the last resort drugs used to treat patients with multi-drug resistant infections. In Kenya, carbapenems are minimally used in public hospitals due to cost and availability but are readily available in private healthcare facilities. At Kilifi County Hospital, carbapenems were introduced for patient care in 2020 but resistant bacteria have been isolated prior to this.

In this thesis, I describe the phenotypic and genomic characteristics of carbapenem resistant Acinetobacter spp, P. aeruginosa, E. coli and K. pneumoniae isolated from patients in Kenya, and the effect of resistance on the fitness and virulence of these bacteria. First, I determined that majority of the infections likely occurred in hospital and resistant bacteria are also carried in the gut of some of the admitted patients. Secondly, the strains were resistant not only to commonly known drugs but to newer drugs such as cefiderocol and eravacycline, which are currently not available in the country. Third, my findings showed that metallo-beta-lactamases are the main carbapenemases present in the resistant strains with blaNDM variants being the most common enzyme detected. Fourth, whole genome sequencing showed that the strains harboured various resistant genes and mutations and were of different sequence types suggesting multiple introductory events of the bacteria into hospital. Finally, I demonstrated that while overall carbapenem resistant bacteria were less fit compared to fully susceptible bacteria, this was not always the case for all strains.

In conclusion, diverse strains of carbapenem resistant bacteria causing clinical infections are present and could easily be disseminated countrywide given their carriage in the gut and increasing use of antibiotics such as cephalosporins. Therefore, several measures such as antimicrobial stewardship, infection prevention control, increased access to clean water and improved sanitation are also needed in this country to prevent an increased prevalence of these bacteria.
Acknowledgements

To my supervisors, Dr Nicola Claire Gordon, Dr David Wareham and Professor James Berkley, I am grateful for the guidance and mentorship throughout my PhD journey and in particular for your support during the challenging times faced as a result of the COVID-19 pandemic.

I would also like to acknowledge and express my gratitude to the many colleagues who have contributed in one way or another towards the completion of this PhD project. I thank all the staff at KEMRI Wellcome Trust Research Programme who were involved in data and sample collection, microbiological analysis and archiving of strains and clinical data used in the thesis. I would like to specifically thank Alfred Mwanzu and Edwin Wanjala on answering my questions and meaningful discussions on antimicrobial resistance and Zaydah De Laurent and staff at MicrobesNG on the sequencing of the bacterial strains.

I would like to thank the Initiative to Develop African Research Leaders (IDeAL) training team: Sam, Dorcas, Liz, Rita, Florence, Solomon, David and Caroline for their support through the years. I thank my fellow PhD colleagues and office mates in microbiology for sharing this journey with me and offering advice and support where need be.

A special thanks to Ivy Kombe, Jacqueline Mutai, Shillah Simiyu, Sheila Murunga, Elizabeth Mwatata, Angela Karani, Martin Mutunga, Edna Ooko and David Collins who provided encouragement and moral support when facing challenges and a relaxing tea and water break to decompress during my candidature.

Finally, I would like to thank my family, brothers Joseph and Linus and mum Phyllis for the support in prayers and encouragement and my husband Lucian Mshote and daughter Nuru Wanjala for being the best support system during this journey.

Asanteni sana!
Declarations

The work contained within this thesis is my own which I undertook at Kenya Medical Research Institute (KEMRI)- Wellcome Trust Research Programme (KWTRP) under the supervision of Dr Nicola Claire Gordon, Dr David Wareham and Professor James Berkley in fulfilment of the requirements for the degree of Doctor of Philosophy at the Open University (UK). I can confirm that the dissertation is the result of my own work but could not have been undertaken without the direct and indirect involvement of many people.

Chapter 2

In general patient consenting, collection of samples and initial microbiological analysis and isolate storage was conducted as part of larger studies on invasive bacterial disease surveillance and clinical trials on malnutrition and run by KWTRP. Dr David Wareham performed the antimicrobial susceptibility testing and interpretation of the isolates to new drugs (eravacycline, cefiderocol, cefepime-sulbactam, imipenem-relebactam and meropenem-vaborbactam).

Chapter 3

Some of the strains were sequenced at MicrobesNG, Birmingham, UK while the remainder of the strains were sequenced in-house on the Illumina MiSeq instrument by Zaydah De Laurent.
Funding

This work was supported through the Developing Excellence in Leadership, Training and Science (DELTAS) Africa Initiative [DEL-15-003]. The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa's Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust [107769/Z/10/Z] and the UK government.

Ethical approval

The bacterial strains used in this study were obtained and stored under studies previously approved by the ethical approval processes at KEMRI and Oxford, where written informed consent from the patient or parent or guardian of the child is obtained before recruitment into the studies. The research studies were approved by KEMRI’s Scientific and Ethics Review Unit (SERU) and Oxford University’s Oxford Tropical Research Ethics Committee (OxTREC). Strains from the invasive bacterial infections’ surveillance was approved by SERU as protocol SSC 1433 and OxTREC as protocol OxTREC 30-10, and the FLACSAM study was approved as SERU 3399 and OxTREC 1-17. SERU approvals are valid for one year and renewed annually while OxTREC approvals are valid for five years before renewal. Ethical approval to utilize the bacteria as well as the clinical and demographic data was approved by SERU on 29th November 2018 as protocol number SERU 3748 (Appendix 1).
Manuscripts and Conference proceedings

Manuscripts in preparation (to be submitted pre-viva)


Manuscripts in draft


Oral presentations


Poster presentations

2. Clinical characteristics, antimicrobial resistance profile and resistance mechanisms of carbapenem resistant bacteria from patients admitted to hospital in rural coastal Kenya. 31st ECCMID, Virtual, 2021.
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List of abbreviations and acronyms

AMR  Antimicrobial resistance
AmpC  Cephalosporinases
AST  Antimicrobial susceptibility testing
CDC  Centre for Disease Control
CFU  Colony forming units
CLSI  Clinical and Laboratory Standards Institute
ESBL  Extended spectrum beta-lactamases
EDTA  Ethylenediaminetetraacetic
EUCAST  European Committee on Antimicrobial Susceptibility testing
FAO  Food and Agriculture Organization
GAP  Global Action Plan on AMR
3-GC  Third generation cephalosporin
GLASS  Global Antimicrobial Resistance and Use Surveillance System
GOK  Government of Kenya
HIC  High income countries
IPC  Infection prevention control
KCH  Kilifi County Hospital
KEMRI  Kenya Medical Research Institute
KHDSS  Kilifi Health and Demographic Surveillance System
KWTRP  KEMRI Wellcome Trust Research Programme
LB  Lysogeny broth
LMIC  Low- and middle-income countries
MBL  Metallo beta-lactamase
MDR  Multi-drug resistance
MIC  Minimum inhibitory concentration
MLST  Multi-locus sequence typing
NAP  National Action Plan
<table>
<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>NHS</td>
<td>Normal human serum</td>
</tr>
<tr>
<td>OIE</td>
<td>World Organization for Animal Health</td>
</tr>
<tr>
<td>SBL</td>
<td>Serine β-lactamases</td>
</tr>
<tr>
<td>UK NEQAS</td>
<td>United Kingdom based National External Quality Assessment Service</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
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Introduction

1.1 Discovery of early chemotherapeutic agents

The ‘germ theory of disease’ which implicates micro-organisms as the culprits for disease was developed in the 19th century and some of its well-known advocates included John Snow, Louis Pasteur, John Lister and Robert Koch. Its acceptance resulted in doctors and scientists trying to prevent and cure disease often with dismal success. In the early 1900s, Paul Ehrlich, a physician, working with chemical dyes noted that some bacteria could be stained with the dyes while others could not (selective cell staining). He therefore reasoned that a substance could be obtained that could selectively bind and kill bacteria without harming the human host. He referred to this substance as a “magical bullet” and this would eventually lead to the discovery of arsphenamine, an arsenic-containing compound, used in the treatment of syphilis, a disease that had ravaged Europe for years and had ineffective and dangerous treatment. The compound was first marketed in 1910 as Salvarsan and marked the first use of the term “chemotherapy”, however, the compound was toxic and had side effects including pain on injection, rashes and liver damage (Williams, 2009).

The first broad spectrum truly effective antibacterial compounds derived from dyes were sulphonamide derivatives and discovered in the 1930s, after years of trial and error, by a team of scientists led by Gerhard Domagk working in the laboratories of IG Farben (Otten, 1986). The work continued to build on Ehrlich’s “magic bullet” concept of a drug that could preferentially target the bacteria with little or no harmful effects on the human host. The discovery was made by working with mice infected with a virulent strain of haemolytic streptococci and the dye was found to be safe and effective, however, the compound Prontosil was a pro-drug, which was active in animal experiments but ineffective on streptococci in vitro and the reason for this was unknown. The mystery was eventually solved by a team of scientists at the Institute Pasteur led by Ernest Fourneau who showed that the drug was metabolized in the animals releasing a smaller active compound, sulfanilamide, that was active both in vitro and in vivo against pathogenic bacteria. This led to a “sulfa craze” with many derivatives of the compounds produced, as sulfanilamide had been synthesized in 1908 and was not covered by a patent by the company and was it was easier and cost-effective to produce and did not dye patients (van Miert, 1994). Domagk received a Nobel Prize in 1939 “for the discovery of antibacterial effects of Prontosil”.

When Alexander Fleming left culture plates of Staphylococci on the bench for a holiday, he did not anticipate that this action would revolutionize medicine. Upon return, he noticed that fungus had
contaminated one of the plates left open accidentally and the bacteria growing near the vicinity of the fungi were lysed and dying while those far from the fungi were growing normally. He identified the fungus as *Penicillium* and grew it in pure culture noting that the broth had a substance, which he called penicillin, with antibacterial activity against pathogenic bacteria such as pneumococcus, meningococcus, gonococcus and diphtheria bacillus but not others such as *Haemophilus influenzae* and *Bacillus coli* (*Escherichia coli*) (Fleming, 1929). Fleming’s findings generated almost no interest mainly because he was unable to isolate the active compound, undeterred however, he continued to share the fungus with several scientists hoping to isolate it and enable further research on penicillin.

It was not until a decade later that a team of Oxford scientists, led by Ernst Boris Chain and Howard Florey, were able to isolate the active compound, penicillin. Up until then the focus on chemotherapeutic agents had been on dyes and other synthetic compounds despite it being known that some bacteria and fungi were able to inhibit or kill competing microbial species. Chain and Florey having worked on lysozyme, another one of Fleming’s discoveries, thought it worthy to investigate the chemical, pharmacological and therapeutic properties of antibacterial substances produced by bacteria and moulds, beginning with Fleming’s findings. They produced substantial amounts of penicillin and showed that not only was it safe but it was effective in inhibiting bacterial growth *in vitro* and its therapeutic effects in infected mice (Chain et al., 1940). The team produced penicillin via fermentation vessels before extracting and purifying it for clinical trials in patients. The first recipient was a 43-year old policeman who was infected through a scratch while pruning roses and had developed life-threatening abscesses. Within days of receiving intravenous penicillin, he showed remarkable recovery until the penicillin stock ran out and he died several days later (Abraham et al., 1941). Florey and Chain tested penicillin on several other cases and saw better outcomes, including some cases that had previously been unsuccessfully treated with sulphonamide (Abraham et al., 1941). The success prompted interest in generating large amounts of penicillin for use by British troops in World War II as infections were known to be more lethal than battle injuries, however, this was hampered by war-time conditions prompting the scientists to travel to the United States of America (USA) for the successful mass production of penicillin and therapeutic use in military and civilians (Gaynes, 2017, International Historic Chemical Landmarks). Fleming, Chain and Florey jointly received a Nobel Prize in 1945 “for the discovery of penicillin and its curative effect in various infectious diseases”.

1.2 Golden Age of antibiotic discovery

The field of antibacterial discovery was accelerated by Selman Waksman, a soil microbiologist, whose team at Rutgers University discovered several antibiotics from actinomycetes, a group of soil microorganisms. Waksman sought to build his research based on a well-known phenomenon referred to as antibiosis, where two micro-organisms existing in the same environment are antagonistic with one micro-organism adversely affecting the other organism. Waksman’s group is known particularly for the discovery of streptomycin from *Actinomyces* (later named *Streptomyces griseus*) by a student, Albert Schatz, which was effective against Gram-negative bacteria unlike penicillin and other known agents (Schatz et al., 1944) and was the first active drug in treating tuberculosis, the Great White Plague. Waksman coined the term antibiotic, referring to a ‘natural compound produced by an organism against another organism’ (Waksman, 1947), and his group went on to discover another notable compound, neomycin (Waksman and Lechevalier, 1949) among many other compounds. He received a Nobel prize in 1952 “for ingenious, systematic and successful studies of the soil microbes that led to the discovery of streptomycin” amid protests by Schatz for being left out of recognition by the Nobel committee for the discovery of the drug.

The work by Waksman and colleagues led to the “Golden Age” of antibiotic discovery of the 1940s to 1960s prompting pharmaceutical companies to seek and obtain soil samples from across the globe for the discovery of antibiotics using his technique. Initial screening of natural products from soil microorganisms focused solely on the genus *Streptomyces* which were abundant producers of natural products or secondary metabolites active against bacteria as well as fungi and viruses. The genus has yielded several important compounds in several antibacterial classes known today: macrolides (spiramycin), aminoglycoside (streptomycin, kanamycin, neomycin), beta-lactams (cephamycin, carbapenems), tetracyclines and chloramphenicol. Thereafter companies began to screen other actinomycetes, fungi and bacteria for active natural compounds leading to further discoveries. More than half of the natural product antibiotics known were discovered from filamentous actinomycetes with the rest made by bacteria and fungi (figure 1) and a few compounds being synthetic (Gould, 2016, Hutchings et al., 2019, Lyddiard et al., 2016, Katz and Baltz, 2016). This era resulted in the discovery of more than half of the antibacterials known today, revolutionising modern medicine and facilitating the development of life-saving medical procedures such as organ transplants, joint and hip replacements among other advanced surgeries. The discovery and use of antibiotics were one of the public health
interventions (others include improved sanitation and hygiene and vaccination) introduced in the 1990s that helped reduce mortality due to infectious diseases and increase life expectancy (MMWR, 1999).

Figure 1.1 Timeline showing discovery and clinical use of new classes of antibacterials as well as first reports of resistance to key antibacterials. The classes are coloured according to the source of the antibiotic with green from actinomycetes, blue from other bacteria, purple from fungi and orange are synthetic. NP=natural products. Image from (Hutchings et al., 2019).

1.3 History of antibacterial resistance

While the field of antibiotic discovery is littered with success and Nobel laureates, the case is not true for antibacterial resistance and it is only recently that the issue has been recognized internationally as a ‘silent pandemic’ with serious health and economic implications. In the beginning, reports of resistance followed the pattern of early discovery and clinical use of antibacterial compounds and were mainly observed in hospitals. Resistance to arsphenamine was first documented in the 1910s with reduced susceptibility demonstrated in vitro and animal models as well as documented clinically in patients, however, clinical resistance was erroneously ascribed to host factors rather than pathogen determinants (Miller, 1931). Resistance to penicillin was demonstrated before clinical use by Fleming when he showed
that certain bacteria were intrinsically resistant to the drug compound (Fleming, 1929). In 1940, the mechanism of resistance was confirmed to be mediated by an enzyme named penicillinase and was absent in susceptible strains indicating that resistance was a result of the pathogen (Abraham and Chain, 1940). Regardless, such findings were of little concern because despite the use of arsenicals to treat syphilis for 40 years, resistance levels had been steady and it was assumed the case would be similar for penicillin as well (Rollo et al., 1952).

Penicillin resistant *Staphylococcus* species from localized infections in patients were first documented in 1942 (Rammelkamp and Maxon, 1942) and by the 1950s, resistant strains were implicated in increasing outbreaks of hospital-related resistant infections and mortality due to indiscriminate widespread use of penicillin (Barber, 1947, Podolsky, 2018). The clinical importance of resistance was forewarned by Fleming in his 1945 Nobel prize lecture when he stated that resistance in bacteria could be induced by subjecting it to sub-optimal concentration of the drug (Fleming, 1945). Several scientists heeded Fleming’s warning and tried to emphasize the importance of bacterial resistance however, the understanding of this was delayed due to a lack of standardization in resistance testing resulting in different laboratories defining resistance differently (Gradmann, 2013). On the other hand, the pharmaceutical industry released new drug compounds rapidly (Figure 1.1) so that the issue of bacterial resistance was ignored or was a rationale for the introduction and uptake of new compounds, as it was assumed that new compounds would always be available to replace those to which organisms had become resistant.

In the early years of antibiotic use, it was assumed that resistance was only spread vertically to a bacterium’s descendants after direct drug exposure, however, this view began to change when multi-drug resistance was observed in enteric bacteria. In the 1950s Japanese scientists reported an increased prevalence of *Shigella* strains with multi-drug resistance (MDR) profiles to streptomycin, chloramphenicol, tetracycline and sulphonamide isolated from patients with dysentery in Japan (Watanabe, 1963). They observed patients with susceptible *Shigella* strains belonging to the same dysentery epidemic and the same serological type as patients with MDR *Shigella* strains. Other patients were reported to have both susceptible and MDR strains, while in some the administration of one of the antibiotics in patients with susceptible strains later resulted in excretion of MDR strains. In one of the epidemics, Matsuyama and colleagues also isolated *E. coli* harbouring multi-drug resistance in these patients. There was no reasonable explanation for these observations until 1959 when Tomoichiro Akiba suggested that the MDR trait was transferred from *Escherichia coli* to *Shigella* in the intestine of patients.
and this transfer was demonstrated in vitro and in vivo. It was revealed that the MDR trait was transferrable to other genera in Enterobacteriaceae by conjugation, transfer of resistance traits through the direct cell to cell contact between bacteria, and was mediated by ‘episomes’ (Watanabe, 1963). These findings were corroborated by Naomi Datta in 1962 when she observed susceptible and resistant Salmonella typhimurium from the same patients in 1959 in an outbreak in a London hospital that was of the same phage type and the resistant trait was transferrable to other enteric bacteria (Datta, 1962). She suggested that the resistant determinant was transferred through a transmissible non-chromosomal vector referred to as a ‘plasmid’.

Simultaneously in the 1960s, Ephraim Anderson and colleagues working with Salmonella typhimurium in the United Kingdom observed strains with similar drug resistance patterns in both humans and animals and postulated that the use of antibiotics as additives in animal feeds resulted in resistant bacteria and traits in livestock that could be transferable in humans, resulting in disease that could not easily be treated and that was sometimes fatal (Anderson and Datta, 1965, Anderson, 1968a, Anderson, 1968b, Anderson and Lewis, 1965b, Anderson and Lewis, 1965a). He advocated for the ban of non-therapeutic use of antibiotics in animal husbandry and its replacement with improved hygiene and sanitation in animal husbandry as well as in the production and handling of food for consumption to reduce the transfer of resistant bacteria and traits from animals to humans. His efforts earned him strong criticisms from the pharmaceutical and veterinary sector alike, nevertheless, it resulted in the Swann Report of 1969 that proposed a ban on the use of antibiotics of human importance as growth promoters (use of antibiotics to prevent infections in animals rather than for treatment of disease) (Watts, 2006). Even though the report did not fully recognize the implications of horizontal gene transfer of drug resistance it was a key milestone in initiating precautionary measures on the use of antibiotics (Lees et al., 2021).

Despite evidence of transferrable drug resistance its impact was assumed to have minimal health implications as it was initially reported in enteric organisms. This began to change when transferrable multi-drug resistance was reported in other organisms such as Haemophilus influenzae and Neisseria gonorrhoeae (Levy and Marshall, 2004) and in the 1970s plasmid resistance was reported in a range of species and diseases. In 1981, Stuart B Levy, a microbiologist at Tufts University, USA who had studied antimicrobial resistance for several years, founded the Alliance for the Prudent Use of Antibiotics (APUA) and together with colleagues was prominent in advocating for the prudent use of antibiotics. In his work, Levy made several notable discoveries, for example, that tetracycline resistance was mediated by an energy-dependent efflux pump. He also discovered the multiple antibiotics resistance operon
(marAB) which regulates genes involved in multi-drug resistance and virulence and third, the mechanism of resistance to triclosan in cleaning products and the linked cross-resistance with clinical antibiotics. Levy understood the consequences of imprudent use of antibiotics and the need for public education at all levels and policy change to curb resistance and published the book *The Antibiotic Paradox: How Miracle Drugs Are Destroying the Miracle* in 1992 discussing this and the threat of a post-antibiotic era (Stafford, 2019).

1.4 Era of the ‘superbug’ and antimicrobial resistance as a ‘super wicked’ problem

Though slow at the beginning, concern about antimicrobial resistance (AMR), which also includes resistance in viruses, fungi and parasites, began to gain traction as a result of the AIDS epidemic, as scientists outlined emerging microbial threats to humankind. It was further supported by increasing resistance in gonorrhoea, tuberculosis and malaria and the shift in focus by pharmaceutical companies from the less profitable antibiotic production to the more profitable chronic disease drugs and the renewed interest by the WHO in resistance. Several scientists, clinicians and organisations called for action especially at the political level to avoid reversing the gains made in modern medicine if resistance to bacterial infections is not tackled both nationally and internationally (Podolsky, 2018).

In 2014, the UK Prime Minister tasked Lord Jim O’Neill, an economist, to analyse the global problem of drug resistance and to propose concrete measures to tackle it. The report, funded by the UK government and Wellcome Trust, estimated that though currently, approximately 700,000 deaths occur annually as a result of infections by drug-resistant micro-organisms, the figure is set to rise to 10 million deaths a year (Figure 1.2), occurring mainly in Asia and Africa, by 2050 if no action is taken (O’Neill, 2014). The report also estimated that 2 to 3.5% of the world’s gross domestic product (GDP), an equivalent of up to 100 trillion dollars would be lost in 2050 due to AMR. These deaths are estimated to be greater than cancer related deaths in the same period, and while the report has been criticized as too broad and lacking in caveats or confidence intervals (de Kraker et al., 2016) there is an agreement that the threat of a post-antibiotic era is looming should the current situation not be addressed.
Figure 1.2 Estimated annual mortality attributed to antimicrobial resistance compared to other diseases or accidents by 2050.

Image from (O'Neill, 2014)

In 2015, the WHO prepared a global action plan (GAP) (WHO, 2015) on AMR that was adopted by the Food and Agriculture Organization (FAO), World Organization for Animal Health (OIE) and by the World Health Assembly in 2015. The key objectives of the plan are:

- to improve awareness and understanding of AMR in the public through training, education and communication
➢ to strengthen knowledge and evidence through research and surveillance
➢ to reduce infections through effective sanitation and hygiene
➢ to optimize the use of antimicrobials in human and animal health
➢ to develop the economic case for sustainable investment that considers the needs of all the countries and increase investment in new drugs, diagnostics tools, vaccines among other measures.

In September 2016, a political declaration on AMR was made during a high-level meeting on AMR held by the United Nations (UN) General Assembly in New York (UN, 2016). Member states agreed to develop national action plans (NAPs) based on the GAP and pledged to raise awareness through education and implement a broad range of approaches targeted towards human, animal, plant and environmental sectors. The Tripartite Joint Secretariat on Antimicrobial Resistance, a consortium consisting of WHO, FAO and OIE, working together with the UN and other organizations, was tasked with leading the global response to AMR from a One Health perspective recognizing the complexity of AMR between and within the different sectors (Figure 1.3).

In order to preserve the effectiveness of antibiotics through stewardship and to tackle resistance, the WHO in 2019 released the AWaRe classification database that grouped 180 antibiotics into Access, Watch and Reserve groups (WHO, 2019a). Access group included antibiotics that are affordable, readily available and used as first and second line choices for certain infectious syndromes. These drugs are active against a wide range of susceptible bacteria and have a lower resistance potential than other groups. The Watch category included drugs with a higher risk of bacterial resistance and are defined as critically important for human medicine. Drugs in this category have been prioritized as targets for stewardship and monitoring to preserve their use in humans. The Reserve antibiotics are deemed as “last resort” options to be used when all other treatment options fail and there are no alternatives for specific patients.
Figure 1.3: An illustration of the complex transmission of AMR between different sectors in human health, animal and plant health and the environment.

Image from (Walsh, 2018).

WHO listed AMR as one of the top ten threats to global health in 2019 (WHO, 2019b) and described it as a ‘super wicked problem’ and an ongoing silent pandemic. Wicked problems (like climate change) in their nature are social or cultural problems that are difficult or impossible to solve and comprise of four main characteristics (Littmann and Simonsen, 2019):

- **time is running out.** No new class of antibiotic has been discovered for several decades with new drugs belonging to existing classes which are in danger of exploitation by existing resistance mechanisms. There is the imminent danger of lacking effective antibiotics against difficult to treat infections and a possibility of entering a post-antibiotic era (Reardon, 2014)

- **persons responsible for solving the problem are also its main contributors.** Several professions are tasked with reducing the effect of AMR however even they are biased against their responsibility in the problem. The pharmaceutical industry not only discovers and produces drugs, but also markets them which is a potential conflict source for AMR. Different healthcare
professionals tend to list patients and the veterinary world as the main contributors of AMR with minimal acknowledgment of the contribution from their part (Muloi et al., 2019, Rusic et al., 2021)

➢ weak or no mandate from those charged with addressing the issue. Efforts to control AMR require central authority mainly at the national level to drive the process. In sub-Saharan Africa (sSA) for example, many countries lack national plans to tackle AMR or the funds to implement control measures such as surveillance or prevention activities. And despite their role, international bodies such as the WHO are unable to effectively help such countries to meet their needs.

➢ political actions discount the future irrationally. Though AMR is recognized as an issue there are doubts regarding its true impact which has the potential to downplay the seriousness of the threat in the public and delay control measures (de Kraker et al., 2016, Yu et al., 2019)

AMR is therefore a complex problem requiring sustained efforts to tackle it. Though many countries have developed national action plans, in many LMICs, effective implementation as well as monitoring and evaluation and funding for these activities are the major challenges (Chua et al., 2021, Wesangula et al., 2020, Frumence et al., 2021). Indeed, a subsequent UN meeting held in April 2021 to discuss the progress made since the first meeting worried that little progress has been made despite the level of threat, especially in LMICs, with calls for increased support for resource-limited nations to implement measures to meet the set targets (Dall, 2021).

1.5 Surveillance and trends in bacterial resistance

The Centers for Disease Control and Prevention (CDC) in 2019 reported that “2.8 million antibiotic resistant infections occur in the USA each year and more than 35,000 people die as a result” (CDC, 2019). Such estimates are based on established research and surveillance systems, the second key objective of the WHO’s GAP. In contrast, many sub-Saharan African and Southeast Asian countries do not know the true burden of AMR within their countries which is a rough estimate at best, due to data from hospitals especially private facilities, (Figure 1.4) with the situation thought to be far much worse due to uncontrolled access to antibiotics, poor sanitation and hygiene conditions and poor governance and infrastructure (Collignon et al., 2018).

Two reviews on antibacterial resistance in Africa spanning articles ranging from 1990 to 2016 concluded that while almost a third of sub-Saharan African countries lacked published data on resistance, there was significant resistance to commonly prescribed and available antibiotics such as penicillin,
amoxicillin, tetracycline and gentamicin in both Gram-negative and positive bacteria (Leopold et al., 2014, Tadesse et al., 2017). The authors also reported that many of the studies were cross-sectional hospital-based studies with quality control measures in microbiological identification and antibiotic susceptibility testing wanting thus making comparison of results between studies and monitoring trends in resistance challenging. Without proper surveillance systems in Africa, the true burden of antibacterial resistance is unknown, and this lack of estimates coupled with inadequate funding and poor infrastructure hampers many sub-Saharan African countries implementation of NAPs. In the latest WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) report of 2020, only 21 out of 47 countries in the region were enrolled in GLASS by July 2019 and only 9 countries contributed AMR data in addition to information on national surveillance systems (WHO, 2020) meaning that the situation is still dire in the region and effective control strategies cannot be implemented.


Figure 1.4 Global aggregate resistance for E. coli, Klebsiella spp and Staphylococcus aureus. Data is missing for the majority of African countries and resistance estimates for key pathogens cannot be determined. Image from (Roope et al., 2019).

1.5.1 β-lactam antibiotics and resistance

Since the accidental discovery of its first member in 1928, β-lactams are arguably one of the most important and widely consumed antibiotic group globally (Klein et al., 2018). They are so named because of the β-lactam ring in their nucleus, which irreversibly binds to transpeptidases (penicillin binding proteins (PBPs)) preventing the crosslinking of the growing peptidoglycan layer, leading to loss in structural integrity and increased susceptibility to osmotic pressure. Penicillins, cephalosporins (including cephemycins), carbapenems and monobactams are antibiotic classes that are members of this group. The main resistance mechanism to the antibiotic group in both Gram negative and positive bacteria is the expression of enzymes known as β-lactamases that hydrolyse the β-lactam ring before it can bind to transpeptidases. Other mechanisms to overcome β-lactams include reduced affinity of the PBPs through mutations or over-expression of PBPs in Gram positive bacteria and active removal of drug through efflux pumps or decreased permeability of the drug through loss of outer membrane porins in Gram negative bacteria (Figure 1.5).
Figure 1.5 Mechanisms of resistance to β-lactam antibiotics in Gram negative and positive bacteria.

Given their wide usage and broad spectrum of activity, β-lactams are the first and main treatment options for infections caused by bacteria such as *S. aureus*, Enterococci, Enterobacterales and *A. baumannii* and therefore increasing rates of multidrug resistance globally is of major concern (Tang et al., 2014). In 2017, following up on a pathogen priority list by CDC focused on increasing scientific, political and public awareness of AMR in USA (CDC, 2013), the WHO, through a team of experts, published a global pathogen priority list to guide discovery, research and development of new antibiotics (Tacconelli et al., 2018). Twenty bacteria were identified through ten criteria that included mortality, health-care burden, community burden, prevalence of resistance, 10-year trend of resistance, transmissibility, preventability in the community setting, preventability in the health-care setting, treatability, and antibiotic pipeline. The bacteria were divided into three tiers:

- **Critical priority**: carbapenem resistant *Acinetobacter baumannii, Pseudomonas aeruginosa* and carbapenem resistant and third generation cephalosporin (3-GC) resistant Enterobacterales
- **High priority**: vancomycin resistant *Enterococcus faecium*, methicillin resistant and vancomycin non-susceptible *S. aureus*, clarithromycin resistant *Helicobacter pylori*, fluoroquinolone resistant *Campylobacter* and *Salmonella* and 3-GC resistant and fluoroquinolone resistant *Neisseria gonorrhoeae*
- **Medium priority**: penicillin non-susceptible *Streptococcus pneumoniae*, ampicillin resistant *Haemophilus influenzae* and fluoroquinolone resistant *Shigella.*
In the above list concerns over β-lactam resistance is listed in more than half of the pathogens with resistance to carbapenems ranked as critical priority as these drugs are considered one of the last line options for the treatment of infections caused by multidrug resistant Gram negative bacteria that are well tolerated with comparatively fewer adverse effects. These highest priority pathogens pose particular risk to critically ill patients on medical devices in healthcare facilities and may be fatal during severe or invasive infections such as pneumonia and bloodstream infections.

Resistance to 3-GC, a second line treatment option for most Gram-negative bacteria, is facilitated by extended spectrum β-lactamases (ESBLs) that also hydrolyse monobactams but not cephamycins. ESBL genes (blaSHV and blaTEM) were first identified in Enterobacteriales in Europe and were associated with hospital acquired infections particularly in Klebsiella pneumoniae, however, soon afterwards ESBL producing E. coli (blaCTX-M) from community acquired infections emerged especially among urinary tract infections (UTI) and subsequently in bloodstream infections (BSI) in Europe (Cantón et al., 2008, Pitout and Laupland, 2008). Currently variants of blaCTX-M in community and hospital acquired isolates predominate in several European countries with variant blaCTX-M-15 being the most abundant (Galas et al., 2008, Voets et al., 2012, Brigante et al., 2005, Empel et al., 2008). In Africa, the proportion of ESBL producing bacteria among Enterobacteriales range from 0.7 – 76% (Table 1.1) depending on the study design and country with varying proportions of genes where it was tested, nevertheless it is clear that there were more ESBLs in hospital acquired infections than in community acquired infections, mirroring the situation globally (Tansarli et al., 2014, Toy et al., 2019, Sangare et al., 2015, Kpoda et al., 2018). For such infections the preferred treatment option is carbapenems.
Table 1.1 Prevalence of extended spectrum producing β-lactamase (ESBL) Enterobacterales in selected studies from Africa

<table>
<thead>
<tr>
<th>Country</th>
<th>Year</th>
<th>Study design</th>
<th>Infection type</th>
<th>Acquisition</th>
<th>Population</th>
<th>Percentage of ESBL</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malawi</td>
<td>2004 – 05</td>
<td>Prospective</td>
<td>BSI</td>
<td>CA</td>
<td>all</td>
<td>0.7%</td>
<td>(Gray et al., 2006)</td>
</tr>
<tr>
<td>Morocco</td>
<td>2004 – 09</td>
<td>Prospective</td>
<td>UTI</td>
<td>CA</td>
<td>U</td>
<td>1.5%</td>
<td>(Barguigua et al., 2011)</td>
</tr>
<tr>
<td>Morocco</td>
<td>2010</td>
<td>Prospective</td>
<td>UTI (lab based)</td>
<td>CA</td>
<td>U</td>
<td>7.5%</td>
<td>(Barguigua et al., 2013)</td>
</tr>
<tr>
<td>Central African Republic</td>
<td>2003 – 05</td>
<td>Retrospective (lab based)</td>
<td>all</td>
<td>U</td>
<td>U</td>
<td>4%</td>
<td>(Frank et al., 2006)</td>
</tr>
<tr>
<td>Senegal</td>
<td>2004 – 06</td>
<td>Prospective</td>
<td>UTI</td>
<td>CA</td>
<td>all</td>
<td>3.8%</td>
<td>(Sire et al., 2007)</td>
</tr>
<tr>
<td>Senegal</td>
<td>2001 – 03</td>
<td>Prospective</td>
<td>UTI</td>
<td>CA</td>
<td>all</td>
<td>6.3%</td>
<td>(Dromigny et al., 2005)</td>
</tr>
<tr>
<td>South Africa</td>
<td>2006</td>
<td>Surveillance (lab based)</td>
<td>BSI</td>
<td>U</td>
<td>U</td>
<td>9.7%</td>
<td>(Brink et al., 2007)</td>
</tr>
<tr>
<td>South Africa</td>
<td>2005 - 06</td>
<td>Retrospective</td>
<td>UTI</td>
<td>HA/CA</td>
<td>U</td>
<td>15%</td>
<td>(Habte et al., 2009)</td>
</tr>
<tr>
<td>South Africa</td>
<td>2004 – 09</td>
<td>Prospective</td>
<td>IAI</td>
<td>U</td>
<td>U</td>
<td>9%</td>
<td>(Brink et al., 2012)</td>
</tr>
<tr>
<td>Nigeria</td>
<td>2005 – 07</td>
<td>Prospective</td>
<td>all</td>
<td>U</td>
<td>U</td>
<td>20%</td>
<td>(Ogbolu et al., 2011)</td>
</tr>
<tr>
<td>Tanzania</td>
<td>2001 – 02</td>
<td>Prospective</td>
<td>BSI</td>
<td>CA</td>
<td>children</td>
<td>15%</td>
<td>(Blomberg et al., 2005)</td>
</tr>
<tr>
<td>Tanzania</td>
<td>2009 – 10</td>
<td>Prospective</td>
<td>all</td>
<td>HA</td>
<td>all</td>
<td>50.3%</td>
<td>(Mshana et al., 2013)</td>
</tr>
<tr>
<td>Country</td>
<td>Year</td>
<td>Study Type</td>
<td>Site(s)</td>
<td>Infection(s)</td>
<td>Setting(s)</td>
<td>Percentage</td>
<td>Reference</td>
</tr>
<tr>
<td>---------</td>
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<td>--------------</td>
<td>-------------</td>
<td>------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Egypt</td>
<td>2006-07</td>
<td>Prospective</td>
<td>BSI</td>
<td>HA</td>
<td>all</td>
<td>76%</td>
<td>(Saied et al., 2011)</td>
</tr>
<tr>
<td>Egypt</td>
<td>2007-08</td>
<td>Prospective</td>
<td>all</td>
<td>HA/CA</td>
<td>adults</td>
<td>16%</td>
<td>(Fam et al., 2011)</td>
</tr>
<tr>
<td>Algeria</td>
<td>2009</td>
<td>Retrospective</td>
<td>all (surgical wards)</td>
<td>HA</td>
<td>all</td>
<td>31%</td>
<td>(Nedjai et al., 2012)</td>
</tr>
<tr>
<td>Ghana</td>
<td>2007-12</td>
<td>Prospective</td>
<td>BSI</td>
<td>CA (HA in neonates)</td>
<td>all</td>
<td>9.7%</td>
<td>(Eibach et al., 2016)</td>
</tr>
<tr>
<td>Kenya</td>
<td>1992-2010</td>
<td>Prospective</td>
<td>UTI, BSI and diarrhoea</td>
<td>CA/HA</td>
<td>U</td>
<td>27%</td>
<td>(Kiiru et al., 2012)</td>
</tr>
<tr>
<td>Kenya</td>
<td>2001-11</td>
<td>Retrospective</td>
<td>BSI, CNS</td>
<td>CA/HA</td>
<td>all</td>
<td>55%</td>
<td>(Henson et al., 2017)</td>
</tr>
</tbody>
</table>


CA – community acquired, HA – hospital acquired, U – undefined
1.5.2 Carbapenems and resistance in Gram negative bacteria

Carbapenems are synthetic derivatives of thienamycin, obtained from *Streptomyces cattleya*, which had a high affinity for PBPs with broad spectrum activity against both Gram negative and positive bacteria. Thienamycin, discovered in the 1970s, differed from other β-lactams in that it substituted carbon for sulphur at the first position and had a hydroxyethyl side chain, unlike penicillin and cephalosporin that have an acylamino substituent on the β-lactam ring. However, the compound was unstable in solution which inspired the search for more stable synthetic derivatives and imipenem was the first to be developed with success in 1985. Its structure enabled it to be stable against inactivation by many β-lactamases including ESBLs. Other compounds developed included meropenem, biapenem, ertapenem, and doripenem which unlike imipenem are protected against deactivation by the human dehydropeptidase 1 in the kidney and do not require co-administration with cilastin, an enzyme inhibitor (Papp-Walace et al., 2011).

As a β-lactam antibiotic, carbapenem resistance is mainly through hydrolytic enzymes known as carbapenemases which are included in three of four classes (A, B, C and D) based on the Ambler classification system (Hall and Barlow, 2005) or three groups (1, 2 and 3) based on the Bush-Jacoby-Medeiros system (Bush and Jacoby, 2010) (Table 1.2). The well-known and globally distributed representatives of each class include *K. pneumoniae* carbapenemases (*bla*KPC) of Class A; Verona integron-encoded metallo-β-lactamases (*bla*VIM), New Delhi metallo-β-lactamase (*bla*NDM) and IMP-type (active on imipenem) carbapenemases (*bla*IMP) of Class B and oxacillinases types (*bla*OXA) of Class D. Class A and D require serine at the active site to function while class B requires a zinc ion for hydrolysis and are referred to as metallo-β-lactamase (MBL).

Table 1.2 Classification of β-lactamases

<table>
<thead>
<tr>
<th>Ambler class</th>
<th>Bush-Jacoby-Medeiros group</th>
<th>Inhibited by</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2 (a, b, c, e, f)</td>
<td>yes (except for some 2b and variable for 2f)</td>
<td>no TEM, SHV, CARBA, KPC, IMI</td>
</tr>
<tr>
<td>B (B1, 2 and 3)</td>
<td>3 (a and b)</td>
<td>no</td>
<td>yes NDM, IMP, VIM</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>no</td>
<td>no CMY, FOX</td>
</tr>
<tr>
<td>D</td>
<td>2 (d)</td>
<td>variable</td>
<td>no OXA</td>
</tr>
</tbody>
</table>
1.5.2.1 Enterobacterales

Carbapenem resistance in Enterobacterales is a concern in *K. pneumoniae, E. coli, Enterobacter, Serratia, Proteus, Providencia* and *Morganella* species but in this work, I will focus on the first two organisms. Increased prevalence of ESBL among *E. coli* and *K. pneumoniae* has resulted in increased use of carbapenems, driving resistance with the former associated with community acquired infections and the latter with hospital acquired infections. Though *E. coli* and *K. pneumoniae* are part of the normal human intestinal microflora, they are also common human pathogens that easily spread between individuals and readily acquiring resistance genes. Two reviews (Logan and Weinstein, 2017, Nordmann et al., 2011) have reviewed the emergence and spread of carbapenemases in Enterobacterales globally and are summarized below (Figure 1.6).

https://academic.oup.com/jid/article/215/suppl_1/S28/3092084

Figure 1.6 The spread and distribution of carbapenemases in Enterobacterales. The image shows endemicity, outbreaks and sporadic reports of different carbapenemases globally. Image from (Logan and Weinstein, 2017).

KPC was first identified in *K. pneumoniae* isolates in the USA and is endemic in parts of the country as well as in Greece, Israel and parts of Latin America. Its global spread is associated with clonal expansion of the *K. pneumoniae* clonal complex 258 which causes major outbreaks in other countries. KPC producers are not common in *E. coli* or in community acquired infections but are associated with high mortality in infected individuals. KPC can hydrolyse all β-lactams but can be inhibited by some β-lactamase inhibitors. MBL hydrolyse all β-lactams except monobactams and are inhibited by metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) but not by β-lactamase inhibitors. The first reported carbapenemase was IMP-1 MBL during an outbreak in seven hospitals in Japan in 1991 and it is currently endemic in Japan and Taiwan with occasional reports or outbreaks in other countries. On the other hand, VIM carbapenemases first described in Italy and France are currently endemic in Greece in *E. coli* and *K. pneumoniae* with sporadic reports or outbreaks in other countries. There was increased focus in MBLs in 2008 when NDM was first discovered in a Swedish patient of Indian origin who had travelled to India (Yong et al., 2009). It was soon realized that unlike KPC, the global dissemination of NDM was not associated with any single clone and was rapidly transferred between unrelated clones and species. The gene is endemic to the Indian sub-continent where nearly 2 – 14 % of the patient
population carries NDM producing Enterobacterales and the Middle East and Balkan countries are thought to be secondary reservoirs for the gene. Also of concern is that the gene can occur in the presence of another carbapenemase gene in a single isolate and can easily be spread in the community through environmental sources where it is present (Walsh et al., 2011). OXA-48 variants, which are present in Enterobacterales, have weak hydrolysing capacity against carbapenems, 3-GC or monobactams but high level against penicillin. They are not inhibited by EDTA or β-lactamase inhibitors and in the presence of ESBL or permeability defects, they have a higher level of resistance against carbapenems. The gene family was first identified in K. pneumoniae in Turkey in 2003 where it was associated with hospital outbreaks in the country and is endemic to the country and Morocco with outbreaks in parts of Europe, North Africa and India (Logan and Weinstein, 2017, Nordmann et al., 2011).

The distribution of carbapenemases in sub-Saharan African countries has not been well understood as there is limited data on carbapenem resistance in Enterobacterales. The Logan et al (figure 1.6) review show a gap for all sub Saharan African countries except South Africa, Senegal and Kenya and in the case of Kenya the data used was from a few published reports from a tertiary hospital and would qualify for sporadic occurrence and not necessarily an outbreak based on data. A 2018 review on carbapenem resistant E. coli and K. pneumoniae in Africa reported that susceptibility data was available from 66% (31/47) of WHO African countries of which 71% (22/31) reported resistance to any of the species. Median resistance levels of <1% (calculated for nations with >100 representative isolates) were observed in 61% (11/18) of the nations for E. coli and in 67% (10/15) for K. pneumoniae and only one (Uganda) and two (Uganda and Madagascar) countries respectively had >5% median resistance levels in E. coli and K. pneumoniae respectively (Mitgang et al., 2018). Genotypic testing for carbapenemase genes was not usually done but where the results were available for 10 countries, OXA-48 was present in eight countries, NDM in six, VIM in five and KPC and IMP in two each and GES and DIM in one each (Figure 1.7). North African countries were represented in earlier review where an abundance of OXA-48 was present in the region’s countries with some VIM and NDM. KPC was reported only in Egypt (Manenzhe et al., 2015). These reviews show that there is an abundance of OXA-48 followed by NDM and VIM in Africa but very few reports of KPC in the continent suggesting it is not prevalent, however majority of the studies do not perform genotyping with data lacking from a third of the region which could provide a different picture of carbapenem resistance in Enterobacterales.
1.5.2.2 *Acinetobacter baumannii*

Is an opportunistic pathogen that usually infects severely ill patients and immune compromised individuals whose care requires medical devices such as ventilators and catheters. *A. baumannii* unlike other members of the genus is an important nosocomial pathogen in intensive care units that readily acquires virulence and resistance genes (Antunes et al., 2014, McConnell et al., 2013). It is estimated that the pathogen causes approximately one million infections annually, half of which are carbapenem resistant resulting in a global annual incidence of 75,000 carbapenem resistant *A. baumannii* infections (Spellberg and Rex, 2013). The pathogen causes ventilator associated pneumonia (VAP) and BSI with a high mortality but also UTI and skin and soft tissue infections. A high mortality is associated with carbapenem resistant *A. baumannii* though this is thought to be as a result of the underlying severe illness in patients and inappropriate empirical treatment rather than a causality (Lemos et al., 2014, Du et al., 2019, Son et al., 2020).
The global spread of carbapenem resistant strains has been as a result of two major clones, global clone (GC) 1 and 2 (also referred to as international clone 1 and 2) which are responsible for the spread of the oxacillinases ($bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$ and $bla_{\text{OXA-58}}$). OXA-23 is the most widespread oxacillinase in this species since its first identification in Scotland in 1985 having originated from $A.\ radioresistens$ through insertion element ISAba1 (Evans and Amyes, 2014). OXA-24/40 was initially identified in Spain and is carried on small plasmids and is responsible for hospital outbreaks worldwide while OXA-58, first identified in France in 2003, has been associated with hospital outbreaks in Europe, USA, South America, Australia and Africa (Da Silva and Domingues, 2016). An intrinsic $bla_{\text{OXA-51}}$ though not carbapenem hydrolysing is associated with this species with the variants OXA-69 and OXA-66 associated with members of GC1 and 2 respectively (Hamidian and Nigro, 2019). Though other carbapenemase resistance genes have been found in $A.\ baumannii$, they are not identified as frequently as in Enterobacterales (Da Silva and Domingues, 2016).

1.5.2.3 *Pseudomonas aeruginosa*

*P. aeruginosa* on the other hand, is known to infect patients with cystic fibrosis but can also cause wound and soft tissue infections, UTI and BSI in other patients (Gellatly and Hancock, 2013). In Europe it is estimated that 9% of all healthcare related infections are due to *P. aeruginosa* making it the fourth most common pathogen in hospitals while in USA it accounts for 7% of such cases (Ruiz-Garbajosa and Canton, 2017). The burden of carbapenem resistant *P. aeruginosa* is similar in different regions (data from Africa not included) and together with $A.\ baumannii$, the proportion of these two organisms is higher than that of carbapenem resistant Enterobacterales with majority of the strains obtained from respiratory infections and UTI than from BSI (Nordmann and Poirel, 2019). High mortality in patients with bloodstream infections with *P. aeruginosa* is associated with inappropriate initial treatment (Micek et al., 2005) and in some studies, the mortality is higher in patients where the bacteria was carbapenem resistant compared to susceptible (Buehrle et al., 2017, Zhang et al., 2016).

There are 10 global high risk clones which are also MDR and extensively drug resistant (XDR) and include the sequence types ST235, ST111, ST233, ST244, ST357, ST308, ST175, ST277, ST654 and ST298 (del Barrio-Tofiño et al., 2020). ST235 is associated with a wide diversity of resistance genes and at least 60 β-lactamase variants and possess the $dprA$ gene which allows it to acquire and maintain foreign resistant elements (Treepong et al., 2018). The clone is also highly virulent thanks to the exotoxin ExoU which is associated with higher mortality in BSI (Peña et al., 2014). In *P. aeruginosa*, MBLs are the most prominent type of carbapenemases with VIM being the most disseminated having been first identified in
the species in Italy and has been identified in countries in all continents. IMP, also first identified in the species in Japan in 1991, is the second most common carbapenemase in *P. aeruginosa* with NDM producers occurring mainly in Asia, Europe and Africa. Among class A carbapenemases, KPC and GES (Guiana extended spectrum β-lactamase) are widespread and have been identified in several countries of all continents except in Africa despite limited data, while oxacillinases are quite rare in the organism (Yoon and Jeong, 2021).

Similar to Enterobacterales, there are limited data on these pathogens in Africa and most of the currently available data is overly represented from the North Africa region. Therefore the estimated pooled prevalence of carbapenem resistant *A. baumannii* (57%) and *P. aeruginosa* (21%) is likely to be biased and not reflective of the entire continent (Kindu et al., 2020). However, the data suggest a high proportion of carbapenem resistance in these organisms with OXA type genes being abundant in *A. baumannii* isolates and VIM in *P. aeruginosa* isolates with KPC being rarely reported in these species in the continent (Kindu et al., 2020, Manenzhe et al., 2015).

### 1.5.3 Current treatment options for carbapenem resistant and multi-drug resistant infections

Carbapenems were the last resort for the treatment of infections caused by MDR bacteria, however as increased consumption drove up resistance, antibiotics that were previously ‘forgotten’ were re-evaluated for patient management. Colistin (polymyxin E), a secondary metabolite of *Paenibacillus polymyxa* subsp. *colistinus*, was discovered in 1947 in Japan and was approved for intravenous use in 1959. It was abandoned for clinical use by the mid-1970s due to nephrotoxicity and neurotoxicity issues but was retained for cystic fibrosis patients and was widely used in the veterinary world for prophylaxis and treatment (Michalopoulos and Karatza, 2010). In 1990s, despite its toxicity, colistin was reintroduced due to lack of options for the treatment of MDR and XDR *P. aeruginosa, A. baumannii* and *K. pneumoniae* infections. However, its misuse and overuse especially in animal farming has led to widespread resistance reducing its effectiveness (Wang et al., 2020). Tigecycline, a glycyclycline derivative of tetracycline was approved for clinical use in 2005 and has been used for the treatment of carbapenem resistant *A. baumannii* and Enterobacterales but has no effect on *P. aeruginosa* which is intrinsically resistant. The drug is less toxic compared to colistin but is not recommended for bacteraemia patients and has a Food and Drug Administration (FDA) warning for use in VAP due to high mortality as a results of pooled data from clinical trials (Sheu et al., 2019). Fosfomycin was discovered in 1969 and has been used for UTI, however it is not indicated against *A. baumannii* and is proposed to be
used in combination therapy. The drug has also been proposed as a promising option in treating MDR bacteria in young children due to less toxicity and adverse effects (Williams, 2020).

In addition to the above drugs, several new drugs have recently been approved (between 2015 -2020) for clinical use in complicated infections or in cases where there are no other alternatives for patient management. None of these new drugs are new drug classes and they are not approved for use in children and have been reviewed previously and will be summarised (Doi, 2019, Sheu et al., 2019, Bassetti et al., 2019). Ceftazidime-avibactam is a new β-lactamase inhibitor combined with a cephalosporin that has recently been approved for complicated UTI (cUTI), complicated intra-abdominal infections (cIAI), hospital acquired pneumonia (HAP) and ventilator associated pneumonia (VAP). Avibactam, unlike most β-lactamase inhibitors, is a non β-lactam inhibitor that is active against class A, C and D β-lactamases but not MBL and is effective against Enterobacterales and P. aeruginosa (Sharma et al., 2016). Other novel β-lactam inhibitors recently approved to be used in combination with carbapenems include vaborbactam and relebactam. Vaborbactam, is a novel boron containing β-lactamase inhibitor that is combined with meropenem with clinical indication against cUTI and VAP and was highly effective and well tolerated. It is effective against Class A but not B or D and is not effective against A. baumannii or P. aeruginosa (Wunderink et al., 2018). Relebactam, also a new β-lactamase inhibitor with a chemical structure like avibactam (diazabicyclooctane core) has been approved for use in combination with imipenem-cilastatin. It inhibits class A and C β-lactamases but not class B or D. Like the former two the drug combination is indicated for cUTI, cIAI, VAP and HAP and is active against Enterobacterales and P. aeruginosa (Mansour et al., 2021) (Zhanel et al., 2018). Eravacycline is a fluorinated tetracycline closely related to tigecycline that is approved for treatment of cIAI (Scott, 2019). It is effective against Enterobacterales and A. baumannii but not P. aeruginosa. Plazomicin is a next generation aminoglycoside active against aminoglycoside-modifying enzymes and approved for cUTI as it does not reach adequate tissue concentrations to be used on its own for severe infections (Eljaaly et al., 2019). Cefiderocol is a novel siderophore cephalosporin which enters the bacterial periplasmic space by exploiting the cross-membrane iron transport system. The drug displayed activity against Enterobacterales, P. aeruginosa and A. baumannii and unlike all other new drugs described, it is stable against all classes of β-lactamases including MBLs and it is the latest drug to be approved for cUTIs (Zhanel et al., 2019).
1.6 Carbapenem resistance in Gram negative bacteria in Kenya

In Kenya carbapenems are barely used for patient management in many public hospitals due to cost and lack of access (Maina et al., 2020, Momanyi et al., 2019), however these drugs are readily used in private healthcare facilities (Kizito et al., 2018). In many LMICs, AMR has been attributed to the easy access of drugs without prescription and in a survey of antibiotic sales in human and veterinary drug stores in Nairobi, Kenya’s capital city, 15% of antibiotics sales for humans were carbapenems (Muloi et al., 2019). While this is alarming, given that it is administered intravenously, it is also likely that the drugs may have been purchased by prescription for use in hospitals where carbapenems may not be readily available from the hospital pharmacy. Given the limited access to carbapenems in public sector hospitals, it is possible that there would be low levels of carbapenem resistance in Kenya in the community or in public hospitals.

There are few studies in Kenya that report on the prevalence of carbapenem resistance among Gram negative bacteria and those that do are several years old and may not accurately represent current trends. Resistance in *K. pneumoniae* ranged from 2 – 30% (Maina et al., 2016, Ayoyi et al., 2017, Apondi et al., 2016), 7 – 13% in *E. coli* (Ndungu C et al., 2014, Wangai et al., 2019) and 13.7% in *P. aeruginosa* (Pitout et al., 2008). The first carbapenemase gene detected in Kenya was NDM-1 in *K. pneumoniae* from patients admitted to a tertiary hospital in Nairobi between 2007 and 2009 (Poirel et al., 2011a). The isolates were identical with similar pulse field gel electrophoresis (PFGE) profiles and were highly similar to the PFGE profile of the first reported NDM isolate from the Swedish patient with history of travel to India (Kumarasamy et al., 2010). Other carbapenemase genes reported from isolates from the same tertiary hospital include NDM-1 and OXA-23 in *A. baumannii* (Revathi et al., 2013, Huber et al., 2014), SPM in *K. pneumoniae* (Maina et al., 2017) and VIM-2 in *P. aeruginosa* (Pitout et al., 2008). Only one other recent study has specifically investigated the presence of carbapenemases from different parts of Kenya focusing on non-invasive isolates from UTI and skin and soft tissue infections (Musila et al., 2021). The study reported predominance of NDM-1 and some NDM-5, VIM (only in *P. aeruginosa*), OXA-181, OXA-24, OXA-58. Interestingly KPC genes have not yet been reported in Kenya like in Uganda though KPC-producers have been reported in a study in Tanzania (Mushi et al., 2014).

1.7 Aims of thesis

At Kilifi County Hospital (KCH), carbapenem resistance has been detected in Gram negative bacteria prior to the introduction of carbapenems in the patient formulary in 2020. The aim of this thesis was to investigate the resistance mechanisms in carbapenem resistant bacteria – in particular in *E. coli*, *K.*
pneumoniae, A. baumannii and P. aeruginosa – identified at KCH, to determine the molecular epidemiology and relatedness of strains causing infection and carried by patients and to assess the impact of resistance on the fitness and virulence of organisms in the absence of the selective pressure. In addition, I explored the clinical impact of the resistant bacteria on admitted patients. This is with an aim of understanding the impact of infection prevention control and/or antimicrobial stewardship practices will have on the level of resistance on introduction of carbapenems in the patient formulary.

1.8 Study setting
The study was conducted using E. coli, K. pneumoniae, Acinetobacter species (baumannii and non-baumannii) and P. aeruginosa isolates obtained from clinical samples of patients admitted to KCH in Kilifi County and carriage E. coli and K. pneumoniae isolated from patients admitted at KCH, Coast General Teaching and Referral Hospital (CGH) in Mombasa County and Mbagathi County Hospital (MCH) located in Nairobi County. The patients were participants of research studies conducted by the Kenya Medical Research Institute (KEMRI) Wellcome Trust Research Programme (KWTRP) located in Kilifi County and based at KCH.

1.8.1 KEMRI Wellcome Trust Research Programme (KWTRP)
The Programme is based within the KEMRI Centre for Geographical Medicine-Coast (CGMRC) and conducts health and social research activities majorly within KCH and among communities living within Kilifi county. The county is located on the northern coastal region of Kenya with a predominantly rural and semi-urban population of 1,453,787 individuals with an average household size of 4.8 individuals (2019 census) (2019). The county experiences a tropical climate with an average annual rainfall of 300 mm to 1300 mm and temperature ranges of 21°C to 34°C. Tourism, fishing and farming are the main economic activities in the county.

The Programme runs the Kilifi Health and Demographic Surveillance System (KHDSS) which covers part of Kilifi County and was established in 2000 including an area of 891 km² and extends 35km North and South of Kilifi town (Scott et al., 2012) (Figure 1.8). The area is centred around Kilifi County Hospital (formerly Kilifi District Hospital) the main public referral health facility and includes 85 healthcare facilities offering outpatient services. Enumerated every four months and with a population of over 300,000 individuals (2019 KHDSS census), the KHDSS was initially set up to monitor births, deaths and migration rates to determine incidence of major diseases such as malaria and invasive bacterial infections, evaluate the impact of community-based intervention measures such as bed nets and
provide a sampling frame for cross-sectional and case-control studies. Since inception, the KHDSS has been used to determine causes and trends in mortality (Deribew et al., 2016, Ndila et al., 2014) effect of interventions such as bed nets and vaccines on hospital admission cases (Hammitt et al., 2019, Kamau et al., 2017) and determine the epidemiology and burden of disease in the region (Etyang et al., 2014, Uyoga et al., 2019).

Longitudinal clinical surveillance in children admitted to KCH was developed in the early 1990s for malaria (Marsh et al., 1995) and invasive bacterial infections (Berkley et al., 2005) and in 2000s for viral infections. A similar longitudinal surveillance programme in adults was initiated in 2007 to investigate the indirect effects of the introduction of pneumococcal conjugate vaccine on adult diseases (Etyang et al., 2014).

Figure 1.8 Map showing location of KCH and KHDSS boundaries (right) within Kilifi county.
In addition to longitudinal surveillance, the Programme conducts a wide range of research activities including vaccine clinical trials, controlled human infection studies, human genetic factors in disease, malnutrition and feeding studies and health systems and research ethics. Other research locations of the programme include Mtwapa and Malindi in Kilifi County, Mombasa county, Nairobi County and Mbale, Uganda as well as collaborative research within Africa. There are four Good Clinical Laboratory Practice (GCLP) accredited laboratories in the Programme that analyse clinical specimens collected from hospital patients and research participants: clinical trials laboratory, short-turnaround laboratory, microbiology laboratory, and immunology basic research laboratory (Gumba et al., 2019). The vast amount of data generated by research and surveillance activities is captured by the Kilifi Integrated Data Management System (KIDMS), a database which links demographic to clinical and research data through unique personal identifiers. All pathogenic bacteria isolated from all hospitalised patient samples collected as part of patient management as well as from various research activities have been stored within the biobank facility from as early as 1990s. This together with the bacterial surveillance studies and the linked longitudinal demographic and clinical data from the community and the hospital provided an ideal environment to conduct this study.
Carbapenem resistant bacteria: clinical characteristics, antimicrobial susceptibility profile and phenotypic detection of β-lactamases

Key points

- Carbapenem resistant *E. coli, K. pneumoniae, Acinetobacter* species and *P. aeruginosa* isolated from clinical and carriage specimens of patients were retrieved and phenotypically characterised by antimicrobial susceptibility testing and production of β-lactamases.
- Infection with carbapenem resistant bacteria was more likely to be acquired in hospital than in the community.
- The strains were resistant not only to commonly used antibiotics but also to recently approved newer drugs such as cefiderocol and eravacycline.
- Metallo-β-lactamase production was the main resistance mechanism detected in majority of the strains.

2.1 Introduction

Carbapenems are grouped in the Watch category by WHO indicating that they are critically important for human medicine and should be monitored given their higher potential for resistance in bacteria. Of all carbapenems only meropenem has been listed in the WHO 2019 list of essential medicines. In Kenya, the carbapenems ertapenem and meropenem have been listed as Reserve antibiotics to be used only in level 5 and 6 hospitals under the guidance of a specialist with close monitoring (MoH, 2019). Therefore carbapenems are minimally used in public hospitals in Kenya compared to cephalosporins which are listed in the access (ceftriaxone, cefixime) and watch groups (ceftazidime) (Maina et al., 2020, Momanyi et al., 2019). In cases where they are used, it is usually not driven by patient microbiological data but by patient response or lack of response to prescribed treatment. Carbapenems are one of the last resort drugs used for the treatment of multi-drug resistant infections such as those that are caused by ESBL-producing bacteria in Africa. In Kenya, admission to hospital increases a patient’s risk for acquisition of carriage of ESBL bacteria resulting in an increased risk of infection with such bacteria especially in the immune-compromised, malnourished and the severely unwell (Henson et al., 2017, Kagia et al., 2019). There are few studies that have reported on the proportion of ESBL producing bacteria causing infections in the country with 27% in *E. coli* (Kiiru et al., 2012) and 55% in *K. pneumoniae* (Henson et al., 2017) with a higher prevalence among hospital acquired infections than community acquired. However, this does not represent the whole country and given the high usage of extended spectrum...
cephalosporins and the reports of resistance to these drugs (Maina et al., 2016, Wangai et al., 2019, Ndungu C et al., 2014), it points to an increased reliance on carbapenems in treatment of multi-drug resistant infections in the country in coming years.

At KCH, carbapenems were recently introduced (2020) as part of the patient formulary, however, carbapenem resistant bacteria have been isolated from patient samples prior to this (unpublished data). Resistance to carbapenems in A. baumannii, P. aeruginosa and Enterobacterales as well as production of ESBL in the latter has been listed as critical priority by WHO requiring urgent development of new drugs as well as increased monitoring and surveillance for resistance trends (Tacconelli et al., 2018). Such data is lacking in many African countries including Kenya, which has yet to provide surveillance data on key pathogens and drugs to the WHO GLASS report since its inception though it has set up surveillance programs in the country (WHO, 2020). Nevertheless, such bacteria should be investigated to understand the resistance mechanisms they possess, and the clinical characteristics of patients infected by such bacteria and the impact on wider usage of carbapenems in patient treatment.

In this chapter, I describe a retrospective study to identify patients who were admitted at KCH and from whom a carbapenem non-susceptible bacteria was isolated from a clinical specimen. I describe their clinical characteristics, the antimicrobial susceptibility profiles of the isolates to commonly known antibiotics and to newer drugs recently approved by the USA FDA and Europe Medical Agency (EMA) for difficult to treat infections, and the phenotypic detection of β-lactamases in these bacteria.

2.2 Methodology

2.2.1 Sampling frame

Carbapenem non-susceptible Gram-negative bacteria strains analysed in this thesis were obtained from the surveillance/studies described below.

Invasive bacterial infections surveillance study

Since 1998, all children up to 14 years of age admitted to KCH have a blood sample collected on admission for culture, except those admitted for elective procedures or minor accidents (Berkley et al., 2005). A cerebrospinal fluid (CSF) culture is also performed on or during admission for suspected meningitis cases. A similar set up was established from January 2007 for patients aged ≥15 years who are admitted to hospital with signs and symptoms suggestive of invasive bacterial infections or meningitis: these patients have a blood or CSF culture performed on admission (Etyang et al., 2014).
Basic haematological and biochemical tests are also conducted on admission for all patients as per clinical guidelines and additional samples such as urine, induced sputum, pleural aspirates and pus are collected for culture or additional tests at the discretion of the clinical team.

Blood cultures are performed using an automated blood-culture system (BACTEC 9050/FX, Beckton Dickinson, USA) and positive blood cultures and other samples are cultured on standard microbiology media (Oxoid, UK) as per local standard operating procedures. Up to 2018, cultured bacteria were identified mainly using standard biochemical testing methods including API strips (BioMérieux, France) and serologic testing for serotypes. Since 2018, bacterial identification is through matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (Bruker Daltonics, Germany) with supplementary biochemical and serological testing if required. All pathogenic bacteria undergo routine clinical antimicrobial susceptibility testing (AST) by disc diffusion method (Bauer et al., 1966) and are stored for future research purposes as consented by patients. The British Society for Antimicrobial Chemotherapy (BSAC) testing guidelines (Andrews, 2001) were implemented up until 2012 with Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2019, CLSI, 2018) used subsequently until to date. All results are fed back to the clinical team for patient care and management.

**First-Line Antimicrobials in Children with Complicated Severe Acute Malnutrition (FLACSAM) trial**

This clinical trial set out to assess the efficacy of ceftriaxone versus penicillin plus gentamicin, and metronidazole versus placebo on mortality and nutritional recovery in sick, severely acute malnourished children in a 2x2 factorial design (that is ceftriaxone with metronidazole or placebo, or penicillin and gentamicin with metronidazole or placebo). The rationale is that children with severe acute malnutrition (SAM) have a higher mortality than non-severe malnourished children and have an increased risk of mortality post-discharge. The aim of the study was to determine if a change to broader spectrum first line antibiotics (ceftriaxone and metronidazole) will reduce the risk of death, both in-hospital and post-discharge, and improve nutritional recovery in SAM children compared to the currently recommended World Health Organization (WHO) first line antibiotics penicillin or ampicillin and gentamicin). The study was conducted at KCH, Coast General Teaching and Referral Hospital (CGH) in Mombasa County, Mbagathi County Hospital (MCH) in Nairobi County, and Mbale Regional Referral Hospital in Uganda. The trial recruited children aged 2 months to 13 years with SAM defined as presence of kwashiorkor at any age; or middle upper arm circumference (MUAC) <11.5cm or weight-for length Z (WLZ) score <-3 for children aged 6 to 59 months; or MUAC <11cm or WLZ score <-3 for children aged 2 to 5 months; or BMI-for-age Z score <-3 or MUAC-for-age Z score <-3 for children aged 5 to 13 years.
A sub-study sought to determine the frequency of faecal carriage of bacteria producing extended spectrum β-lactamases (ESBLs) at admission, discharge and days 45 and 90 follow up in SAM patients involved in the trial and in a parallel cohort of non-SAM patients admitted to hospital during the same time period. Surveillance rectal swabs at admission, discharge and 45 and 90 days after discharge were obtained from study participants and were screened for ESBL producing bacteria using 8% gentamicin MacConkey agar (Oxoid) and ceftriaxone and ceftazidime discs on blood agar. Bacterial colonies on the MacConkey- gentamicin agar and within 25 mm and 22 mm zone size of ceftriaxone and ceftazidime discs respectively were sub-cultured on blood agar before bacterial identification with antibiotic susceptibility testing and ESBL production confirmed using CLSI guidelines (CLSI, 2019).

2.2.2 Bacterial collections

Clinical carbapenem resistant organisms (“Clinical CRO” set)

The database was searched for all microbiological data pertaining to Acinetobacter species, Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumoniae cultured from clinical specimens of patients admitted from January 2005 to October 2020. Resistant isolates were identified by retrieving organisms which had be recorded as having reduced susceptibility to imipenem or meropenem, which is routinely tested by imipenem disc (10 µg) for hospital surveillance clinical isolates with the addition of meropenem disc (10 µg) for Acinetobacter species in 2019. Additional susceptibility testing for invasive isolates (blood and cerebrospinal fluid) as part of an investigation on antimicrobial resistance trends (Ngure Kagia, personal communication) was done using imipenem and meropenem discs and the results were obtained from the study investigators to ensure capture of all available isolates. The inhibition zone sizes recorded in the database for the drugs was re-analyzed using the 2019 CLSI breakpoints (CLSI, 2019) and all isolates with an interpretation of intermediate or resistant (henceforth referred to as resistant) were included in the study. These isolates are hereby referred to as “clinical CRO”.

Clinical carbapenem susceptible organisms (“Clinical CSO” set)

To obtain a control set to enable comparison of the biological fitness and pathogenicity to resistant bacteria described in chapter four, carbapenem susceptible Escherichia coli and Klebsiella pneumoniae from clinical samples were matched to the clinical CRO set described above. Isolates were matched by species, from paediatric or adult surveillance, and then, in descending order, by specimen type, antimicrobial susceptibility profile (susceptible, intermediate and resistant) and time frame, to obtain carbapenem susceptible organisms of the same species, from a similar patient and specimen type, and
as close to the date of isolation of the CRO as possible. Each clinical CRO was matched to two carbapenem susceptible bacteria, one of which was matched as similar as possible to the antimicrobial susceptibility profile of the resistant bacteria other than carbapenems while the second matched bacteria was chosen to be susceptible to as many of the antibiotics tested as possible. The matching was done within a maximum time frame of 1 year before or after the isolation of the corresponding CRO. Where matching on specimen type was not possible, blood culture isolates were used as the default. As for the CRO collection, zone sizes recorded in routine testing were re-analyzed using 2019 CLSI breakpoints prior to selection. These isolates are henceforth referred to as “clinical CSO”.

Carriage carbapenem resistant organisms (“Carriage CRO” set)

All carbapenem non-susceptible Enterobacterales isolated from surveillance rectal swabs from FLACSAM study participants on admission were included in the study, except those from Uganda. Isolates were tested using imipenem as per the FLACSAM study protocol, and all intermediate and resistant bacteria were included in the study and are referred to as “carriage CRO”. Only Enterobacterales were included as this FLACSAM sub-study did not include non-fermenting organisms.

2.2.3 Confirmatory bacterial species identification and susceptibility testing

For all study strains, confirmatory identification and AST were performed to ensure no mislabelling/ misstoring of isolates prior to further analysis. The strains were retrieved from storage and re-cultured on blood agar at 35°C ± 2°C overnight. For identification, a single colony was applied onto the MALDI 96 target ground steel plate well using a toothpick and air dried at room temperature. The sample was overlaid with 1 µl of 70% formic acid (Sigma-Merck, USA) for in situ cell lysis on plate and dried before 1 µl of α-cyano-4-hydroxycinnamicacid (HCCA) (Bruker) matrix solution was added and dried. The bacterial mass spectra were generated using the MicroFlex Analysis LT mass spectrometer (Bruker) and analyzed using the MALDI BioTyper software package (version 4.1) against the reference database. A score value of ≥2 was accepted for reliable species identification while a score of 1.7 to 1.99 was accepted for identification to genus level. Scores of ≤ 1.69 were considered non-reliable and repeat testing was performed with freshly grown colonies. Daily quality checks (QC) were performed using the bacterial test standard as per the manufacturer’s protocol.

For the reliable identification of A. baumannii, all stored Acinetobacter spp isolated from clinical samples from 2005 to 2018 were analysed using the blaOXA-51-like polymerase chain reaction (PCR) to detect the chromosomally encoded oxacillinase gene usually used to distinguish the species from other
genus members (Turton et al., 2006). Crude DNA extracts were obtained by boiling a loopful colony of the sample in 1X Tris ethylenediaminetetraacetic acid (EDTA) buffer for 30 minutes and the \textit{bla}\textsubscript{OXA-51-like} gene was amplified using GoTaq G2 DNA Polymerase master mix (Promega, USA) under the following cycling conditions: 94\(^\circ\)C for 3 minutes, 35 cycles of 94\(^\circ\)C for 45 seconds, 57\(^\circ\)C for 45 seconds and 72\(^\circ\)C for 1 minute and finally 72\(^\circ\)C for 5 minutes. The amplified product was visualized using 2\% agarose gel with Red Safe staining (Intron Biotechnology, South Korea). Results obtained using PCR were compared with identification using MALDI TOF mass spectrometry.

AST confirmatory testing was performed for all isolates in the collections using the disc diffusion method (Bauer et al., 1966, CLSI, 2018) where colonies from a fresh overnight culture plate were suspended in normal saline (0.85\% sodium chloride) to a turbidity of \(\approx 0.5\) McFarland and swabbed on Mueller Hinton agar (MHA). Standardized antimicrobial-containing discs (Oxoid) were placed on the agar surface aseptically, incubated overnight and the resultant zone of inhibition interpreted using 2019 CLSI breakpoints (CLSI, 2019). The drugs used were (most commonly available): ampicillin (10 \(\mu\)g), amoxillicin-clavulanate (augmentin) (20/10 \(\mu\)g), cefoxitin (30 \(\mu\)g), ceftriaxone (30 \(\mu\)g), ceftazidime (30 \(\mu\)g), cefotaxime (30 \(\mu\)g), gentamicin (10 \(\mu\)g), amikacin (30 \(\mu\)g), ciprofloxacin (5 \(\mu\)g), trimethoprim-sulphamethoxazole (co-trimoxazole) (1.25/23.75 \(\mu\)g), chloramphenicol (30 \(\mu\)g), imipenem (10 \(\mu\)g), ertapenem (10 \(\mu\)g), meropenem (10 \(\mu\)g).

The minimum inhibitory concentration (MIC) values of imipenem, meropenem, ertapenem and colistin were determined for the study strains. Meropenem (PHR 1772, calculated potency 613.94 \(\mu\)g/mg) and imipenem (PHR 1796, calculated potency 807.2 \(\mu\)g/mg) (Sigma) MICs were determined by agar dilution method as per 2015 CLSI guidelines (CLSI). The drug powder was prepared to a stock concentration of 1280 \(\mu\)g/ml and diluted to a final concentration ranging from 32 to 0.5 \(\mu\)g/ml in MHA plates for testing. Test bacteria in suspensions of 0.5 McFarland turbidity were diluted ten-fold in normal saline and 2 \(\mu\)l was spotted on the drug plates, air dried and incubated overnight. The plates were examined the following day and MIC taken as the lowest concentration in which there was no growth of bacteria. Ertapenem MIC was determined using Epsilometer test (Etest) strips (BioMérieux) with bacterial suspension prepared and plated as per testing by disc diffusion as described above. Colistin MIC (PHR 1605, potency 746 \(\mu\)g/mg) (Sigma) was determined by broth micro-dilution method where a stock solution with a concentration of 1280 \(\mu\)g/ml was diluted to a concentration ranging from 16 to 0.5 \(\mu\)g/ml in cation adjusted Mueller Hinton broth (CAMHB) (Sigma). A hundred microlitres of each dilution was added to individual wells in a microtiter plate. Test bacteria prepared to a turbidity of 0.5 McFarland was
diluted 20-fold in CAMHB and 10 µl of test organism added to each drug-containing drug and the plate incubated overnight.

The strains were investigated further with newer drugs and drug-inhibitor combinations available in Europe; eravacycline (20 µg), cefiderocol (30 µg) (MAST, UK), cefepime-sulbactam (60 µg) (LiofilChem, Italy), imipenem-relebactam (10/25 µg) and meropenem-vaborbactam (30 µg). Drug susceptibility was interpreted according to CLSI guidelines (CLSI, 2019) except for colistin which was interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2020 breakpoints (EUCAST, 2020) AST testing for these drugs and subsequent interpretation was performed by Dr Wareham (supervisor) at QMUL laboratory, London. Isolates that were intermediate (by CLSI breakpoints) or “increased dose” (by EUCAST breakpoints) for any antibiotic tested were classified as resistant.

2.2.4 Phenotypic detection of β-lactamase activity

β-lactamase activity was detected using multiple methods relevant to the organisms tested (table 2.1).

Table 2.1 Phenotypic detection of β-lactamase activity in carbapenem resistant bacteria

<table>
<thead>
<tr>
<th>Test</th>
<th>Acinetobacter spp</th>
<th>P. aeruginosa</th>
<th>Enterobacterales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbapenemase</td>
<td>THT</td>
<td>mCIM, THT</td>
<td>mCIM, THT</td>
</tr>
<tr>
<td>MBL</td>
<td>Imipenem-EDTA test</td>
<td>Imipenem-EDTA test</td>
<td>Imipenem-EDTA test, eCIM</td>
</tr>
<tr>
<td>ESBL</td>
<td>ND</td>
<td>ND</td>
<td>CLSI ESBL confirmatory test</td>
</tr>
<tr>
<td>AmpC</td>
<td>MAST Combi AmpC kit</td>
<td>MAST Combi AmpC kit</td>
<td>MAST Combi AmpC kit</td>
</tr>
</tbody>
</table>

THT – Triton Hodge test, mCIM – modified carbapenemase inactivation method, eCIM – EDTA supplemented mCIM

MBL – metallo- β-lactamase, AmpC – cephalosporinase, ESBL – extended spectrum β-lactamase

a) Modified carbapenemase inactivation method (mCIM)

Bacteria were revived on blood agar overnight at 35°C ± 2°C and carbapenemase activity was detected by suspending either 1 µl loopful of Enterobacterales or 10 µl of P. aeruginosa in 2 ml tryptic soy broth (TSB) (Oxoid). A 10 µg meropenem disc was added and the suspension incubated at 35°C ± 2°C for 4 hours ± 15 minutes for drug inactivation by carbapenamase. Just before or immediately following completion of incubation, a 0.5 McFarland suspension of E. coli ATCC 25922 (fully susceptible indicator strain) was prepared in normal saline and plated on MHA plate. The meropenem disc was removed from
the suspension and placed on the inoculated plate and incubated overnight at 35°C ± 2°C. (Pierce et al., 2017, Lisboa et al., 2018). Bacteria was positive for carbapenemase activity if the zone diameter was 6-15 mm or the presence of pinpoint colonies within a 16-18 mm zone observed. The result was negative if diameter was ≥ 19 mm (clear zone) and indeterminate if diameter was 16-18 mm or ≥ 19 mm with presence of pinpoint colonies. Known carbapenemase producing and non-producing strains from previous UK based National External Quality Assessment Service (UK NEQAS) material were used as positive and negative controls. This method was recommended by CLSI 2019 for Enterobacterales and *P. aeruginosa* where testing has been conducted.

b) EDTA supplemented mCIM (eCIM)

For Enterobacterales with a positive mCIM, EDTA supplemented testing was performed to differentiate serine based- from zinc based- carbapenemases. The assay was performed as for the mCIM assay described, except 20 µl of 0.5 M EDTA (Sigma) was added to the TSB media to obtain a concentration of 5 mM EDTA (Sfeir et al., 2019) prior to addition of the bacterial suspension and meropenem disc. The test was positive (metallo-β-lactamase producing isolates) if there was a ≥5 mm increase in diameter for eCIM versus mCIM. The method has been tested only in Enterobacterales with known carbapenemase producing and non-producing strains from EQA used as controls.

![Figure 2.1 Carbapenemase inactivation method (mCIM and eCIM) for detection of carbapenemases.](image-url)
Strain A was negative for carbapenemase activity, while strain B was positive for carbapenemase but had a negative eCIM (lower disc) and has a serine based carbapenemase (SBL). Strain C was positive for both carbapenemase activity (upper disc) and eCIM (lower disc) and has a zinc based carbapenemase (MBL).

c) Triton Hodge test (THT)

The assay is an alteration of the modified Hodge test to increase identification of strains bearing NDM especially in *Acinetobacter* spp. While the modified Triton Hodge test is able to detect class A and D carbapenemases effectively, it performs poorly in the detection of NDM producers due to the anchoring of the lipoprotein in the outer membrane and supplementation of Triton in agar media solubilises this protein increasing detection (Pasteran et al., 2016). 50 µl of Triton X-100 (Sigma) was spread on MHA plate to obtain a concentration of 0.2% v/v. A 0.5 McFarland suspension of *E. coli* ATCC 25922 (fully susceptible indicator organism) was diluted ten-fold and inoculated on the MHA-triton plate. The plate was air-dried, and then a 10 µg meropenem disc was placed at the center of the plate and a 10 µl loop used to streak 3-5 colonies of test bacteria in a straight line from the edge of the meropenem disc to the plate edge. Known carbapenemase producing and non-producing strains from EQA material were used as positive and negative controls. Each assay was conducted with three different strains per plate. The plate was incubated overnight at 35°C ± 2°C. Growth of the indicator strain toward the carbapenem disc in a clover leaf appearance was interpreted as positive for carbapenemase activity if the magnitude of enhanced growth was >3 mm. The assay was performed for all species.
Figure 2.2 Triton Hodge test for detection of carbapenemases.

Appearance of a clover leaf like enhanced growth around the test strain indicates carbapenemase activity. Strain A was negative for carbapenemase activity (no clover leaf) while strains B and C were positive with an enhanced growth that can be measured (as indicated by arrow).

d) Imipenem EDTA combined disc test

Zinc based carbapenemase activity was also detected using the imipenem EDTA combined disc test. Two imipenem discs were placed on opposite zones of an MHA plate streaked with 0.5 McFarland turbidity of the test bacteria and 5.13 µl of 0.5 M EDTA was added to one of the discs to achieve a concentration of 750 µg EDTA. The plate was incubated overnight at 35°C ± 2°C. An increase of ≥7 mm in zone diameter of imipenem / EDTA versus imipenem only was interpreted as positive for MBL activity. However, given that false positives and negative results can occur in *Acinetobacter* species, an imipenem / EDTA inhibition zone size of ≤14 mm in strains where a difference between the two discs was ≥7 mm was considered negative. Similarly, an imipenem / EDTA inhibition zone size of ≥17 mm was considered positive where the difference between the disc was either 5 or 6 mm (Yong et al., 2002). The assay was
performed for all species. Known carbapenemase producing and non-producing strains from EQA were used as positive and negative controls for each assay.

Figure 2.3 Imipenem EDTA test for detection of zinc based β-lactamases. Strain A was positive for zinc-based enzymes while strain B was negative.

e) Cephalosporinase (AmpC) overproduction

The overproduction of AmpC was detected using MASTDISCS® Combi AmpC Detection set D69C (Halstead et al., 2012). The test was conducted as per the manufacturer instructions. Briefly, the kit contains three types of antimicrobial disc. Disc A contains cefpodoxime (10 µg) and an AmpC inducer compound, disc B contains cefpodoxime (10 µg), an AmpC inducer and an ESBL inhibitor compound, and disc C contains cefpodoxime (10 µg), an AmpC inducer, an ESBL inhibitor and an AmpC inhibitor compound. The assay was performed using the disc diffusion method where a 0.5 McFarland suspension of test organism was inoculated on to surface of MHA and one each of discs A, B and C applied to the surface and incubated overnight at 35°C ± 2°C. An increase of ≥5 mm in zone diameter of both disc C versus disc A and disc C versus disc B was interpreted as positive for AmpC activity. Known AmpC producing and non-producing strains from EQA (UK NEQAS) were used as positive and negative controls for each assay.
Figure 2.4 Cephalosporin overproduction as detected using MASTDISCS®Combi AmpC Detection set. Strain A was negative as the difference between disc C and B is less than five millimetres even though the difference between disc C and A is greater than five. Strain B is positive for AmpC overproduction.

f) ESBL production

ESBL activity was detected using ceftazidime and ceftazidime-clavulanate (30/10 µg), and cefotaxime and cefotaxime-clavulanate (30/10 µg) (Oxoid)combined disc tests. The assay was performed using the standard disc diffusion method where a 0.5 McFarland suspension of test organism was inoculated on to surface of MHA and the disc aseptically placed on the surface and the plate incubated overnight at 35°C ± 2°C an increase of ≥ 5 mm in zone diameter of either of the cephalosporin/clavulanate combination discs compared to the respective cephalosporin alone was considered positive as per (CLSI, 2019). The assay was performed for Enterobacterales only as recommended by CLSI guidelines with E. coli ATCC 25922 and K. pneumoniae ATCC 700673 used as positive and negative controls.

Figure 2.5 Detection of ESBL activity.
Strain A was negative for ESBL production as there is no difference between the cephalosporin and cephalosporin with clavulanate discs while strain B was positive.

2.2.5 Data collection and statistical analysis

All data collected as part of the surveillance and study procedures and all data microbiological data pertaining to the samples were obtained from the stored electronic records. Clinical data obtained included dates of birth, admission, discharge and death; sex; duration of hospitalization; HDSS enumeration status; prior hospitalization (data available for child patients only and limited to hospitalization in the previous 14 days); clinical signs and symptoms (temperature, heart rate, respiratory rate, blood pressure, Glasgow coma score for adults (GCS) and Blantyre coma score (BCS) for paediatrics); prior admissions, nutritional status indicators, discharge diagnosis and any additional clinical information. For clinical isolates (i.e. from non-FLACSAM patients) haematological and clinical chemistry test results were also obtained.

For adults, where the information was available, the quick Sepsis Related Organ Failure Assessment (qSOFA) score was calculated. The qSOFA is used to identify patients with suspected infection who are at greater risk for a poor outcome outside the intensive care unit (ICU). A point is assigned for low blood pressure (SBP≤100 mmHg), high respiratory rate (≥22 breaths per min), or altered mentation (Glasgow coma scale (GCS) <15: a qSOFA scored of ≥2 near the onset of infection is associated with a greater risk of death or prolonged intensive care unit stay (Singer et al., 2016).

Discharge diagnosis, additional clinical notes and all laboratory data was used to describe the patient’s presenting complaint, primary bacterial infection and comorbidities. An infection was considered community acquired if the carbapenem resistant bacteria was isolated from a sample collected up to 48 hours after admission and healthcare associated if isolated more than 48 hours after admission or within 48 hours if the patient had prior admission to hospital in the previous 14 days. Mortality was calculated for in-hospital deaths; the 30-day all-cause mortality was calculated from date of collection of sample to date of death in hospital.

Given the small numbers of carbapenem resistant bacteria and to explore if there were difference in other types of resistance, I obtained clinical data of patients from whom a carbapenem susceptible bacteria was isolated from blood, CSF and urine samples admitted between 2007 and 2020. Clinical and outcome data for patients with infections due to third generation cephalosporin (3GC) susceptible and resistant Enterobacterales with susceptibility determined using cefotaxime. Data was also obtained for
carbapenem susceptible *Acinetobacter* species and *P. aeruginosa* with susceptibility to third generation cephalosporins determined using cefotaxime (2007-2010) and ceftriaxone (2012-2020) in *Acinetobacter* and ceftazidime in *P. aeruginosa*.

Results were expressed as median and interquartile range (IQR) for continuous variables and as proportions for categorical variables. Continuous variables were compared using Wilcoxon ranksum test while categorical variables were compared using Fischer’s exact test (two-sided). Logistic regression analysis was used to examine the relationship between outcome and bacterial infection and place of acquisition while adjusting for confounders. A P value of <0.05 was considered statistically significant.

Statistical analysis was performed using STATA v15 (StataCorp, Texas).

2.3 Results

2.3.1 Sampling frame

2.3.1.1 Clinical and demographic characteristics of patients with a carbapenem resistant organism (clinical CRO)

During the study time frame, 2118 bacteria of the target species (*Acinetobacter* species, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*) were isolated from 1880 admitted patients. Imipenem susceptibility results were available for 1649 isolates, of which 72 were non-susceptible (resistant and intermediate) to imipenem and / or meropenem where tested. All 72 were successfully retrieved for further testing, and reduced susceptibility to one or more carbapenem was confirmed in 61/72 (85%) of the isolates, representing 54 patients. Of the 61 isolates, 41 (67%) were *Acinetobacter* species, 9 (15%) *P. aeruginosa*, 7 (11%) *K. pneumoniae* and 4 (7%) *E. coli*. More than half of the isolates were from blood cultures (34 (56%)) (Table 2.2). Six patients (11%) had more than one organism isolated during their stay with four individuals having duplicates of the same species (*K. pneumoniae*, *E. coli*, *P. aeruginosa* and *A. baumannii*) during the same episode; one patient had *P. aeruginosa* and *A. baumannii* and another had triplicate *A. baumannii* isolated during their stay. The duplicate and triplicate *A. baumannii* were isolated from the same specimen type (pus) more than a month apart and but had similar AST profiles. The duplicate *E. coli* and *P. aeruginosa* were isolated on the same day but from different specimen types while the *K. pneumoniae* were isolated from aspirate less than two weeks apart but with similar AST profile.

Table 2.2 Distribution of carbapenem resistant organisms isolated from clinical samples obtained from children and adult patients admitted to Kilifi County Hospital from 2005 to 2020.
Species | Blood n (%) | Urine n (%) | Pus n (%) | Others* n (%)
--- | --- | --- | --- | ---
*A. baumannii* (n=20) | 9 (45) | 1 (5) | 9 (45) | 1 (5)
*Acinetobacter* (non-baumannii) (n=21) | 19 (90) | 2 (10) | 0 (0) | 0 (0)
*E. coli* (n=4) | 1 (25) | 1 (25) | 1 (25) | 1 (25)
*K. pneumoniae* (n=7) | 1 (14) | 4 (57) | 0 (0) | 2 (29)
*P. aeruginosa* (n=9) | 4 (44) | 2 (22) | 3 (33) | 0 (0)

* induced sputum (1 *A. baumannii*), CSF (1 *E. coli*), aspirate (2 *K. pneumoniae*)

The median age of patients with a clinical CRO was 18 months (IQR: 4 – 65 months); 32 (59%) of the patients were aged less than two years old while 61% (33) were male (Table 2.3). Five paediatric patients had been admitted to hospital in the past 2 weeks (17%). Lower respiratory tract infection (20%) and sepsis (including neonatal sepsis) (18%) were the two main presenting clinical syndromes during admission. Bloodstream infection (57%) was the main bacterial infection in patients with a resistant strain followed by urinary tract infection (17%) and skin and soft tissue infection (9%). The infection was considered to have been acquired in the community in 19 (35%) individuals with the rest of the patients considered to have acquired the bacterial infection in hospital. The median overall length of hospitalization was 14 days (IQR: 4 – 31) with a median stay of 7 days (IQR: 0 – 16) prior to isolation of organism. Inpatient mortality was 33% (18) while the 30-day all-cause mortality was 31% (17) for all patients from whom a resistant organism was cultured.

Table 2.3 Overall demographic and clinical characteristics of all patients admitted at Kilifi County Hospital with carbapenem resistant Gram-negative bacteria isolated from clinical specimens collected from 2005 to 2020.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 (61)</td>
</tr>
<tr>
<td>Female</td>
<td>21 (39)</td>
</tr>
<tr>
<td><strong>Age group</strong></td>
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<tr>
<td>&lt; 1 month</td>
<td>12 (22)</td>
</tr>
<tr>
<td>1 – 23 months</td>
<td>19 (35)</td>
</tr>
<tr>
<td>24 – 59 months</td>
<td>8 (15)</td>
</tr>
<tr>
<td>5 – 17 years</td>
<td>6 (11)</td>
</tr>
<tr>
<td>Presenting syndrome</td>
<td></td>
</tr>
<tr>
<td>----------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>≥ 18 years</td>
<td>9 (17)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Presenting syndrome</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 18 years</td>
<td>9 (17)</td>
</tr>
<tr>
<td>Lower respiratory tract infection (LRTI)</td>
<td>11 (20)</td>
</tr>
<tr>
<td>Sepsis/ neonatal sepsis</td>
<td>10 (18)</td>
</tr>
<tr>
<td>Malaria</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Gastroenteritis</td>
<td>4 (7)</td>
</tr>
<tr>
<td>Burns</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Wound infection</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Birth asphyxia</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Pre-term delivery</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Others</td>
<td>15 (28)</td>
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<table>
<thead>
<tr>
<th>Bacterial infection</th>
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</thead>
<tbody>
<tr>
<td>Bloodstream infection (BSI)</td>
<td>31 (57)</td>
</tr>
<tr>
<td>Urinary tract infection (UTI)</td>
<td>9 (17)</td>
</tr>
<tr>
<td>Skin &amp; soft tissue infection (SSTI)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Bacterial meningitis</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Intra-abdominal infection (IAI)</td>
<td>2 (4)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>qSOFA score *</th>
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<tbody>
<tr>
<td>&lt;2</td>
<td>6/9 (67)</td>
</tr>
<tr>
<td>≥2</td>
<td>3/9 (33)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Prior admission †</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>yes</td>
<td>5/30 (17)</td>
</tr>
<tr>
<td>no</td>
<td>25/30 (83)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Infection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Community associated</td>
<td>19 (35)</td>
</tr>
<tr>
<td>Healthcare associated</td>
<td>35 (65)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Outcome</th>
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</tr>
</thead>
<tbody>
<tr>
<td>30-day all-cause mortality</td>
<td>17 (31)</td>
</tr>
<tr>
<td>In-hospital fatality</td>
<td>18 (33)</td>
</tr>
</tbody>
</table>

* q-SOFA score available for adult patients only
† admission in hospital 14 days prior to current admission, available for child patients only
Comparison of patient characteristics: carbapenem resistant and susceptible

Enterobacterales

Nine patients had a carbapenem resistant Enterobacterales: 3 with \textit{E. coli} and 6 with \textit{K. pneumoniae}. Six carbapenem susceptible \textit{E. coli} and 12 carbapenem susceptible \textit{K. pneumoniae} were identified through the matching process and were all within the one-year time frame, species and paediatric or adult surveillance. Three isolates could not be matched to the specimen type of the CRO and were matched to a blood isolate (two from aspirate and one from urine). Of the 18 CSO, six fully matched the selection criteria for antimicrobial susceptibility (given intrinsic resistance to ampicillin in \textit{K. pneumoniae}) with differences observed mainly in cefoxitin and amikacin for the first match with similar AST profile with CRO and in cotrimoxazole and ampicillin / augmentin in the second match that was susceptible to the antibiotics tested. More male patients had a resistant bacterium (67\%) than a susceptible one (44\%) (Table 2.4). There was no significant difference in the age group distribution despite selection of match by bacteria characteristic ($p=0.457$). All resistant Enterobacterales bacteria isolated from patients were considered healthcare associated compared to only 44\% (8/18) of the susceptible group ($p=0.009$). Patients from whom a resistant bacterium was isolated had a median length of hospital stay of 13 days (IQR: 9 – 20) prior to positive sample compared to less than 2 days (IQR: 0 – 4) in patients with a susceptible strain ($p=0.0036$).

The 30-day all-cause mortality was 11\% (1/9) in the resistant group and 22\% (4/18) in the susceptible group. Inpatient case fatality rate was 22\% in both groups. In a multiple logistic regression analysis, the odds for death from any cause at 30 days was 63\% lower in resistant cases than in susceptible ones (OR: 0.375, 95\% CI: 0.27 – 5.17; $p=0.464$) while there was no evidence of a difference in 30-day all-cause mortality between community and hospital acquired infections.

Table 2.4 Demographic and clinical characteristics of patients admitted at Kilifi County Hospital with carbapenem resistant- (CRE) and carbapenem susceptible- Enterobacterales (CSE) isolated from clinical specimens collected from 2005 to 2020.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CRE (N=9)</th>
<th>CSE (N=18)</th>
<th>$p$-value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6 (67)</td>
<td>8 (44)</td>
<td>0.42</td>
</tr>
<tr>
<td>Female</td>
<td>3 (33)</td>
<td>10 (56)</td>
<td></td>
</tr>
<tr>
<td>Age group</td>
<td>Overall</td>
<td>3GC-S</td>
<td>3GC-R</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>&lt; 1 month</td>
<td>1 (11)</td>
<td>2 (11)</td>
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</tr>
<tr>
<td>1 – 23 months</td>
<td>3 (33)</td>
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<tr>
<td>≥ 18 years</td>
<td>3 (33)</td>
<td>6 (33)</td>
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<tr>
<th>qSOFA score</th>
<th>Overall</th>
<th>3GC-S</th>
<th>3GC-R</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2</td>
<td>3/3 (100)</td>
<td>4/6 (67)</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>≥2</td>
<td>0/3 (0)</td>
<td>2/6 (33)</td>
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<thead>
<tr>
<th>Prior admission</th>
<th>Overall</th>
<th>3GC-S</th>
<th>3GC-R</th>
<th>p-value</th>
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<tbody>
<tr>
<td>yes</td>
<td>1/6 (17)</td>
<td>1/12 (8)</td>
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<td>no</td>
<td>5/6 (83)</td>
<td>11/12 (92)</td>
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<tr>
<th>Infection</th>
<th>Overall</th>
<th>3GC-S</th>
<th>3GC-R</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Healthcare</td>
<td>9 (100)</td>
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<td>Community</td>
<td>0 (0)</td>
<td>10 (56)</td>
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<table>
<thead>
<tr>
<th>Outcome</th>
<th>Overall</th>
<th>3GC-S</th>
<th>3GC-R</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-day all-cause mortality</td>
<td>1 (11)</td>
<td>4 (22)</td>
<td></td>
<td>0.636</td>
</tr>
<tr>
<td>In-hospital fatality</td>
<td>2 (22)</td>
<td>4 (22)</td>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

There were 1059 patients from whom a carbapenem susceptible Enterobacterales was cultured and 57% (606) were less than 2 years old (Table 2.5). Overall, 66% (700) of patients acquired the infection in the community, however the infection in 91% (497) of patients with a 3GC susceptible bacteria was community acquired compared to 40% (203) in cases with 3GC resistant bacteria ($p<0.001$).

In-hospital mortality was significantly higher among patients with 3GC resistant bacteria (45%) compared to those with susceptible strain (38%) ($p<0.021$). There was a significantly higher odds for in-hospital death in patients with a 3GC resistant bacteria compared to those with a susceptible one (OR: 1.36, 95% CI: 1.02 – 1.83; $p=0.036$) in a multiple logistic regression model while the odds of death associated with hospital acquisition were similar for the two groups in the same model (OR: 0.955, 95% CI: 0.70 – 1.3 ; $p=0.773$).

Table 2.5 Demographic and clinical characteristics of patients admitted at Kilifi County Hospital with carbapenem susceptible- Enterobacterales (CSE) isolated from clinical specimens collected from 2007 to 2020.
<table>
<thead>
<tr>
<th></th>
<th>(N=1059)</th>
<th>(N=549)</th>
<th>(N=510)</th>
</tr>
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<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>568 (54)</td>
<td>286 (52)</td>
<td>282 (55)</td>
</tr>
<tr>
<td>Female</td>
<td>491 (46)</td>
<td>263 (48)</td>
<td>228 (45)</td>
</tr>
<tr>
<td><strong>Age group</strong></td>
<td></td>
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</tr>
<tr>
<td>&lt; 1 month</td>
<td>347 (33)</td>
<td>129 (23)</td>
<td>218 (43)</td>
</tr>
<tr>
<td>1 – 23 months</td>
<td>259 (24)</td>
<td>143 (26)</td>
<td>116 (23)</td>
</tr>
<tr>
<td>24 – 59 months</td>
<td>87 (8)</td>
<td>41 (7)</td>
<td>46 (9)</td>
</tr>
<tr>
<td>5 – 17 years</td>
<td>60 (6)</td>
<td>26 (5)</td>
<td>34 (6)</td>
</tr>
<tr>
<td>≥ 18 years</td>
<td>306 (29)</td>
<td>210 (38)</td>
<td>96 (19)</td>
</tr>
<tr>
<td><strong>Infection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthcare</td>
<td>356 (34)</td>
<td>51 (9)</td>
<td>305 (60)</td>
</tr>
<tr>
<td>associated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community</td>
<td>700 (66)</td>
<td>497 (91)</td>
<td>203 (40)</td>
</tr>
<tr>
<td>associated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-hospital fatality</td>
<td>436 (41)</td>
<td>207 (38)</td>
<td>229 (45)</td>
</tr>
</tbody>
</table>

3GC-S & -R: third generation cephalosporin susceptible and resistant

2.3.1.3 Carbapenem resistant A. baumannii and other Acinetobacter spp

There were 258 Acinetobacter species isolated from clinical specimens obtained from 2005 to 2018, of these 70 (27%) were identified as A. baumannii by PCR and 70 (27%) by MALDI-TOF. On comparing the performance of MALDI-TOF to PCR there were only two discrepancies, 69/70 (99%) of the PCR positive isolates were positively (score ≥2) identified to species level as A. baumannii and one isolate was identified as Acinetobacter species (best match identified as A. baumannii but with a score of <2 which is low for species confirmation). Of the 188 PCR negative isolates, one was identified as A. baumannii by MALDI-TOF but had a non-specific band above 1500 bp by PCR. The performance of MALDI-TOF in the identification of A. baumannii was found to be 99% specific and 99% sensitive (Fischer’s exact P-value <0.001) and can therefore be used for a faster and simpler identification of the pathogen from clinical samples.

Thirty-eight patients had a carbapenem resistant Acinetobacter spp of which 17 had the clinically significant A. baumannii (Table 2.6). Only two child patients had prior hospital admission and were infected with CRAB. There was a significant difference (p <0.001) in the source of the bacterial infection with 94% (16/17) and 24% (5/21) of CRAB and CRAN patients respectively considered to have acquired it
in hospital. Patients with CRAB were hospitalized for a median duration of 10 days (IQR: 6 – 18) before isolation of resistant bacteria compared to 0 days (IQR: 0 – 1) in patients with CRAN ($p <0.0001$).

There were more deaths at 30 days (any cause) in patients with CRAB with 8 (47%) compared to 4 (19%) in CRAN infected patients. The odds for death (30 day all-cause) was higher in patients infected with *A. baumannii* than in those with other *Acinetobacter* spp though not significant (OR: 2.52, 95% CI: 0.35 – 18.12; $p=0.36$). Acquiring infection in hospital was not associated with risk of death.

Table 2.6 Demographic and clinical characteristics of patients admitted at Kilifi County Hospital with carbapenem resistant *A. baumannii* (CRAB) and other carbapenem resistant *Acinetobacter* species (CRAN) isolated from clinical specimens collected from 2005 to 2020.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CRAB (N=17)</th>
<th>CRAN (N=21)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7 (41)</td>
<td>15 (71)</td>
<td>0.099</td>
</tr>
<tr>
<td>Female</td>
<td>10 (59)</td>
<td>6 (29)</td>
<td></td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 month</td>
<td>7 (41)</td>
<td>4 (19)</td>
<td></td>
</tr>
<tr>
<td>1 – 23 months</td>
<td>3 (18)</td>
<td>11 (52)</td>
<td></td>
</tr>
<tr>
<td>24 – 59 months</td>
<td>2 (12)</td>
<td>4 (19)</td>
<td><strong>0.029</strong></td>
</tr>
<tr>
<td>5 – 17 years</td>
<td>4 (23)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>≥ 18 years</td>
<td>1 (6)</td>
<td>2 (10)</td>
<td></td>
</tr>
<tr>
<td>qSOFA score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>1/1 (100)</td>
<td>0 (0)</td>
<td>0.333</td>
</tr>
<tr>
<td>≥2</td>
<td>0 (0)</td>
<td>2/2 (100)</td>
<td></td>
</tr>
<tr>
<td>Prior admission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>2/13 (15)</td>
<td>0/8 (0)</td>
<td>0.505</td>
</tr>
<tr>
<td>no</td>
<td>11/13 (85)</td>
<td>8/8 (100)</td>
<td></td>
</tr>
<tr>
<td>Infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthcare associated</td>
<td>16 (94)</td>
<td>5 (24)</td>
<td><strong>&lt;0.001</strong></td>
</tr>
<tr>
<td>Community associated</td>
<td>1 (6)</td>
<td>16 (76)</td>
<td></td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-day all-cause mortality</td>
<td>8 (47)</td>
<td>4 (19)</td>
<td>0.087</td>
</tr>
<tr>
<td>In-hospital fatality</td>
<td>8 (47)</td>
<td>4 (19)</td>
<td>0.087</td>
</tr>
</tbody>
</table>
One hundred and sixty-five patients had a carbapenem susceptible *Acinetobacter* of which 40 (24%) had *A. baumannii* (Table 2.7). Of those with *A. baumannii* infection, 17 (43%) were considered to have acquired it in hospital compared to 17 (14%) with non-*baumannii* (p<0.001). There was a higher proportion of resistance to 3GC in *A. baumannii* (85%) compared to non-*baumannii* (25%) with significantly more deaths among patients with *A. baumannii* (45%) than non-*baumannii* (15%) (p<0.001). The odds for in-patient mortality were higher in patients with *A. baumannii* (OR: 3.71, 95% CI: 1.33 – 10.32; p=0.012) and in those with a hospital acquired infection (OR: 3.4, 95% CI: 1.41 – 8.17; p=0.006) however resistance to 3GC was not associated with risk of death in a multiple logistic model.

Table 2.7 Demographic and clinical characteristics of patients admitted at Kilifi County Hospital with carbapenem susceptible- *Acinetobacter* spp isolated from clinical specimens collected from 2007 to 2020.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>A. baumannii</em> (N=40)</th>
<th>Other <em>Acinetobacter</em> (N=125)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16 (40)</td>
<td>84 (67)</td>
<td>0.003</td>
</tr>
<tr>
<td>Female</td>
<td>24 (60)</td>
<td>41 (33)</td>
<td></td>
</tr>
<tr>
<td><strong>Age group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 month</td>
<td>18 (45)</td>
<td>37 (30)</td>
<td></td>
</tr>
<tr>
<td>1–23 months</td>
<td>13 (33)</td>
<td>40 (32)</td>
<td></td>
</tr>
<tr>
<td>24–59 months</td>
<td>2 (5)</td>
<td>16 (13)</td>
<td>0.348</td>
</tr>
<tr>
<td>5–17 years</td>
<td>3 (7)</td>
<td>17 (13)</td>
<td></td>
</tr>
<tr>
<td>≥18 years</td>
<td>4 (10)</td>
<td>15 (12)</td>
<td></td>
</tr>
<tr>
<td><strong>Infection</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Healthcare associated</td>
<td>17 (42.5)</td>
<td>17 (14)</td>
<td></td>
</tr>
<tr>
<td>Community associated</td>
<td>23 (57.5)</td>
<td>108 (86)</td>
<td></td>
</tr>
<tr>
<td><strong>3GC</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-susceptible</td>
<td>34 (85)</td>
<td>31 (25)</td>
<td></td>
</tr>
<tr>
<td>Outcome</td>
<td>In-hospital fatality</td>
<td>18 (45)</td>
<td></td>
</tr>
</tbody>
</table>

2.3.1.1 Carbapenem resistant *P. aeruginosa*

There were eight patients infected with a carbapenem resistant *P. aeruginosa* (CRPA) of which five (62.5%) were children less than 5 years while among carbapenem susceptible *P. aeruginosa* (CSPA) 66 (79%) were less than five (Table 2.8). Majority of the infections in patients with CRPA were acquired in
hospital (75%) while among those with a susceptible strain it was community acquired (65%). A high rate of in-patient mortality was observed for pseudomonal infections regardless of resistance (63% in CRPA and 67% in CSPA).

Table 2.8 Demographic and clinical characteristics of patients admitted at Kilifi County Hospital with carbapenem resistant (CRPA) and susceptible (CSPA) *Pseudomonas aeruginosa* isolated from clinical specimens.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CRPA (N=8)</th>
<th>CSPA (N=83)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>5 (62.5)</td>
<td>43 (52)</td>
</tr>
<tr>
<td>Female</td>
<td>3 (37.5)</td>
<td>40 (48)</td>
</tr>
<tr>
<td><strong>Age group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 month</td>
<td>0 (0)</td>
<td>27 (32.5)</td>
</tr>
<tr>
<td>1 – 23 months</td>
<td>2 (25)</td>
<td>27 (32.5)</td>
</tr>
<tr>
<td>24 – 59 months</td>
<td>3 (37.5)</td>
<td>12 (14)</td>
</tr>
<tr>
<td>5 – 17 years</td>
<td>0 (0)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>≥ 18 years</td>
<td>3 (37.5)</td>
<td>13 (16)</td>
</tr>
<tr>
<td><strong>Infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthcare associated</td>
<td>6 (75)</td>
<td>29 (35)</td>
</tr>
<tr>
<td>Community associated</td>
<td>2 (25)</td>
<td>54 (65)</td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-hospital fatality</td>
<td>5 (62.5)</td>
<td>55 (67)</td>
</tr>
</tbody>
</table>

2.3.1.4  **Overall clinical and demographic characteristics of patients with carriage of carbapenem resistant organism (carriage CRO)**

During the FLACSAM study period in Kilifi, Nairobi and Mombasa, a total of 1351 children participated in the trial while 1299 children in the parallel cohort of non-malnourished children of which 24 (1.8%) and 20 (1.5%) respectively had an imipenem non-susceptible strain isolated from rectal swabs. Resistance was confirmed in 32 isolates from 31 patients of which 24 were *E. coli*, 6 *K. pneumoniae* and 1 *Enterobacter cloacae* and 1 *Citrobacter freundii*. Majority of the isolates were isolated from patients in Mombasa (20) while Kilifi had the least (4).
Majority of the study participants were male in all sites with a median age of 9 (IQR: 7 – 12) months in Nairobi and 11 months in Kilifi and Mombasa (IQR: 6 – 18.5 and 8 – 17.5 respectively) (Table 2.9). Lower respiratory tract infections and gastroenteritis were the common clinical diagnosis in participants. Eighteen participants (58%) had a history of previous hospital admission with 16 (89%) admitted within six months prior to recruitment in the study. Only five (16%) patients died in hospital, 1 in Nairobi and 4 in Mombasa. There was no significant difference between the different sites.

Table 2.9 Demographic and clinical characteristics of malnourished and non-malnourished patients admitted to three county hospitals with carbapenem resistant Enterobacterales bacteria isolated from rectal swabs collected from 2017 to 2019.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Kilifi (N=4) n (%)</th>
<th>Nairobi (N=7) n (%)</th>
<th>Mombasa (N=20) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3 (75)</td>
<td>5 (71)</td>
<td>15 (75)</td>
</tr>
<tr>
<td>Female</td>
<td>1 (25)</td>
<td>2 (29)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 – 23 months</td>
<td>3 (75)</td>
<td>6 (86)</td>
<td>17 (85)</td>
</tr>
<tr>
<td>24 – 59 months</td>
<td>1 (25)</td>
<td>1 (14)</td>
<td>2 (10)</td>
</tr>
<tr>
<td>5 – 12 years</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Clinical indication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>low respiratory tract infection (LRTI)</td>
<td>3 (75)</td>
<td>7 (100)</td>
<td>12 (60)</td>
</tr>
<tr>
<td>malnutrition</td>
<td>1 (25)</td>
<td>3 (43)</td>
<td>10 (50)</td>
</tr>
<tr>
<td>gastroenteritis</td>
<td>1 (25)</td>
<td>3 (43)</td>
<td>9 (45)</td>
</tr>
<tr>
<td>Prior admission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>1 (25)</td>
<td>4 (57)</td>
<td>8 (40)</td>
</tr>
<tr>
<td>&lt; 1 month</td>
<td>1 (25)</td>
<td>1 (14)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>1 – 6 months</td>
<td>2 (50)</td>
<td>1 (14)</td>
<td>7 (35)</td>
</tr>
<tr>
<td>&gt; 6 months</td>
<td>0 (0)</td>
<td>1 (14)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In hospital mortality</td>
<td>0 (0)</td>
<td>1 (14)</td>
<td>4 (20)</td>
</tr>
</tbody>
</table>

2.3.2 Antimicrobial susceptibility patterns to commonly known and newer drugs

Clinical carbapenem resistant Enterobacterales were resistant to nearly all commonly used antimicrobials except for aminoglycosides (gentamicin and amikacin) and chloramphenicol in E. coli.
(Figure 2.6 A & B). Interestingly, the strains were resistant to newer drugs currently not available in Kenya including to cefiderocol, a novel siderophore cephalosporin active against bacteria with MBL. Likewise, *Acinetobacter* spp were resistant to all commonly available treatment options with only amikacin and ciprofloxacin retaining activity against less than 40% of the other *Acinetobacter* spp (Figure 2.6 C & D). Unlike in Enterobacterales, eravacycline and cefiderocol were active against *Acinetobacter* spp as well as cefepime / sulbactam in other *Acinetobacter* spp. In contrast, more than half (67%) of *P. aeruginosa* strains showed susceptibility to the few anti-pseudomonal options commonly available including meropenem. As expected, the *Pseudomonas* isolates were intrinsically resistant to eravacycline which is closely related to tigecycline. Among the newer drugs tested, *Pseudomonas* isolates were susceptible to cefiderocol only with high percentage of resistance to meropenem / vaborbactam (78%) and imipenem / relebactam (100%) (Figure 2.4 E). None of the clinical strains were resistant to colistin.
Figure 2.6 Antimicrobial susceptibility profile of carbapenem resistant bacteria from KCH patient clinical samples as tested by disc diffusion.

A) *E. coli* (n=4) and B) *K. pneumoniae* (n=7) were non-susceptible (resistant and intermediate) to several antibiotics including newer agents and agent / inhibitor combinations. C) A few *A. baumannii* (n=20) were non-susceptible to eravacycline and cefiderocol while D) non-*baumannii Acinetobacter* (n=21) were susceptible to eravacycline. E) *P. aeruginosa* (n=9) had no resistance to cefiderocol. Resistance to colistin, as tested by MIC, was not observed in any of the organisms.

Antibiotics: ampicillin (AMP), ampicillin / clavulanate (AMC), cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), imipenem (IMI), meropenem (MEM), ertapenem (ETP), gentamicin (CN), amikacin (AK), ciprofloxacin (CIP), chloramphenicol (C), cotrimoxazole (SXT), colistin (COL), eravacycline (ERV), cefiderocol (FDC), cefepime-sulbactam (FPS), imipenem / relebactam (IMR), meropenem / vaborbactam (MEV)

Among matched carbapenem susceptible Enterobacterales, while half were resistant to third generation cephalosporin and ciprofloxacin, they were generally resistant to ampicillin, ampicillin / clavulanate and co-trimoxazole (Figure 2.7). Among the newer drugs, the isolates were susceptible to carbapenem / β-lactam inhibitors combinations with less than 30% of *E. coli* and *K. pneumoniae* showing resistance to cefiderocol. While 25% of *E. coli* were resistant to eravacycline and 70% of *K. pneumoniae* were resistant to it.
Figure 2.7 Antimicrobial susceptibility profile of carbapenem susceptible Enterobacterales from KCH patient clinical samples as tested by disc diffusion.

A) *E. coli* (n=6) had high rates of resistance to ampicillin and cotrimoxazole and some resistance to ciprofloxacin while B) *K. pneumoniae* (n=12) had considerable resistance to ampicillin / clavulanate and cotrimoxazole. Some resistance was exhibited to new agents or agent/inhibitor combinations especially to cefpime / sulbactam.

Carriage Enterobacterales were resistant to commonly known drugs with more than half of *E. coli* susceptible to amikacin and chloramphenicol. Only one *K. pneumoniae* showed resistance to colistin (Figure 2.8).
2.3.3 Mechanism of carbapenem resistance – phenotypic

Carbapenemase activity was detected in all but three of all the carbapenemase resistant Enterobacterales of which one each had ESBL, AmpC and one with no detectable β-lactamase activity (Table 2.10). Among clinical strains all but one (K. pneumoniae) had metallo-β-lactamases (MBL) activity while serine based β-lactamases (SBL) were detected in 6 (19%) of carriage strains. MBL only was detected in 57/83 (69%) of isolates with any carbapenamase activity while 23 (28%) and 1 (1%) had SBL only and MBL and SBL activity respectively. Only one P. aeruginosa isolate had a carbapenamase activity (MBL) while half of A. baumannii had MBL activity and 7 (33%) of non- baumannii had SBLs.

Some of the bacteria that were resistant by ertapenem were susceptible by imipenem and/or meropenem and on comparing MIC, the gold standard with disc diffusion for the detection of carbapenem resistant strains, discordant results were observed when using meropenem for nine isolates, six with imipenem and one with ertapenem (Figure 2.9). Discordance occurred mainly among SBL and non-carbapenemase
producing strains and among a few MBLs which also tended to be sensitive when tested using meropenem or imipenem MIC and therefore ertapenem was more sensitive in detecting low level carbapenemase producing and / or carbapenem resistance compared with meropenem or imipenem. Three bacteria that lacked either carbapenemase activity had either extended spectrum β-lactamase (ESBL), cephalosporinase over production (AmpC) or no β-lactamase activity tested.

Table 2.10 Phenotypic detection of β-lactamase activity in carbapenem resistant bacteria

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>MBL activity</th>
<th>SBL</th>
<th>MBL &amp; SBL</th>
<th>ESBL only</th>
<th>AmpC only</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. baumannii</strong> (n=20)</td>
<td>0 (0)</td>
<td>10 (50)</td>
<td>10 (50)</td>
<td>0 (0)</td>
<td>-</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Acinetobacter spp (n=21)</td>
<td>1 (5)</td>
<td>13 (62)</td>
<td>7 (33)</td>
<td>0 (0)</td>
<td>-</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Clinical Enterobacterales (n=11)</td>
<td>0 (0)</td>
<td>10 (91)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Carriage Enterobacterales (n=32)</td>
<td>1 (3)</td>
<td>23 (72)</td>
<td>6 (19)</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong> (n=9)</td>
<td>8 (89)</td>
<td>1 (11)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>-</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Carbapenemase activity was detected by modified carbapenemase inactivation method (Enterobacterales and *P. aeruginosa*) and Triton Hodge test (all strains). MBL was detected by EDTA supplemented carbapenemase inactivation method (Enterobacterales) and imipenem – EDTA (all strains). ESBL was detected by cephalosporin / cephalosporin – clavulanate test (Enterobacterales) while AmpC overproduction was detected by MAST AmpC detection kit (all strains).

Figure 2.9 β-lactamase activity in Enterobacterales (clinical and carriage isolates).
Comparison between minimum inhibitory concentration (MIC) and disc diffusion for imipenem (A), meropenem (B) and ertapenem (C).
Meropenem and imipenem MIC done using agar dilution method while ertapenem MIC by Etest strips. Dotted lines along the x-axis represent breakpoints, with resistant isolates to the left, sensitive to the right and intermediate in the middle while breakpoint lines along the y-axis separate sensitive isolates at the bottom, resistant at the top and intermediate in the middle.
Carbapenemase activity by Triton Hodge test (THT) was detected in 18 (90%) A. baumannii and 19 (90%) Acinetobacter spp. Among A. baumannii, SBL only was detected in 10 strains while MBL was detected in 10 strains. Alternatively, among other Acinetobacter, SBL was detected in 7 strains and MBL in 13 strains. In two strains carbapenemase activity was not detected by THT though one had MBL detected by Imipenem – EDTA test and the other was negative for all β-lactamases tested. Discordance between MIC and disc diffusion was not observed when testing using imipenem though it was observed in four strains with MBL activity when using meropenem (Figure 2.10)
Carbapenamase activity (MBL) was detected in only one *P. aeruginosa* strain: this was the only isolate that was not susceptible to meropenem when tested by MIC (Figure 2.11).
Figure 2.11 β-lactamase activity in *P. aeruginosa*.
Comparison between MIC and disc diffusion for imipenem (A) and meropenem (B).

2.4 Discussion

Carbapenems are minimally used in Kenyan public hospitals (Maina et al., 2020) due to cost and availability as they are currently classified in the reserve group of antibiotics yet they are readily accessible in private healthcare settings (Kizito et al., 2018). At KCH, a rural public hospital, carbapenems were introduced for treatment of infections resistant to all other available treatment options in 2020 yet, as my study shows, carbapenem resistant bacteria have been isolated from patient samples prior to this. Additionally, carriage studies demonstrate the presence of carbapenem resistant bacteria in the gut of individuals in three counties in Kenya even with limited usage of the drugs. This implies widespread use of the drug could result in a selection pressure that drives up the incidence of carbapenem resistant infections in the country, leaving patients with infections for which there are no available antimicrobials.

More than half of the infections were in children less than two years of age whose immune system is still developing and are therefore prone to infections resulting in misuse and over use of antibiotics in this group with implications for resistance (Romandini et al., 2021). Lower respiratory tract infections and sepsis were the leading patient presenting syndromes while resistant bacteria were isolated mainly from bloodstream and urinary tract infections. This reflects that the resistant bacteria were hospital acquired and were not related to the original presenting syndrome. Apart from non-`baumannii` *Acinetobacter*, infections caused by other species were predominantly considered hospital acquired with a longer median length of stay prior to isolation of the organisms compared to infections with susceptible strains. This was expected as longer duration of hospitalizations has been reported as one of the risk factors for infection with carbapenem resistant bacteria (Stewardson et al., 2019, Priyendu et al., 2016, Palacios-Baena et al., 2021).

There was no difference in the proportion of mortality between carbapenem resistant and susceptible *Enterobacterales* with no association between resistant bacteria and outcome in multivariate analysis and this lack of difference is attributed to the small numbers. A larger sample size would enable a detection in the differences in mortality between patients infected with resistant versus susceptible bacteria but it is important to note that other factors such as appropriateness of antibiotics given and presenting time to hospital will also have an effect (Dramowski et al., 2021). However, it is important to point out however, that the study was not designed to provide comparative patient cohort but rather a comparative bacteria for downstream *in vitro* analyses and were exploratory only and, while there was
no significant difference in baseline patient characteristics (age, gender etc), patients were not matched for presenting complaint or comorbidities. The observation of lack of mortality differences is interesting, but a larger sample size is needed to investigate this. However, it is likely that patients with susceptible infections may not be receiving adequate treatment either due to widespread resistance to first line drugs (Leopold et al., 2014, Tadesse et al., 2017) or late presentation to hospitals by patients who do not receive timely treatment, or patients receiving substandard medications compromising their treatment. While my analysis reports a significant difference in the crude in-patient mortality between third generation cephalosporin resistant and susceptible Enterobacterales infections, bias due to co-morbidity or other underlying factors were not factored which could affect the results observed. Indeed, a recently published retrospective study reported that third generation cephalosporin resistance had no impact on mortality in Africa, a study in which KCH was one of the participating centres (Dramowski et al., 2021). The study attempted to reduce confounding and bias due to underlying disease and competing events by adjusting for matched un-infected controls prior to comparison of resistant and susceptible bacteria. However, the authors suggest that early appropriate antibiotic treatment is an important modifier on the impact of AMR on clinical outcome, and this data was not obtained for their study and could have an impact on the results. It is therefore still unclear on the impact of AMR especially in LMIC settings.

There was no difference in in-patient mortality between carbapenem resistant \textit{A. baumannii} and other \textit{Acinetobacter} and this likely to be due to the small numbers which could not enable a detection of a difference between the two groups. A significant difference in the in-patient mortality between patients with carbapenem susceptible \textit{A. baumannii} and other \textit{Acinetobacter} was observed but as explained earlier these differences could change if co-morbidities and underlying factors were factored in the analysis. Previous studies have reported that \textit{A. baumannii} infections are more likely to be hospital acquired with a higher case fatality than other \textit{Acinetobacter} infections (Wareham et al., 2008, Park et al., 2013, Wisplinghoff et al., 2012), however, there is still debate about whether infection with \textit{A. baumannii}, carbapenem resistant or not, is independently associated with mortality or is simply a marker for underlying disease severity in patients, with colonization reflecting the prolonged hospitalisation and use of broad spectrum antimicrobials. This indicates that identification of \textit{A. baumannii} from other \textit{Acinetobacter} is clinically important in the management of patients in order to reduce mortality. MALDI-TOF was highly specific and sensitive in identifying \textit{A. baumannii} in my study and therefore, where available, could easily be adopted for routine diagnostic laboratories compared to molecular techniques such as PCR (Gözalan et al., 2020), however it high installation cost will be challenge for implementation in Kenya and many LMICs with limited resources for public healthcare.
There was a high in-patient mortality for patients infected with *P. aeruginosa* (more than 60%) regardless of carbapenem sensitivity which was not surprising given that it is difficult to treat and is intrinsically resistant to many of the first line drugs. Inappropriate antibiotic therapy and clinical severity have been identified as some of the risk factors for mortality with pseudomonal infections (Montero et al., 2020). Thus, the rapid identification of pseudomonal infection and timeliness of effective therapy for at risk individuals, who may have underlying conditions, could reduce the high mortality associated with this organism.

Carbapenem resistant Enterobacterales are usually resistant to most other antibiotics making infections by such bacteria difficult to treat. This was observed in this both clinical and carriage isolates in the study. The case was similar for *A. baumannii*, however other Acinetobacter spp showed some sensitivity to amikacin and ciprofloxacin. Only a third of *P. aeruginosa* strains were resistant to the commonly known anti-pseudomonal drugs suggesting that prompt care and keen monitoring of patients with such infections may reduce the negative impact of infection. In 2017 the World Health Organization (WHO) published a list of priority pathogens for which new antibiotics were urgently needed (Tacconelli et al., 2018). Since then new drugs albeit within existing antibacterial classes have been approved for use in difficult to treat infections with no alternatives. One such drug, cefiderocol, a novel siderophore cephalosporin, utilizes the iron transport channel to gain access to the bacteria due to its catechol moiety (Ito et al., 2017). Its structure also makes it stable against the activity of most β-lactamases including MBLs and is effective against the WHO’s critical priority pathogens. Other drugs tested include carbapenems combined with novel β-lactamase inhibitors, relebactam and vaborbactam (Zhanel et al., 2018) and eravacycline, a novel fluorocycline similar to tigecycline (Sutcliffe et al., 2013).

Currently, these drugs are not available in Kenya and are not part of the Essential Medicines lists and thus it was surprising that the strains, especially the clinical Enterobacterales, were resistant to them, including to cefiderocol which is reported to be relatively stable to the action of MBLs. Most Acinetobacter species and *P. aeruginosa* were susceptible to cefiderocol indicating that the drug could be used for such strains. Carriage of strains with reduced susceptibility to cefiderocol has been reported elsewhere in paediatric population where the drug is not approved for use (Mariani et al., 2021). Given that a few of the carbapenem susceptible clinical Enterobacterales were also resistant to cefiderocol it suggests that a proportion of the bacteria already possess resistance mechanisms towards this new drug which has been hypothesized to be due to existing resistance mechanisms to β-lactams or within the iron transport system utilised for the uptake of the drug into the bacteria (Hackel et al., 2017). The
isolates, both susceptible and resistant, showed considerable resistance to eravacycline and this may be as a result of existing mechanisms or other modifications targeting tetracycline family compounds (Langford et al., 2020). Resistance towards carbapenem / β-lactamase inhibitor combination is expected given that many of the strains possess MBLs which are unaffected by β-lactamase inhibitors. These findings indicate that there is reduced potential for these new drugs should they be introduced in the region and susceptibility testing of new drugs should be routine particularly for Enterobacterales.

Carriage carbapenem resistant Enterobacterales showed similar susceptibility trends to clinical strains indicating a source for resistant determinants for the group. Only one isolate (K. pneumoniae) was resistant to colistin as well as carbapenems and was found in the gut of a 9-month-old non-malnourished child admitted to KCH. The colistin resistant bacteria was still present in the gut of the patient at discharge. There is limited data on colistin use and resistance in the country, however a study showed that the drug was mainly purchased for veterinary use rather than human use (Muloi et al., 2019) and it is possible that the original source of the strain was animal and not human. There has only been one previously reported case of a colistin resistant strain in Kenya, from a hospitalized patient with hip wound infection in Nairobi. This isolate possessed the plasmid borne mcr8 (Kyany’a and Musila, 2020), but was carbapenem susceptible.

A comparison of the gold standard, MIC, and disc diffusion showed that ertapenem was better at detecting low MIC carbapenem resistant isolates especially among those with serine-β-lactamases (SBLs), AmpC overexpression, ESBL or other resistance mechanism while meropenem was the least even among Acinetobacter and P. aeruginosa. There was an abundance of MBLs (69%) compared to SBL (28%) among the resistance strains with SBLs detected mainly in Acinetobacter species and among carriage isolates in Enterobacterales. Data on characterization of carbapenemases is limited in Africa however in east Africa, there are more members of MBLs detected in carbapenem resistant strains than SBLs except in A. baumannii in Uganda (Ssekatawa et al., 2018). Given the proximity of the region to the Indian sub-continent it is expected that MBLs would be abundant in the region however additional surveillance data is required to confirm this. Interestingly, carbapenemase activity was detected in only one P. aeruginosa isolate which was MBL. This points to carbapenem resistance in the other isolates being mediated by efflux pump or loss of porins and given that the isolates were generally susceptible to meropenem it is highly likely that resistance is due to mutation in porin (oprD gene) which is seen in imipenem resistant / meropenem susceptible P. aeruginosa while overexpression of efflux pump is likely in imipenem susceptible / meropenem resistant strains (Pragasam et al., 2016).
The are several limitations in this study, and the most important is the low sample size of resistant bacteria which could not enable an effective comparison with susceptible bacteria. Secondly, while the study protocols in KCH ensure consistent sampling at admission, there are no standardised criteria for post-admission blood cultures, which may mean that the true burden of carbapenem resistance is underestimated, as culture are not consistently performed for those with lack of improvement or deteriorating prognosis. Third, antibiotic use prior to admission, a risk factor for infection with resistant bacteria, was not available as was data on antibiotic treatment on some of the patients as this is an effect modifier of clinical outcome. Low blood volumes in paediatric populations and prior antibiotic use could result in low sensitivity of blood cultures in isolation of bacteria. Co-morbidities and underlying factors such as HIV, TB and malnutrition was not factored in mortality analysis and could affect the results obtained.

In conclusion, patients with carbapenem resistant bacteria are likely to acquire the infection in hospital. There was no difference in the outcome of patients which may be due to small numbers, or poor treatment options for susceptible infections, or fitness cost of multi-drug resistance in bacteria leading them to cause less severe infections. The strains show reduced susceptibility to newer drugs which have only been recently approved for clinical use and which are not yet available in Kenya, which could potentially compromise their usefulness when introduced in the market. MBLs were the main resistance mechanism detected. In the next chapter, I will outline the molecular detection of resistance determinants and correlate the findings with the phenotypic results.
3 Genomic characterization of carbapenem resistant bacteria

**Key points**

- $\textit{Bla}_{\text{NDM}}$ was the dominant carbapenemase gene detected by PCR with $\textit{bla}_{\text{NDM}-1}$ as the major variant. $\textit{Bla}_{\text{OXA-23}}$ was the main oxacillinase in \textit{Acinetobacter} spp.
- $\textit{Bla}_{\text{KPC}}, \textit{bla}_{\text{VIM}}$ and $\textit{bla}_{\text{IMP}}$ genes were not detected in any of the carbapenem resistant strains.
- Diverse sequence types and resistance traits were detected in the sequenced isolates implying multiple introduction events of resistant bacteria.
- Presence of a gene may not necessarily confer resistance to a drug as was observed in the case of amikacin, ciprofloxacin and cotrimoxazole and variations in the functional regions around the gene may affect the expression or activity of the product and needs to be considered in WGS analysis.

3.1 Introduction

Genomic characterization, especially whole genome sequencing (WGS), is emerging as a crucial technique for understanding the transmission dynamics of infectious pathogens. For example, WGS has been able to explain the history of cholera introduction into Africa and the reason that the epidemics have persisted in the continent for three decades (Weill et al., 2017). Likewise, WGS solved the mystery of cholera outbreak introduction in Haiti in 2010 by Nepalese troops sent to assist the country after it was affected by a devastating earthquake in the same year (Orata et al., 2014). Infection with MDR bacteria poses a challenge in healthcare as there are limited options for treatment of infected individuals, it is therefore important to track such bacteria in as near real time as possible to avoid hospital wide outbreaks that could have serious implications (Guducuoglu et al., 2018, Snitkin et al., 2012, Lee et al., 2020). Genomics has also enabled the understanding of the spread of important resistance determinants globally (Logan and Weinstein, 2017, Pitout et al., 2005) and the easier identification of novel mutations or genes associated with treatment failure in patients (Klein et al., 2021, Shi et al., 2020).

Currently there are several technologies available for whole genome sequencing and the cost of sequencing a single bacterium has reduced considerably over the last two decades. Sequencing technologies are categorized into short read sequencing which sequence DNA fragments of up to 500 bp at one go and long read sequencing which can sequence fragments size of up to 10 kbp at one go. Short read sequencing is performed by first generation sequencing machines such as the Sanger sequencing...
machine and the next generation (or second generation) sequencing instruments such as Solexa, Ion Torrent and Illumina’s MiSeq and HiSeq. Long read sequencing technologies include the PacBio instrument and the portable Oxford Nanopore instruments (Besser et al., 2018). In LMICs, genomic analysis is still a challenge mainly due to the high cost of purchase and installation of sequencing technologies, consistent supply of reagents due to procurement issues and the high cost of shipment of the organisms for sequencing in HICs if preferred. Additionally, many LMICs have limited skilled bioinformaticians for the in-depth analysis of sequence data, although there some free web based sequence analysis tools such as those by the Centre for Genomic Epidemiology (http://www.genomicepidemiology.org/) that provide information such as the sequence type, plasmids, resistance and virulence genes present as well as presence of mutations conferring resistance with little or no technical skills.

In Kenya, very few studies have reported on the genetic basis of carbapenem resistance in Gram negative bacteria with the early reports of carbapenemase genes from isolates from patients admitted at a large tertiary public hospital in Nairobi. These were the first published reports of \textit{bla}\textsubscript{NDM} in \textit{K. pneumoniae} and \textit{A. baumannii}, \textit{bla}\textsubscript{OXA-23} in \textit{A. baumannii} (Poirel et al., 2011a, Revathi et al., 2013), \textit{bla}\textsubscript{VIM} in \textit{P. aeruginosa} (Pitout et al., 2008) and \textit{bla}\textsubscript{SPM} in \textit{K. pneumoniae} (Maina et al., 2017) identified by PCR. A recent review on carbapenem resistance in East Africa region suggests that carbapenem resistance and its genes are more prevalent than previously estimated (Ssekatawa et al., 2018) and more data is required to determine its true prevalence and potential implications. Remarkably, there have been no further studies that have described the distribution of carbapenemase genes in Kenya until recently (Musila et al., 2021). In the paper, they described carbapenemase genes in 27 \textit{A. baumannii}, 2 \textit{E. cloacae}, 3 \textit{E. coli}, 2 \textit{K. pneumoniae} and 14 \textit{P. aeruginosa} isolated from skin and soft tissue infections and urinary tract infections from patients admitted in six hospitals from 2015 to 2018 in five counties: Nairobi, Kismu, Kisihi, Kericho and Kilifi. They identified \textit{bla}\textsubscript{NDM-1} in all strains except \textit{E. coli} which had \textit{bla}\textsubscript{NDM-7}, \textit{bla}\textsubscript{VIM-1} (1) and \textit{bla}\textsubscript{VIM-6} (2) in \textit{P. aeruginosa} with the only \textit{bla}\textsubscript{OXA-48-like} identified was \textit{bla}\textsubscript{OXA-181} in \textit{K. pneumoniae}. In \textit{A. baumannii}, \textit{bla}\textsubscript{OXA-23} was the main oxacillinase gene with only one \textit{bla}\textsubscript{OXA-58} gene identified.

In this chapter, I describe the molecular mechanism of carbapenem resistance in all strains and the specific resistance determinants, sequence types, virulence genes and plasmid types found in some selected clinical and carriage strains. All isolates were screened for commonly known carbapenemases
by PCR and based on the results and AMR profile, some clinical and carriage strains underwent WGS by Illumina MiSeq.

3.2 Methodology

3.2.1 Detection of carbapenemase genes

I looked for the presence of some of the commonly known carbapenemase genes by PCR using previously published primers: \textit{bla}$_{\text{KPC}}$, \textit{bla}$_{\text{NDM}}$, \textit{bla}$_{\text{OXA-48}}$, \textit{bla}$_{\text{VIM}}$, \textit{bla}$_{\text{IMP}}$ (Poirel et al., 2011b) \textit{bla}$_{\text{OXA-23}}$, \textit{bla}$_{\text{OXA-24}}$, \textit{bla}$_{\text{OXA-51}}$ and \textit{bla}$_{\text{OXA-58}}$ (Woodford et al., 2006) and were performed in three multiplex assays (Table 3.1). \textit{Bla}$_{\text{OXA-51-like}}$ are chromosomally encoded oxacillinases present in \textit{A. baumannii} usually used to distinguish the species from other members of the genus but they do not necessarily confer resistance to carbapenems (Coelho et al., 2006) (chapter 2). Bacteria were cultured on blood agar plates at 35°C ± 2°C overnight and DNA extracted by boiling 2 µl loopful of bacteria in 1X Tris EDTA (TE) buffer at 100°C for 10 min. The supernatant was separated by centrifugation at 13000 rpm for 10 min. Target gene amplification was done using Multiplex PCR kit (Qiagen, UK) according to manufacturer’s instruction. Each reaction comprised 1X PCR Master Mix, 0.2 µM concentration of each primer and 2 µl of template DNA in a final volume of 25 µl. DNA was amplified in a thermocycler (Veriti, Applied Biosystems, USA) under the following cycling conditions: 95°C for 15 min, followed by 30 amplification cycles of 94°C for 30 s, 57°C for 90 s, and a final extension of 72°C for 10 min. Amplified DNA fragments were separated on a 2% agarose gel with Red Safe staining (Intron Biotechnology, South Korea) at 100 V for one hour in 1X Tris Borate EDTA buffer (TBE) (Sigma, USA) and visualized under UV.

<table>
<thead>
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<th>Primer</th>
<th>Sequence (5' – 3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
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<td></td>
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<tr>
<td>\textit{bla}$_{\text{VIM}}$ F</td>
<td>GATGGTGTTTGGTCGCATA</td>
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</tr>
<tr>
<td>R</td>
<td>CGAATGCGCAGCACCAG</td>
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<tr>
<td>\textit{bla}$_{\text{IMP}}$ F</td>
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<td>232</td>
</tr>
<tr>
<td>R</td>
<td>GGTTAAAYAAAAACAACCACC</td>
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</tr>
<tr>
<td>Multiplex 2</td>
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<td>\textit{bla}$_{\text{NDM}}$ F</td>
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</table>
3.2.3 Whole genome sequencing

Isolates were exported to the UK with the intention to WGS at MicrobesNG (https://microbesng.com/), and facilitated through Queen Mary University of London. However, due to the COVID-19 pandemic and collaborator illness, only one batch was completed and, the rest of the samples were sequenced at the KEMRI Wellcome trust Research Programme (KWTRP) on the MiSeq instrument (Illumina, USA) by a full-time experienced sequencing team. I included all invasive carbapenem resistant Enterobacterales and some selected Acinetobacter species, *P. aeruginosa* with extensive drug resistance including to newer drugs and some carriage Enterobacterales for whole genome sequencing. In addition, three *E. coli* strains, two carbapenem resistant (from CSF and blood) and one carbapenem susceptible (from CSF) from a meningitis patient were sequenced as it was a unique case of hospital acquired infection (detailed review of the patient in chapter 5). I also included a carbapenem resistant environmental *A. baumannii* for comparison with *A. baumannii* clinical strains.

3.2.3.1 Sequencing at KWTRP

DNA was extracted using Quick-DNA™ Miniprep Kit (Zymo research, USA) where bacteria were cultured on blood agar plates at 35°C ± 2°C overnight and 2 µl loopful of bacteria suspended in 200 µl of 1X TE buffer. 800 µl of genomic lysis buffer was added to the sample and the mixture incubated at room temperature for five minutes. The DNA was separated from the lysate by centrifugation at 10,000 x g for one minute. The bound DNA was washed twice with 200 µl of DNA pre-wash buffer and 500 µl of g-DNA buffer.
wash buffer respectively at 10,000 rcf for one minute each. DNA was eluted using 80 µl of DNA elution buffer heated to 70°C and incubated at room temperature for five minutes and centrifuged at 15,000 x g for 30 s. DNA was quantified using Qubit DNA quantification assay (Invitrogen, USA). Briefly, the Qubit working solution was prepared by diluting the Qubit reagent 1:200 in Qubit buffer enough for two standards and the samples. The assay was prepared by mixing 10 µl of each standard with 190 µl of working solution and 1 µl of each sample with 199 µl of working solution and incubated at room temperature for two min. The standards, used to generate a standard curve, and samples were read using the DNA quantification assay on a Qubit 2.0 fluorometer.

DNA was sequenced using the Illumina DNA Prep sequencing kit on a MiSeq Next generation sequencer as per instructions (Illumina, USA). Briefly, 15 µl of DNA at a total concentration of 100 – 500 ng was mixed with 10 µl of mixed equal volumes of bead-linked transposomes and tagmentation buffer 1 in a well and run in the thermocycler at 55°C for 15 min. The reaction was cleaned by mixing 5 µl of tagment stop buffer with the tagmentation reaction and heated in a thermocycler at 37°C for 15 min and the supernatant discarded. The beads were washed twice with 50 µl of tagment wash buffer and the tagmented DNA mixed with 10 µl of PCR master mix comprising of equal volumes of enhanced PCR mix and nuclease free water and 5 µl of 96 plex pre-paired i7 and i5 index adapters. The DNA was amplified in a thermocycler with the following conditions: 68°C for 3 min, 98°C for 30 min followed by 12 cycles of 98°C for 45 s, 62°C for 30 s and 68°C for 2 min.

The libraries were cleaned by adding 20 µl of nuclease free water and 22.5 µl of sample purification beads to supernatant in each well and shaken at 1600 rpm for one minute and incubated at room temperature for five minutes. In a clean well, 62.5 µl of the mixture was added to 7.5 µl of undiluted sample purification beads and incubated at room temperature for five minutes. The supernatant was discarded, and the beads washed twice with 80% ethanol and air dried before 16 µl of resuspension buffer was added and incubated at room temperature for three minutes. Fifteen microliters of the supernatant were transferred into a new well and the libraries normalized to 4 nM concentration using Qubit or bioanalyzer and 5 µl of each library pooled together. The libraries were denatured using 5 µl of 0.2N sodium hydroxide and centrifuged at 280 x g at 20°C for one minute and incubated at room temperature for five minutes. The concentration of the denatured pool was reduced to 10 pM by the addition of 990 µl of HT1 buffer to the mix and the further dilution of 300 µl of the mix with 300 µl of HT1 buffer. Finally, 48 µl of 8% PhiX control at the same concentration as the pool was added to 552 µl
of the pool and incubated at 96 for two minutes and on ice for at least five minutes before it was loaded into the MiSeq reagent kit and run on the sequencer

3.2.3.2 Sequencing at MicrobesNG

At MicrobesNG sequencing was performed using Illumina short read sequencing platform with 2X250 bp paired end reads ([https://microbesng.com/microbesng-faq/](https://microbesng.com/microbesng-faq/)).

3.2.4 Data analysis

3.2.4.1 Sequencing data from KWTRP

I assessed the quality of the raw reads assessed using FastQC ([https://www.bioinformatics.babraham.ac.uk/projects/fastqc/](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) and confirmed species identity using Center for Genomic Epidemiology ([http://www.genomicepidemiology.org/](http://www.genomicepidemiology.org/)) KmerFinder 3.2 (Clausen et al., 2018, Larsen et al., 2014). Low quality reads of less than 20 score and adapter regions were trimmed using Trimmomatic v.0.39 (Bolger et al., 2014) and the multilocus sequence type (MLST) identified using MLST 2.0 (Larsen et al., 2012) using data obtained from PubMLST ([https://pubmlst.org/](https://pubmlst.org/)) (Jolley et al., 2018). In cases where two different typing scheme databases are available, both sequence types were obtained. Typing was according to schemes by Institut Pasteur ([https://bigsdb.pasteur.fr/](https://bigsdb.pasteur.fr/)) for *A. baumannii* (Diancourt et al., 2010), *E. coli* (Jaureguy et al., 2008) and *K. pneumoniae*; PubMLST for *A. baumannii* (Bartual et al., 2005) and *P. aeruginosa* and Enterobase ([https://enterobase.warwick.ac.uk/](https://enterobase.warwick.ac.uk/)) (Zhou et al., 2020) for *E. coli* (Wirth et al., 2006). The presence and type of plasmids in *E. coli* and *K. pneumoniae* was detected using PlasmidFinder 2.1 (Carattoli et al., 2014, Clausen et al., 2018). The presence of chromosomal and acquired resistance genes in all species, and point mutations associated with drug resistance in Enterobacteriales only were detected using ResFinder 4.1 (Bortolaia et al., 2020, Clausen et al., 2018, Zankari et al., 2017). Virulence genes in *E. coli* were detected using VirulenceFinder 2.0 (Joensen et al., 2014). *E. coli* serotypes were identified using SerotypeFinder 2.0 (Joensen et al., 2015). The above analysis was performed using default settings of the various databases, namely 60% minimum length coverage and 90% identification threshold (95% for PlasmidFinder and 85% for SerotypeFinder). KmerResistance 2.2 was also used to search for resistance genes in raw reads that would otherwise be missed by ResFinder (Clausen et al., 2016, Clausen et al., 2018). I imported the trimmed reads into Geneious Prime® 2022.0.1 and performed *de-novo* assembly using SPAdes assembler 3.15.2 (Prijibelski et al., 2020). Assembled genomes of *K. pneumoniae* were uploaded into Pathogenwatch ([https://pathogen.watch/](https://pathogen.watch/)) and were also analysed using Kleborate (Lam et al., 2021) to
identify resistance and virulence determinants and to type capsular polysaccharide (K) and lipopolysaccharide (O) antigens using Kaptive (Wyres et al., 2016).

3.2.4.2 Sequence data from MicrobesNG

All raw sequence reads were trimmed using Trimmomatic (Bolger et al., 2014) and the quality assessed using inhouse scripts and Samtools (Li et al., 2009), BedTools (https://bedtools.readthedocs.io/en/latest/) and BWA mem (Li and Durbin, 2009). The reads were assembled de-novo using SPAdes (Prjibelski et al., 2020) and were also assembled using a reference genome using Kraken (Wood and Salzberg, 2014) and BWA mem (Li and Durbin, 2009). All further analysis of the reads and assembled genomes was performed as outlined in section 3.2.4.1.

3.3 Results

3.3.1 blaNDM is the commonly detected carbapenemase gene

I detected the presence of blaNDM in all resistant organisms except P. aeruginosa, blaOXA-48 in Enterobacterales and blaOXA-23 and blaOXA-58 in Acinetobacter species. I did not detect blaKPC, blaVIM and blaIMP in either of the species or blaOXA-24 in Acinetobacter species. The most frequently identified gene was blaNDM detected in 31/61 (51%) and 23/32 (72%) of the clinical and carriage isolates respectively (Table 3.2). For 12 strains, including all nine of P. aeruginosa isolates, none of the carbapenemase genes tested were detected despite MBL activity detected by phenotypic in one Pseudomonas. It is likely that another MBL not tested by PCR could be explain the resistance depicted by the single P. aeruginosa with enzymatic activity, while non-enzymatic based resistance mechanisms such as efflux pumps or altered porin could explain resistance in the other Pseudomonas strains. In 10 (50%) A. baumannii more than one carbapenemase (blaNDM and blaOXA-58) were detected together and where blaOXA-23 was detected (nine strains) it was always in isolation.

In non baumannii Acinetobacter, 12 (57%) had multiple genes with blaOXA-58 detected together either with blaOXA-23 (5) or blaNDM (5), and in 2 strains, all the three genes were detected. In nine non baumannii, either blaNDM (4) or blaOXA-23 (5) was present alone. Two strains had detectable MBL activity but none of the MBLs tested were detected while one strain was negative by Triton Hodge test but had blaOXA-23 and blaOXA-58 by PCR.

Among Enterobacterales, 10/11 clinical strains had blaNDM. Interestingly, the oxacillinase most commonly found in Enterobacterales, blaOXA-48, was observed only in six carriage isolates. None of the genes tested
for were present in two *K. pneumoniae* and one *E. coli* which was consistent with phenotypic results where they had ESBL, AmpC and no beta-lactamase activity respectively.
Table 3.2 Distribution of carbapenemases as detected by PCR

|                   | No gene | $\text{bla}_{\text{NDM}}$ | $\text{bla}_{\text{OXA-48}}$ | $\text{bla}_{\text{OXA-23}}$ | $\text{bla}_{\text{OXA-58}}$ | $\text{bla}_{\text{NDM}}$ & $\text{bla}_{\text{OXA-48}}$ | $\text{bla}_{\text{NDM}}$ & $\text{bla}_{\text{OXA-58}}$ | $\text{bla}_{\text{OXA-23}}$ & $\text{bla}_{\text{OXA-58}}$ | $\text{bla}_{\text{OXA-23}}$ & $\text{bla}_{\text{OXA-58}}$ |
|-------------------|---------|---------------------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| **Clinical isolates** |         |                           |                             |                             |                             |                               |                               |                               |                               |                               |                               |
| A. baumannii (n=20) | 0 (0)   | 0 (0)                     | -                           | 9 (45)                      | 1 (5)                       | 10 (50)                       | 0 (0)                         | 0 (0)                         |                               |                               |                               |
| Acinetobacter (non-baumannii) (n=21) | 0 (0)   | 4 (19)                    | -                           | 5 (24)                      | 0 (0)                       | 5 (24)                        | 5 (24)                        | 2 (9)                         |                               |                               |                               |
| E. coli (n=4)      | 0 (0)   | 4 (100)                   | 0 (0)                       | -                           | -                           | 0 (0)                         | -                             | -                             |                               |                               |                               |
| K. pneumoniae (n=7) | 1 (14)  | 6 (86)                    | 0 (0)                       | -                           | -                           | 0 (0)                         | -                             | -                             |                               |                               |                               |
| P. aeruginosa (n=9) | 9 (100) | 0 (0)                     | -                           | -                           | -                           | -                             | -                             | -                             |                               |                               |                               |
| **Carriage isolates** |         |                           |                             |                             |                             |                               |                               |                               |                               |                               |                               |
| E. coli (n=24)     | 1 (4)   | 18 (75)                   | 4 (17)                      | -                           | -                           | 1 (4)§                        | -                             | -                             |                               |                               |                               |
| K. pneumoniae (n=6) | 1 (17)  | 3 (50)                    | 2 (33)                      | -                           | -                           | 0 (0)                         | -                             | -                             |                               |                               |                               |
| Others* (n=2)      | 0 (0)   | 1 (50)                    | 1 (50)                      | -                           | -                           | 0 (0)                         | -                             | -                             |                               |                               |                               |
| Others* include 1 E. cloacae and 1 C. freundii |

§strain likely possess a $\text{bla}_{\text{OXA-48}}$ only; (-) indicates not tested
3.3.2 Carbapenem non-susceptible strains possess an array of resistance genes against many antibiotics

Thirty-two strains were selected for sequencing: eight *A. baumannii*, three *P. aeruginosa*, nine *E. coli*, nine *K. pneumoniae* and three *Acinetobacter* species. Two *K. pneumoniae* and one *A. baumannii* failed the sequencing run in Kilifi. The sample distribution of the sequenced strains were blood (9), urine (6), pus (5), rectal (5), CSF (2), pleural aspirate (1), hospital environmental (1) isolated from patients aged one day to 71 years old (Table 3.3). These isolates were selected based on cost and its role in spread of resistance genes. All the clinical Enterobacterales were chosen in order to investigate their potential role in spreading infection in the hospital while the rest of the carriage and clinical isolates were chosen based on the antibiogram profile to represent a diversity.

Table 3.3 Characteristics of patients of from whom bacterial strains were sequenced

<table>
<thead>
<tr>
<th>Sequence Centre</th>
<th>County</th>
<th>Sex</th>
<th>Age at admission</th>
<th>Hospital outcome</th>
<th>Specimen</th>
<th>Carbapenem susceptibility</th>
<th>Collection date</th>
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<tr>
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<td></td>
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<tr>
<td>K_ABA1</td>
<td>KWTRP</td>
<td>Kilifi</td>
<td>F</td>
<td>23 months</td>
<td>died</td>
<td>blood</td>
<td>NS</td>
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<tr>
<td>K_ABA2</td>
<td>KWTRP</td>
<td>Kilifi</td>
<td>F</td>
<td>1 day</td>
<td>alive</td>
<td>urine</td>
<td>NS</td>
</tr>
<tr>
<td>K_ABA3</td>
<td>KWTRP</td>
<td>Kilifi</td>
<td>F</td>
<td>5 years</td>
<td>alive</td>
<td>pus swab</td>
<td>NS</td>
</tr>
<tr>
<td>K_ABA4†</td>
<td>KWTRP</td>
<td>Kilifi</td>
<td>M</td>
<td>1 day</td>
<td>alive</td>
<td>pus swab</td>
<td>NS</td>
</tr>
<tr>
<td>K_ABA5†</td>
<td>KWTRP</td>
<td>Kilifi</td>
<td>M</td>
<td>1 day</td>
<td>alive</td>
<td>pus swab</td>
<td>NS</td>
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<td>KWTRP</td>
<td>Kilifi</td>
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<td>n/a</td>
<td>n/a</td>
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</tr>
<tr>
<td>K_ABA7</td>
<td>KWTRP</td>
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<td>F</td>
<td>5 years</td>
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<td>NS</td>
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<td><strong>Other Acinetobacter spp</strong></td>
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</tr>
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<td>MicrobesNG</td>
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<td>NS</td>
</tr>
<tr>
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<td>MicrobesNG</td>
<td>Kilifi</td>
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<td>3 months</td>
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<td>blood</td>
<td>NS</td>
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</tr>
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### E. coli

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<th>City</th>
<th>Sex</th>
<th>Age</th>
<th>Status</th>
<th>Sample Type</th>
<th>Susceptibility</th>
<th>Date</th>
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<tr>
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<td>KWTRP</td>
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### K. pneumoniae

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* * isolates from the same patient with meningitis; † isolates from the same patient with skin infection

NS indicates non-susceptible and S indicates susceptible
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<th>Antibiotic</th>
<th>A. baumannii</th>
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<th>E. coli</th>
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</table>

**Table 3.4 Antimicrobial susceptibility profile of sequenced isolates**

- **A. baumannii**
  - K_ABA1: AMP<1, AMC18, FOX24, CTX31, CAZ12, CRO<1, MEM6, ETP6, CN<1, AK29, CIP21, SXT16, COL12, ERV16, FDC16, FPS16, IMR16, MEV16
  - K_ABA2: AMP<1, AMC24, FOX14, CTX14, CAZ14, CRO<1, MEM19, ETP24, CN19, AK24, CIP12, SXT9, COL19, ERV12, FDC9, FPS9, IMR9
  - K_ABA3: AMP<1, AMC6, FOX6, CTX6, CAZ6, CRO6, MEM16, ETP6, CN6, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6
  - K_ABA4: AMP<1, AMC19, FOX24, CTX24, CAZ12, CRO<1, MEM6, ETP6, CN6, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6
  - K_ABA5: AMP<1, AMC6, FOX>32, CTX>32, CAZ32, CRO<1, MEM6, ETP6, CN6, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6
  - K_ABA6: AMP<1, AMC24, FOX14, CTX14, CAZ14, CRO<1, MEM19, ETP24, CN19, AK24, CIP12, SXT9, COL19, ERV12, FDC9, FPS9, IMR9
  - K_ABA7: AMP<1, AMC21, FOX19, CTX19, CAZ19, CRO<1, MEM19, ETP21, CN19, AK21, CIP19, SXT9, COL19, ERV19, FDC19, FPS19, IMR19

- **Other Acinetobacter spp**
  - K_ACN1: AMP<1, AMC20, FOX15, CTX15, CAZ18, CRO4, MEM<1, ETP20, CN27, AK16, CIP16, SXT16, COL16, ERV16, FDC16, FPS16, IMR16
  - K_ACN2: AMP<1, AMC20, FOX10, CTX10, CAZ15, CRO4, MEM<1, ETP20, CN27, AK16, CIP16, SXT16, COL16, ERV16, FDC16, FPS16, IMR16
  - K_ACN3: AMP<1, AMC20, FOX10, CTX10, CAZ15, CRO4, MEM<1, ETP20, CN27, AK16, CIP16, SXT16, COL16, ERV16, FDC16, FPS16, IMR16

- **P. aeruginosa**
  - K_PAE1: AMP<1, AMC6, FOX6, CTX6, CAZ6, CRO6, MEM<1, ETP6, CN6, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6
  - K_PAE2: AMP<1, AMC6, FOX4, CTX4, CAZ6, CRO6, MEM<1, ETP6, CN6, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6
  - K_PAE3: AMP<1, AMC6, FOX6, CTX6, CAZ6, CRO6, MEM<1, ETP6, CN6, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6

- **E. coli**
  - K_ECO1: AMP6, AMC6, FOX6, CTX6, CAZ6, CRO6, MEM<1, ETP6, CN7, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6
  - K_ECO2: AMP6, AMC6, FOX6, CTX6, CAZ6, CRO1.5, MEM<1, ETP6, CN6, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6
  - K_ECO3: AMP6, AMC6, FOX6, CTX6, CAZ6, CRO32, MEM<1, ETP6, CN6, AK6, CIP6, SXT6, COL6, ERV6, FDC6, FPS6, IMR6
  - K_ECO4: AMP15, AMC22, FOX29, CTX29, CAZ6, CRO6, MEM<1, ETP20, CN29, AK20, CIP20, SXT22, COL20, ERV18, FDC25, FPS35, IMR29, MEV33
  - K_ECO5: AMP6, AMC20, FOX15, CTX15, CAZ6, CRO6, MEM<1, ETP18, CN15, AK18, CIP18, SXT16, COL16, ERV16, FDC26, FPS31, IMR33
All antimicrobial susceptibility determined by disc diffusion method except imipenem, meropenem, ertapenem and colistin which were determined by minimum inhibitory concentration.

(*) indicates not tested by MIC but by disc diffusion; (-) indicates not tested; red indicates resistance, orange indicates intermediate (CLSI) or increased dosage (EUCAST) and green indicates sensitive to drug.

Antibiotics: ampicillin (AMP), ampicillin/clavulanate (AMC), cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), imipenem (IMI), meropenem (MEM), ertapenem (ETP), gentamicin (CN), amikacin (AK), ciprofloxacin (CIP), chloramphenicol (C), cotrimoxazole (SXT), colistin (COL), eravacycline (ERV), cefiderocol (FDC), cefepime/sulbactam (FPS), imipenem/relebactam (IMR), meropenem/vaborbactam (MEV)
3.3.2.1 Sequence types (STs)

Species identity by KmerFinder 3.2 was concordant with MALDI-TOF identification. *A. baumannii* strains belonged to four STs in the Pasteur scheme: ST32 (K_ABA1), ST25 (K_ABA2, 4 and 5), ST164 (K_ABA3) and ST1 (K_ABA 6 and 7) which corresponded to STs 930, 229, 1418 and 405 respectively in the PubMLST scheme (Table 3.5). For K_ABA3, there was a 24 bp gap in the housekeeping gene *recA* used in both schemes and the STs identified are the nearest match. The two ST 405 strains were unique in that they possessed two variants of the *gdhB* locus, allele 4 and 182 resulting in two different STs: ST405 and 2323. This has previously been reported that in some *A. baumannii* isolates the second allele, 182 and its variants, is often annotated at *gdhB2* and has low affinity for primers for amplification of MLST regions, and the *gdhB* variant, allele 4, was used to assign ST405 to the isolates (Gaiarsa et al., 2019).

Though all sequenced *A. baumannii* were non-susceptible to commonly known drugs, except to ceftazidime and ciprofloxacin in K_ABA1 and to amikacin in K_ABA7, there were differences in susceptibility to newer drugs where tested (Table 3.4). For example, K_ABA2 and 4 are both ST25 isolated from patients in 2011 and 2019 respectively, both isolates had similar AST profile to common drugs but while K_ABA4 was susceptible to all the newer drugs, K_ABA2 was susceptible to eravacycline only. Epidemiologically, the two patients with ST25 were neonates admitted to HDU, one born in the maternity ward (K_ABA2) and the other at a health centre (K_ABA4 and 5).

ST1 strains included a strain isolated from a reception swab sample of the children’s ward in July 2019 (K_ABA6) and from a patient admitted to the high dependency unit (HDU) in Dec 2019 (K_ABA6). The strains had similar AST profile to common drugs except amikacin where K_ABA6 was resistant and K_ABA7 was susceptible. While the environmental swab was not tested against the panel of newer drugs K_ABA7 showed susceptibility to cefiderocol only.

The three *P. aeruginosa* belonged to STs 357 for K_PAE1, and possibly 2343 for K_PAE3 (*mutL* gene had a 19 bp gap). Strain K_PAE2 could not be assigned an existing ST and is novel due to the combination of the allele profiles with the *mutL* variant (116) not observed with the other allelic profiles. K_PAE1 and 2 were sensitive to meropenem while K_PAE3 was intermediate to meropenem. K_PAE2 was sensitive to ceftazidime and both aminoglycosides while K_PAE3 was sensitive to amikacin only. All strains were susceptible to cefiderocol and non-susceptible to imipenem-relebactam and meropenem-vaborbactam apart from K_PAE2 to the latter drug.
The *E. coli* strains represented 7 STs in the Pasteur scheme: ST 471 (K_ECO1), ST692 (K_ECO2 and 3), ST74 (K_ECO4), ST43 (K_ECO5), ST 2 (K_ECO6 and 7), ST635 (K_ECO9) and a novel ST (K_ECO8) due to mutation in *polB* and combination of allelic profiles. These corresponded to sequence types in the Enterobase scheme as ST410 (K_ECO1, 2 and 3), STs 88 (K_ECO4), 131 (K_ECO5), 167 (K_ECO6 and 7), 617 (K_ECO8) and 448 (K_ECO9). The Pasteur scheme has eight alleles; two of the *E. coli* strains, K_ECO2 and 3 had gaps in one or more of the alleles and have been ascribed to the nearest most likely ST. However, the Enterobase scheme uses different alleles and only a single isolate had a gap in one allele resulting in more confidence in sequence type determination.

For one patient, two distinct STs were isolated within 30 days of each other. This was an adult patient who was admitted with meningitis: initial CSF culture grew a carbapenem-susceptible *E. coli* strain ST88 (Enterobase) (K_ECO4) for which he received inpatient treatment. After an initial improvement he developed meningitis again, this time both the CSF sample and a blood culture sample grew a carbapenem MDR strain, ST167 (K_ECO6 and 7) resistant even to newer drugs (case report described in detail in chapter 5).

Isolates K_ECO1, 2 and 3 had the same ST by Enterobase (410) but two distinct types by Pasteur (471 and 692), the first was a clinical isolate from urine sample in KCH in 2013 while the latter two were carriage isolates from patients in Nairobi (K_ECO2) and Mombasa (K_ECO3) county isolated in 2018 and 2019 respectively. The carriage strains had similar AST profile except to imipenem, meropenem and chloramphenicol where K_ECO2 was susceptible. K_ECO1 was susceptible only to meropenem, amikacin and colistin.

*K. pneumoniae* were represented with a diversity of strains: ST101 (K_KPN1 and 2), possibly ST38 (K_KPN3), ST147 (K_KPN4), ST231 (K_KPN5), ST 307 (K_KPN6) and ST530 (K_KPN6). For K_KPN3, there was a 40 bp gap in *rpoB* gene and is ascribed the nearest ST. K_KPN1 was isolated from a urine sample of a 21-month-old child admitted to the HDU in March 2015 while K_KPN2 was from a seven-year-old patient admitted to the general paediatric in April 2015. Both strains were of the same sequence type and had similar AST profiles even to newer drugs suggesting similarity. Despite the diverse STs among the isolates, they were all MDR to commonly used and newer drugs but were sensitive to colistin except K_KPN5, a carriage isolate from a 9-month-old patient in Kilifi (MIC 16 µg/ml).
3.3.2.2 Carbapenemases and other 6-lactamases

An array of plasmids, in Enterobacterales, and resistance factors were detected in the strains which is unsurprising given the MDR nature of the strains (Table 3.5). Carbapenemase genes were detected in all but two *E. coli* and two *P. aeruginosa* strains that were carbapenem non-susceptible. While it was expected for the strains *K*. *Eco8*, *K*. *Pae1* and 2 as it lacked carbapenemase activity, it was surprising for *K*. *Eco9* as *blaNDM* was detected by PCR. Thirty-eight different carbapenemases were detected, within the MBL family these were *blaNDM*-1, *blaNDM*-5, *blaNDM*-7, *blaNDM*-17 and *blaDIM*-1. Among the class D oxacillinases were: *blaOXA-23* and *blaOXA-225* of the *blaOXA-23*-like family; *blaOXA-58* and *blaOXA-420* of the *blaOXA-58*-like family, *blaOXA-91*, *blaOXA-64*, *blaOXA-69* and *blaOXA-100* of the *blaOXA-51*-like family; *blaOXA-50*, *blaOXA-395* and *blaOXA-48* of the *blaOXA-50*-like family, and *blaOXA-181* and *blaOXA-232* of the *blaOXA-48*-like family (Table 3.6). Oxacillinases were the most abundant carbapenemases, found in each species including the naturally occurring oxacillinases *blaOXA-51*-like and *blaOXA-50*-like in *A. baumannii* and *P. aeruginosa* respectively.

*blaNDM*-1 was present in at least one isolate from each species except in *P. aeruginosa* while *blaNDM*-5 and *blaNDM*-7 were found in Enterobacterales. A *blaNDM*-17 was identified in *K*. _ABA6_ which was isolated from the reception of the paediatriic ward reception. The isolate was ST1 similar to *K*. _ABA7_ isolated from a patient in the same ward within the same year and at 79% coverage of the *blaNDM*-17 template used, it is likely that the NDM is of a different variant than identified. An MBL previously not reported in Kenya, *blaDIM*-1 referred to as Dutch imipenemase was identified in *P. aeruginosa* (*K*. _PAE3_) which had displayed carbapenemase activity unlike the other carbapenem non-susceptible *P. aeruginosa*. Notably, *K*. _PAE1_ and 2 had the naturally occurring oxacillinases, *blaOXA-50*-like, though did not display any carbapenemase activity. The two carriage *K. pneumoniae* (*K*. _KPN 3_ and 5) and the two carriage *E. coli* (*K*. _ECO2_ and 3) had *blaOXA-232* and *blaOXA-181* respectively. By PCR and phenotypic testing, *K*. _ECO3_ had both *blaNDM* and *blaOXA-48* but by sequencing the strain had *blaOXA-181* in its genome which was surprising, however during PCR the isolate was placed next to an isolate which possessed *blaNDM* and it is highly likely that cross-contamination occurred during the PCR process resulting in detection of the MBL.

For cephalosporinas, *blaADC-25* were detected in all *A. baumannii*, *blaPAO* in all *P. aeruginosa* and *blaCMY* variants in 4/9 isolates *E. coli*. A variety of extended spectrum β-lactamases (ESBLs) were identified and were specific for the different species with *blaPER*-7 in three *A. baumannii* and *blaVEB*-1 in one

_Acinetobacter_ and a novel *blaPAU* in *P. aeruginosa*. *blaCTX-M-15* was the dominant ESBL found in 6/7 _K. pneumoniae_ and 6/9 *E. coli* while other variants detected were *blaCTX-M-10*, *blaCTX-M-71* in *E. coli* and *blaCTX-M-11* in _K. pneumoniae_. *BlatEM* was detected in 10 Enterobacteriales strains (3 *blaTEM-1A*, 3 *blaTEM-1B* and
\textbf{bla}^{\text{TEM-1D}} \text{ in } K. \textit{pneumoniae} \text{ and } 3 \textbf{bla}^{\text{TEM-1B}} \text{ in } E. \textit{coli}. \text{ All } K. \textit{pneumoniae} \text{ strains had variants of the } \textbf{bla}_{\text{SHV}} \text{ and had more than one ESBL gene present as did three } E. \textit{coli} \text{ strains.}

Phenotypic susceptibility testing results for \(\beta\)-lactams was generally concordant with AMR gene results, discordance was observed for K_ABA1 and K_ACN2 which was susceptible to ceftazidime despite the presence of oxacillinases. K_ECO2 was susceptible to imipenem and meropenem despite detection of \textbf{bla}_{\text{OXA-181}} \text{ and this contrasted with K_ECO3 which despite having the same gene was resistant to all three carbapenems.}

3.3.2.3 \textit{Other resistance genes and point mutations}

A variety of aminoglycoside (AG) modifying enzyme genes were detected in all but one \textit{E. coli} (K_ECO4) and included variants of AG \(N\)-acetyltransferases (AACs), AG \(O\)-nucleotidytransferases (ANTs) and AG \(O\)-phosphotransferases (APHs) (Table 3.5). Most of the genes detected though could not confer resistance to amikacin in many isolates and gentamicin in \textit{E. coli} as they conferred resistance to other aminoglycosides not tested. While \textit{aac(6')-Ib-cr} was predicted to confer resistance to amikacin, the presence of the gene alone did not confer resistance to the drug. Pan-aminoglycoside resistance conferred by 16S ribosomal RNA methyltransferase genes were detected in ST25s \textit{A. baumannii} (Pasteur), and in \textit{K. pneumoniae}, ST101s with \textit{armA}, ST231 with \textit{rmtF} and ST530 with \textit{rmtB}.

Sulphonamide resistance genes (\textit{sul1/2}) were present in all isolates except one of each species while trimethoprim resistance genes (\textit{dfrA} variants) were present in all Enterobacterales except a single \textit{E. coli} (K_ECO4) and \textit{K. pneumoniae} (K_KPN4) and was only present in one non-\textit{baumannii} and one \textit{P. aeruginosa} and. Sulphamethoxazole-trimethoprim (SXT) susceptibility testing was conducted in Enterobacterales only and K_ECO4 and K_KPN4, both with complete \textit{sul1/2} genes were susceptible to the drug. Chloramphenicol resistance genes (\textit{floR}, \textit{cmlA1} or \textit{catA/B}) were lacking in one \textit{A. baumannii}, four \textit{E. coli} and two \textit{K. pneumoniae}. Seven \textit{E. coli} isolates were susceptible to chloramphenicol and in three, a \textit{catB3} gene was identified though an incomplete coverage of the template suggesting an incomplete protein with no definitive function in resistance.

Tetracycline resistance genes (\textit{tetA/B/D/39}) were present in nearly all isolates and in isolates where eravacycline testing was done and were resistant, \textit{tetA} gene was present in \textit{A. baumannii} and \textit{E. coli} and \textit{tetD} in \textit{K. pneumoniae}, though two eravacycline resistant \textit{K. pneumoniae} strains (K_KPN4 and 6) did not have any \textit{tet} gene. Isolate K_KPN7 had a point mutation in \textit{acrR} (F204L) gene that confers resistance to tigecycline though it was susceptible to eravacycline indicating that the mutation is drug specific.
The new fluoroquinolone resistance gene, crpP, was identified in K_PAE1 and 3 however this did not translate phenotypically even in the presence of aac(6’)-Ib-cr in K_PAE1. All ciprofloxacin resistance Enterobacterales had multiple resistant determinants: aac(6’)-Ib-cr (except K_ECO6, 9 and K_KPN5), qnrS1/B1 (present in two E. coli and five K. pneumoniae), mutations in gyrA (S83L, D87N), parC (S80I) and parE (S458A, I529L) in E. coli strains and mutations in gyrA (S83I, F or Y and 87G or A), parC (80I) and acrR (P161R, G164A, F172S, R173G, L195V, F197I, K201M) in K. pneumoniae. Isolate K_KPN7 had reduced susceptibility to ciprofloxacin and though it possessed aac(6’)-Ib-cr and only two mutations in acrR gene (G164A, P161R) instead of the seven present in other strains and no mutations in gyrA or parC and may account for the differences in the susceptibility. Six A. baumannii isolates were resistant to ciprofloxacin though no acquired resistance markers were reported by ResFinder or KmerResistance.

Phenotypic resistance to colistin was observed in K. pneumoniae K_KPN5, no known acquired resistance gene or mutation was identified using ResFinder, however there were gene changes in mgrB and pmrB detected by Kleborate that may result in colistin resistance. Mutations were also detected in mgrB in K_KPN3 and pmrB in K_KPN2 predicted to confer resistance to colistin though the isolates were phenotypically sensitive.

Six K. pneumoniae had point mutations in the gene coding for outer membrane protein ompK37 resulting in resistance to carbapenems and in 4/7, there were additional mutations in the ompK36 gene that reduces susceptibility to cephalosporins. Carbapenem MIC values of K_KPN7 were lower than other species despite the presence of blaNDM-5 and this may be a result of no mutations in ompK37 gene.

3.3.2.4 Plasmids detected in Enterobacterales

On average there were 4.2 plasmids per each Enterobacterales with a minimum of one plasmid in K_ECO4 and 9 and a maximum of seven plasmids in K_KPN1 and 2 (Table 3.5). The dominant plasmids were IncF present in all isolates except K_ECO4 and 8 and included 12 IncFIA, 9 IncFIB and 10 IncFII. While IncFII (pAMA1167-NDM-5) plasmids were identified in E. coli carriage isolates from Nairobi and Mombasa (K_ECO2 and 3 respectively) none of the isolates had blaNDM in their genome, alternatively, IncFIB (pNDM-Mar) (as well as IncHI1B (pNDM-MAR)) was identified in K_KPN6 and blaNDM-7 was detected in the strain on sequencing. The second most abundant plasmids were the colicinogenic plasmids (Col) absent in K_ECO1 and K_KPN6 and 7. The replicons identified included Col(BS512), Col440I and II, ColKP3, ColpVC, Col(pHAD28), Col(8282), Col(MG828) and Col(156). IncR plasmids were present in four K. pneumoniae strains (K_KPN1, 2, 4 and 7). Other plasmids identified were IncC (1), IncV (1), IncHI1B (1), IncX3 (1) and IncB/O/K/Z (1).
Table 3.5 MLST, plasmid and resistance genes and resistance conferring mutations in sequenced isolates

<table>
<thead>
<tr>
<th>A. baumannii</th>
<th>MLST</th>
<th>Plasmids</th>
<th>Carbapenemase</th>
<th>Other β-lactamases</th>
<th>Antimicrobial resistance</th>
<th>Other resistance genes</th>
<th>Point mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_ABA1</td>
<td>32</td>
<td>930</td>
<td>OXA-420, OXA-100</td>
<td>ADC-25, CARB-5</td>
<td>ant(2'')-la, aph(3'')-lb</td>
<td>floR, sul2, mphE, msrE</td>
<td>cmlA1, su1/2, tetB, mphE, msrE, arr-2, qacE</td>
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<td>K_ABA2</td>
<td>25</td>
<td>229</td>
<td>OXA-23, OXA-64</td>
<td>PER-7, ADC-25</td>
<td>arma, aac(6')-lan, aac(3)-lla, aph(3')-lb, aph(6)-ld</td>
<td>cmlA1, su1/2, tetB, mphE, msrE, arr-2, qacE</td>
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<tr>
<td>K_ABA3</td>
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<td>?</td>
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<td>ADC-25, CARB-5</td>
<td>ant(2'')-la</td>
<td>tet39</td>
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<td>229</td>
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<td>25</td>
<td>229</td>
<td>OXA-23, OXA-64</td>
<td>PER-7, ADC-25</td>
<td>arma, aac(6')-lan, aac(3)-lla, aph(3')-lb, aph(6)-ld</td>
<td>cmlA1, su1/2, tetB, mphE, msrE, arr-2/3, qacE</td>
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<tr>
<td>K_ABA6</td>
<td>1</td>
<td>405</td>
<td>OXA-58, NDM-17, OXA-69</td>
<td>ADC-25</td>
<td>aac(3)-la; aph(3')-la; ant(3')-la; aph(3')-VI</td>
<td>catA1, su1, tetA, qacE</td>
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<tr>
<td>K_ABA7</td>
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<td>405</td>
<td>OXA-58, NDM-1, OXA-69</td>
<td>ADC-25</td>
<td>aac(3)-la; aph(3')-la; ant(3')-la; aph(3')-VI</td>
<td>catA1, su1, tetA, qacE</td>
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Other Acinetobacter species

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<thead>
<tr>
<th>Other Acinetobacter species</th>
<th>Plasmids</th>
<th>Carbapenemase</th>
<th>Other β-lactamases</th>
<th>Antimicrobial resistance</th>
<th>Other resistance genes</th>
<th>Point mutation</th>
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<tbody>
<tr>
<td>K_ACN1</td>
<td>OXA-225</td>
<td>VEB-1, CARB-2/5/16/49</td>
<td>aadA2b/A1, aph(3')-lb, aph(6)-ld, ant(2'')-la</td>
<td>sul1/2, dfrA15, floR, tet39/A/G,</td>
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<tr>
<td><strong>K_ACN2</strong></td>
<td>OXA-23, OXA-58</td>
<td>CARB-5/16/49</td>
<td>aph(3')-la, ant(2'')-la, aac(3)-lld, aph(3'')-lb, aph(6)-Id</td>
<td>mphE, msrE, qacE, tet39, sul2, floR, mphE, msrE</td>
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<td></td>
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<tr>
<td><strong>K_ACN3</strong></td>
<td>NDM-1, OXA-58, OXA-225</td>
<td>CARB-5/16/49</td>
<td>aph(3')-la, aac(3)-lld, aph(3'')-lb, aph(6)-Id, aph(3')-Vl</td>
<td>tet39, sul2, floR, msrE/A, mphE/C</td>
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</table>

**P. aeruginosa**

| K_PAE1 | 357 | OXA-50 | TEM-1B, PAU-1, PAO | aadA1, aph(3')-lb, aph(6)-Id, aac(6')-lb-cr, aac(6')-lb/3, aph(3')-llb, ant(3'')-la | catB7, crpP, su1, dfrA1, fosA, msrE, mphE/A, qacE |
| K_PAE2 | new | OXA-395 | PAO | aac(6')-lla, ant(2'')-la, aph(6')-Id, aph(3')-llb | catB7, fosA, catB7, cmlA1, crpP, su1, fosA, arr-2, qacE |
| K_PAE3 | 2343 | DIM-1, OXA-488 | PAO | | |

**E. coli**

| K_ECO1 | 471 | 410 | IncFIB, IncFIA, IncC | NDM-1 | CTX-M-101, CMY-140, OXA-1 | aadA5, aac(6')-lb-cr, ant(3'')-la, aph(3')-lb, aph(6)-ld | catA1, sul2, tetA, dfrA17, floR, mdfA, qacE |
| K_ECO2 | 692 | 410 | Col(B5S12), ColKP3, Col(MG828), IncFIA, IncFIB, IncFII(pAMA1167-NDM-5) | OXA-181 | CTX-M-15, TEM-1B, CMY-2, OXA-1 | aadA5, aac(6')-lb-cr, aac(3)-lld, aph(6)-Id, aph(3'')-lb | qnrS1, su1/2, dfrA17, tetB, mphA, mdfA |
| K_ECO3  | 692   | 410   | Col(BS512), IncFIA, IncFII(pAMA1167-NDM-5) | OXA-181 | CTX-M-15, TEM-1B, CMY-2, OXA-1 | aadA5, aac(6')-Ib-cr, aac(3)-Ild, aph(6)-Ild, aph(3'')-Ib, aac(3)-Ila | qnrS1, catA1, sul1/2, tetB, dfrA17, mphA, mdfA sul2, tetB, mdfA, sitABCD catB3, dfrA14, sul2, tetA, mdfA, sitABCD | ¥
| K_ECO4  | 74?   | 88    | Col8282 | Col(BS512), IncFII | CTX-M-15, OXA-1 | aac(6')-Ib-cr, aph(6)-Ild, aph(3'')-Ib | ¥
| K_ECO5  | 43    | 131   | Col(BS512), IncFIB, IncFIA, IncB/O/K/Z, IncFII | NDM-5 | CTX-M-15, OXA-1 | aac(6')-Ib-cr, aac(3)-Ila | ¥ except parE 1529L
| K_ECO6  | 2     | 167   | Col(BS512), ColpVC, IncFIA, IncFII | NDM-5 | CTX-M-15, OXA-1 | aadA2, aac(6')-Ib-cr, aac(3)-Ila | ¥
| K_ECO7  | 2     | 167   | Col(BS512), ColpVC, IncFIA, IncFII | NDM-5 | CTX-M-15, OXA-1 | aadA2, aac(6')-Ib-cr, aac(3)-Ila | ¥
| K_ECO8  | new   | 617   | Col440I, IncI (Gamma), IncFIA | NDM-1 | CTX-M-15, CMY-42, OXA-1, | aadA5, aac(6')-Ib-cr, aac(3'')-Ib, aph(6)-Ild | dfrA17, sul1/2, tetA, mphA, mdfA, qacE, sitABCD dfrA12, sul1, tetA, mphA, mdfA, qacE | ¥
| K_ECO9  | 635   | 448   | Col(156) | | CTX-M-71, TEM-1B, | aadA2 | | ¥

**K. pneumoniae**

<p>| K_KPN1  | 101   | Col(pHAD28), Col440I, Col440II, ColpVC, IncFIB(K), IncFII(K), IncR | NDM-1 | CTX-M-15, TEM-1A, SHV-11, OXA-9 | armA, aadA1, aac(6')-Ib, aac(3)-Ild, aac(6')-Ib-cr | catA1, tetD, qnrB1, fosA, dfrA12, sul1/2, msrE, gyrA (p.83Y, |
| K_KPN2  | 101 | Col(pHAD28), Col440I, Col440II, ColpVC, IncFIB(K), IncFII(K), IncR | NDM-1 | CTX-M-15, TEM-1A, SHV-11, OXA-9 | armA, adaA1, aac(6’)-lb, aac(3)-lfd, aac(6’)-lb-cr | catA1, tetD, qnrB1, fosA, dfrA12, su1/2, mrsE, mphE/A, OqxA/B, qacE | mphE/A, OqxA/B, qacE | p.87G; parC (p.80I) |
| K_KPN3  | 38? | Col440I, Col440II, ColKP3, IncFIB(K), IncFII(K), Col(MG828) | OXA-232 | CTX-M-15, TEM-1B, SHV-40, OXA-1 | aac(3)-lla, aac(6’)-lb-cr, aph(6)-Id, aph(3’)-Ib | catB3, qnrB1, dfrA14, sul2, tetA, fosA, gyrA (p.83F, p.87A); parC (p.80I); MgrB (83%) |
| K_KPN4  | 147 | IncR, IncFIB(pKPHS1), IncFIB(pQil), Col(pHAD28) | NDM-1 | CTX-M-15, TEM-1A, SHV-11 | aadA1, aac(6’)-lb-cr, aph(3’)-VI, aac(6’)-lb | sul1, fosA, qnrS1, arr-3, OqxA/B, qacE | gyrA (p.83I); parC (p.80I) |
| K_KPN5  | 231 | ColKP3, IncFII(K), IncFIA, Col440I | OXA-232 | CTX-M-15, TEM-1B, SHV-75 | rmtF, adaA2, aac(6’)-lb | catA1, dfrA12, tetB, fosA, arr-2, mphA, ermB, OqxA/B, qacE | gyrA (p.83I, p.87G); parC |</p>
<table>
<thead>
<tr>
<th>Strain</th>
<th>IncFIB(K)(pCAV1099-114), IncFIB(pNDM-Mar), IncHI1B(pNDM-Mar), IncX3</th>
<th>NDM-7</th>
<th>CTX-M-15, TEM-1D, SHV-28/100, OXA-1</th>
<th>aadA2, aac(3)-Ila, aac(6')-lb-cr</th>
<th>catA2/B3, qnrS1, fosA, dfrA12, sul1/2, mphA, OqxA/B, qacE</th>
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<tbody>
<tr>
<td>K_KPN6</td>
<td>307</td>
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<td></td>
<td></td>
<td>(p.80I); MgrB (36%); PmrB (36%)</td>
</tr>
<tr>
<td>K_KPN7</td>
<td>530</td>
<td>NDM-5</td>
<td>CTX-M-114, TEM-1B, SHV-99, OXA-1</td>
<td>rmtB, aadA2, aac(6')-lb-cr, aac(3)-Ila, aph(3’’)-lb, aph(6)-Id</td>
<td>dfrA12, sul1, mphA, ermB, OqxA/B, qacE</td>
</tr>
</tbody>
</table>

MLST databases: Pasteur (Pas), PubMLST (Pub) and Enterobase (Ent)

† presence of both *gdh*B and *gdh*B2, ST determined using *gdh*B

‡ mutations present in *gyr*A: S83L, D87N; *par*C S80I and *par*E S458A for fluoroquinolone resistance


¶ mutations present in *omp*K37: I70M, I128M conferring carbapenem resistance

¶ mutations present in *omp*K36: N49S, L59V conferring cephalosporin resistance
Table 3.6 Distribution of carbapenemase genes detected in non-susceptible strains

<table>
<thead>
<tr>
<th></th>
<th>Metallo-β-lactamases</th>
<th>Oxacillinases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NDM-1</td>
<td>NDM-5</td>
</tr>
<tr>
<td><strong>A. baumannii</strong></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Acinetobacter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>K. pneumoniae</strong></td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>
3.3.2.5  Serotypes and virulence genes

The *E. coli* strains represented different serotypes with only two strains, isolated from the same patient, having the same serotype, O101 H5 (K_ECO6 and 7) (Table 3.7). The diverse serotypes like the diverse STs indicates that the strains are unrelated and are independent introduction events in the hospital. Similarly, different capsular polysaccharide (KL) loci and lipopolysaccharide (O) loci were identified in the *K. pneumoniae* strains. Apart from K_KPN1 and 2 with the same loci, KL17 O1v1, all other strains were of different KL loci. All the strains were of the O1 or O2 loci except for K_KPN3 which was OL101.

Several genes involved in iron uptake and metabolism in *E. coli* were identified and included genes for aerobactin (*iutA, iucC*), enterobactin (*iroN*) and yersiniabactin (*fyuA*) with genes encoding for proteins involved in iron transport (*sitA*) and iron level regulation (*irp2*). In four strains (K_ECO2, 3, 6 and 7) the presence of virulence genes associated with iron uptake and metabolism were not detected. Only genes involved in yersiniabactin (*ybt*) were detected in six of seven *K. pneumoniae* strains. Other virulence genes detected in *E. coli* included genes involved in motility such as fimbriae and pili (*lpfA, papA* and *papC*), outer membrane protease (*ompT*), serum resistance and survival (*traT, iss*), haemolysin (*hylF*), bacterial adherence (*iha*) and toxin production (*sat*). The carbapenem susceptible *E. coli* strains K_ECO4 and 5 had more virulence genes than the carbapenem resistant strains.

Table 3.7 Serotypes and virulence determinants identified in Enterobacterales

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Virulence genes (iron uptake and metabolism)</th>
<th>Virulence genes (other)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_ECO1</td>
<td>O8 H21</td>
<td><em>iutA</em></td>
<td><em>lpfA</em></td>
</tr>
<tr>
<td>K_ECO2</td>
<td>O8 H9</td>
<td></td>
<td><em>lpfA</em></td>
</tr>
<tr>
<td>K_ECO3</td>
<td>H9</td>
<td><em>sitA, ireA, iroN, fyuA, iucC, iutA, irp2</em></td>
<td><em>lpfA, yfcV, hra, eilA</em></td>
</tr>
<tr>
<td>K_ECO4</td>
<td>O8 H17</td>
<td><em>fyuA, iucC, iutA, sitA, irp2</em></td>
<td>*lpfA, papC, etsC, ompT, papA_20, hra, tsh, cvaC, iss, traT, hlyF</td>
</tr>
<tr>
<td>K_ECO5</td>
<td>O25 H4</td>
<td></td>
<td>*papC, papA_F43, yfcV, hra, ompT, chuA, iss; iha, sat, usp</td>
</tr>
<tr>
<td>K_ECO6</td>
<td>O101 H5</td>
<td></td>
<td><em>hra, capU, iss, traT</em></td>
</tr>
</tbody>
</table>
3.4 Discussion

The importance of determining drug resistance and its mechanisms in a clinical setting cannot be stressed enough. Currently, the common practice for the detection of resistance in organisms has always been phenotypic with minimum inhibitory concentration (MIC) as the gold standard and disc diffusion method (preferred in low resource settings due to its affordability and low expertise requirements) or an automated broth dilution method (e.g. Vitek 2, BD Phoenix, Microscan) used for routine testing. While such methods are advantageous in that they provide quantitative or qualitative susceptibility results to guide clinicians and pharmacists, the major drawback is that they require the isolation of the organism and the total duration to results may be three to four days resulting in the use of empirical broad-spectrum antibiotics for treatment while results are awaited. At KWTRP, disc diffusion is the preferred drug susceptibility testing method though recent published data on antimicrobial susceptibility to many of the disease-causing bacteria is limited.

Through PCR, carbapenemases genes were detected in all isolates except three Enterobacterales and all *P. aeruginosa*. *Bla*<sub>NDM</sub> was the most commonly detected gene present in nearly all the Enterobacterales - clinical and carriage – tested. Despite the scarcity of published findings, it is known that *bla*<sub>NDM</sub> has been present in Kenya from as early as 2007 first identified in *K. pneumoniae* and later in a hospital outbreak of *A. baumannii* (Poirel et al., 2011a, Revathi et al., 2013). A whole genome sequencing study of invasive *K. pneumoniae* from blood and CSF from patients in Kilifi from 2001 to 2011 did not detect the presence...
of the gene, though a pNDM-mar-like plasmid backbone was identified in several isolates suggesting that the gene may have been present (Henson et al., 2017). In this study, the first \( \text{bla}_{\text{NDM}} \) clinical carrying strain in KCH was \( \text{E. coli} \) isolated from a urine sample in 2013, thereafter in non-\( \text{baumannii} \) Acinetobacter species before I detected it in a \( \text{K. pneumoniae} \) from urine in 2015. Given that invasive bacterial surveillance has been on-going at KCH since 1997 and the detection of the pNDM-mar-like plasmid backbone in several previous isolates, it is likely the gene carrying plasmids may have been introduced in Kilifi county region in the early 2000s from neighbouring regions or private facilities. However, the lack of carbapenem usage in the hospital may have resulted in the loss of the gene from plasmids in early years, but with the recent increased usage of third generation cephalosporins and carbapenems (in private facilities and tertiary hospitals), the environment is providing a low level selection pressure for maintenance of the gene. The fact that two-thirds of carriage strains from three different parts of the country carry \( \text{bla}_{\text{NDM}} \) suggests that the widespread use of carbapenems will provide a suitable environment for the proliferation of the gene in the country.

Interestingly, half of the Acinetobacter spp had more than one carbapenemase gene with 68% (15) of these having a \( \text{bla}_{\text{NDM}} \) which tended to occur with \( \text{bla}_{\text{OXA-58}} \). Co-occurrence of oxacillinase with an MBL is rare having been first reported in China (Zhou et al., 2015) and Malaysia (Ang et al., 2016) in \( \text{A. pittii} \) and in Vietnam (Tada et al., 2015) and Algeria (Ramoul et al., 2016) in \( \text{A. baumannii} \). In this study this was first seen in two non-\( \text{baumannii} \) isolated from blood and urine in 2013 and it was not until 2018 that it occurred in \( \text{A. baumannii} \) and has been the dominant resistant trait combination since. In two other Acinetobacter species \( \text{bla}_{\text{OXA-23}} \) and \( \text{bla}_{\text{OXA-58}} \) and \( \text{bla}_{\text{NDM}} \) occurred together and, to my knowledge, this is the first report of this and may only be a matter of time before this is observed in \( \text{A. baumannii} \) further complicating treatment of infected patients. \( \text{bla}_{\text{OXA-24}} \) was not observed in any of the Acinetobacter species which is not surprising given that previous studies in Kenya have not reported its presence either (Huber et al., 2014, Revathi et al., 2013, Musila et al., 2021). None of the genes tested were detected in \( \text{P. aeruginosa} \) which is unsurprising for all but one of the strains which was positive for MBL activity. It is likely that the other \( \text{P. aeruginosa} \) strains were resistant to imipenem by non-enzymatic activity either increased efflux pumps or diminished porin activity. Given the strains’ susceptibility to meropenem and resistance to imipenem, it is likely that mutations in the porin \( \text{oprD} \) gene may explain the findings as over-expression of efflux genes has been found to confer resistance to meropenem and susceptibility to imipenem in carbapenemase non-producing \( \text{P. aeruginosa} \) (Pragasam et al., 2016). Previous studies that examined \( \text{P. aeruginosa} \) in the country reported the presence of MBLs \( \text{bla}_{\text{VIM}} \) and \( \text{bla}_{\text{NDM}} \) (Musila et al.,
2021, Pitout et al., 2008) and it was surprising that none of the genes were detected in the one strain with enzymatic activity in Kilifi.

Whole genome sequencing revealed that the strains were of different sequence types and had diverse resistant traits which is suggestive of multiple introduction events of resistant bacteria in the hospital rather than one particularly successful clone. A few cases, however, had similar resistant genes and sequence types such as ST1 and 25 of *A. baumannii* and ST101 of *K. pneumoniae*. This requires a review of infection prevention control practices with the hospital to reduce reservoirs for bacteria to exchange resistance genes especially among *A. baumannii* which presents a challenge in eradication. Three *E. coli* strains, one a clinical isolate and two carriage isolates had similar ST by Enterobase (410) and but were different by Pasteur (471 and 692). The Pasteur scheme has been shown to be more diverse than the Enterobase scheme (Kaas et al., 2012) and as such the clinical *E. coli* strain is different from the carriage strains. Members of high risks clones associated with multi-drug resistance and outbreaks were represented in ST1 of *A. baumannii*, ST357 in *P. aeruginosa* and ST307 in *K. pneumoniae* which when considering findings from Musila et al on carbapenemases in Kenya (Musila et al., 2021), indicates probable country-wide outbreaks.

Whereas there were diverse variants of *bla*<sub>NDM</sub> gene in the strains, the first variant (*bla*<sub>NDM-1</sub>) associated with India was the most abundant similar to Musila et al’s findings and considering PCR results may indicate that *bla*<sub>NDM-1</sub> is prevalent in Kenya with though other variants may be circulating in low levels. In contrast, we did not detect *bla*<sub>VIM</sub> in the single *P. aeruginosa* (K_PA3) with MBL activity but the novel *bla*<sub>DIM-1</sub> (Dutch Imipenemase) first reported in Netherlands in a *P. stutzeri* isolate from a Dutch patient without history of travel (Poirel et al., 2010). The MBL has since been reported in India in a case earlier than the Dutch index case (Deshpande et al., 2014), in 60% of isolates in Sierra Leone (Leski et al., 2013), in Ghana (Janice et al., 2021) and a second variant of the gene in China (Sun et al., 2015). It has been postulated that the gene may have emerged in Indian subcontinent due to the earliest reported isolate, but given the diverse range of families from which the gene was detected in Sierra Leone and its rarity elsewhere implies that gene is prevalent in the region and more research is required to determine origin.

*Bla*<sub>OXA-50</sub> and its variants were detected in all *P. aeruginosa* in sequenced strains but may have an insignificant role in carbapenem resistance given the lack of detection of enzymatic activity in the species. Likewise, there was diversity in the range of oxacillinases identified in this study as compared to Musila et al’s paper with *bla*<sub>OXA-420</sub>, a *bla*<sub>OXA-58</sub> variant, observed in *A. baumannii*, *bla*<sub>OXA-225</sub>, a variant of
bla_{OXA-23}, observed in other Acinetobacter species and bla_{OXA-395} and bla_{OXA-488} of bla_{OXA-50} in P. aeruginosa. Also identified were bla_{OXA-181} and bla_{OXA-232}, variants of bla_{OXA-48} in carriage E. coli and K. pneumoniae strains respectively. Though rarely studied, these genes are prevalent and may present a challenge with the treatment of infected patients with beta-lactams such as penicillins and cephalosporins.

In Uganda, while the majority of the E. coli and K. pneumoniae had a single carbapenemase gene either bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{KPC} or bla_{OXA-48} as detected by PCR (Okoche et al., 2015), a few isolates however, had more than one gene (up to a maximum of three genes) which was in contrast to my findings in Kilifi or in Musila et al.’s paper where usually one carbapenemase gene was detected in Enterobacterales. In Uganda, bla_{OXA-48}, bla_{VIM} and bla_{IMP} are commonly identified in Enterobacterales while bla_{NDM} and bla_{KPC} occur at low prevalence (Ssekatawa et al., 2021, Okoche et al., 2015, Ampaire et al., 2015). Similar to Uganda, multiple carbapenemase genes have been reported in some of the Enterobacterales in Tanzania with a predominance of bla_{VIM} and bla_{IMP} (Mushi et al., 2014). The findings in the two countries are a stark contrast to Kenya where bla_{NDM} predominates with no reports of bla_{KPC}, bla_{VIM} or bla_{IMP} or co-occurrence of carbapenemases in Enterobacterales so far. It is likely that there is a higher usage of carbapenems in the hospitals involved in the study compared to the Kenyan hospitals which could explain the findings however as research and surveillance is limited in the countries, more data is required to understand the prevalence of carbapenem resistance. The lack of bla_{KPC} in Kenya can be explained by a recent study on the Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS) which did not detect the gene in any of the sites in Africa with only one isolate in Asia (Sands et al., 2021). This was attributed to the absence of ST258 among K. pneumoniae strains which is responsible for the dissemination of the gene. This high-risk ST has not been reported in the country but given that travel between the three nations, it may only be a matter of time before it is introduced into the country especially as carbapenem usage increases in public hospitals.

Bla_{OXA-23} is the main carbapenemase gene detected in A. baumannii in Uganda but unlike in my study, bla_{OXA-24} and bla_{VIM} were also detected in the strains which has yet to be reported in Kenyan isolates (Aruhomukama et al., 2019, Kateete et al., 2016). P. aeruginosa isolates from Uganda and Tanzania possessed bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM} (Uganda) and bla_{KPC} (Tanzania) (Mushi et al., 2014, Kateete et al., 2016, Aruhomukama et al., 2019). In Kenya, only bla_{VIM} and bla_{NDM} has been reported in the species (Musila et al., 2021, Pitout et al., 2008) and with the identification of bla_{DIM} in this study, it indicates that MBLs are the predominant carbapenemases in the species in the region.
Several resistance genes were present in sequenced carbapenem resistant bacteria with ESBL and cephalosporinases co-occurring with carbapenemases. Previously unreported ESBL genes in Kenya were identified; *bla*<sub>PER</sub>-7 in *A. baumannii* and the novel *bla*<sub>PAU</sub>-1 in *P. aeruginosa*, first reported in China (Wang et al., 2019b) and India (Khan et al., 2020). The gene was reported to confer resistance to piperacillin and ampicillin and elevating the MIC of ceftriaxone and cefepime, here apart from the *bla*<sub>PAU</sub>-1 possessing strain only two others were resistant to ceftazidime one of which had *bla*<sub>DIM</sub> thus confirming that the gene reduced susceptibility to third generation cephalosporins.

In many of the strains, phenotypic resistance corelated with the genotypic traits except for some drugs in a few cases. Two *E. coli* strains with similar resistance gene traits, *bla*<sub>OXA-181</sub> in K_ECO2 and 3 had different susceptibilities to carbapenems with K_ECO2 susceptible to imipenem and meropenem and intermediate resistance to ertapenem while K_ECO3 was resistant to all these drugs. Both isolates were carriage isolates though from patients from different parts of the country (Nairobi and Mombasa). It is likely that while the full oxacillinase gene was present in K_ECO2 its expression was hindered maybe due to changes in the promoter region resulting in the susceptibility / reduced susceptibility to the carbapenem drugs. A different explanation though less known reason could be the antibiotic resistance gene silencing, a concept previously reported in animal studies for drugs such as tetracycline and sulphonamide (Enne et al., 2006). Though intact reading frames could observed for the genes the transcripts were not detected implying that their expression could be switched off by the bacteria. Most of the strains had multiple aminoglycoside resistance genes however, most of these did not translate into resistance to amikacin. Amikacin resistance was only observed in strains that possessed 16S rRNA methyltransferases (*armA* and *rmtB*), *aph(3')-VI* or *aac(6')-lb*. Phenotypic resistance prediction by ResFinder based on gene presence predicted that *aac(6')-lb-cr* conferred resistance to both amikacin or ciprofloxacin but in instances where it was the only resistance gene present there was no concomitant resistance to the drug in the isolate. Similarly, the presence of *crpP* gene in *P. aeruginosa* did not result in resistance in ciprofloxacin and this has been reported elsewhere in Europe (Hernández-García et al., 2021). The absence of acquired ciprofloxacin resistance genes in *A. baumannii* provides evidence that mutations in the topoisomerase enzymes and efflux pumps (*acrR* in the case of *K. pneumoniae*) were the main mechanisms of resistance to fluoroquinolones in the absence of quinolone resistance gene (*qnr*). In two Enterobacterales strains susceptible to co-trimoxazole, only sulphonamide resistance genes (*sul1/2*) were observed while trimethoprim resistance genes (*dfr*) were absent, however in cases where the reverse occurred, the bacteria was resistant to the drug implying that *dfr* genes are required for
resistance to cotrimoxazole. A point mutation that confers resistance to tigecycline was reported in a single *K. pneumoniae* isolate, however the isolate was not tested for tigecycline susceptibility.

Colistin is a reserve drug in the Kenya and is mainly used in animal practice (Muloi et al., 2019), it is therefore expected that colistin susceptibility testing and resistance in human strains in Kenya is rare. I identified a carbapenem resistant, colistin resistant *K. pneumoniae* isolate (K_KPN5) in the gut of a 9-month-old admitted to KCH in 2018. The isolate was confirmed to have been present during discharge of the patient. Through WGS, the strain was found to have mutation in the *mgrB* gene which encodes a negative feedback regulator protein that regulates the two-component regulatory system (PhoQ-PhoP) that modifies the bacterium’s response to environmental stimuli such as low magnesium cation levels (Cannatelli et al., 2014b). In addition, there was a mutation in the *pmrB* gene of the PmrA/PmrB signalling system which is the major regulator of lipopolysaccharide (LPS) modification (Cannatelli et al., 2014a). Colistin acts by binding to LPS and phospholipids in the outer cell membrane and displaces magnesium and calcium cations from the phosphate group of the lipids disrupting the integrity of the outer membrane. Modifications in the *mgrB* (Haeili et al., 2017, Cannatelli et al., 2014b, Poirel et al., 2015) and *pmrB* (Phan et al., 2017, Jayol et al., 2014) genes has been shown to confer resistance to colistin in isolates lacking the plasmid *mcr* mediated resistant gene. The mechanism of resistance suggests that mutations occurred in the strain in the presence of colistin and given that it is mostly used in animal practice in Kenya, it likely that the organism was transmitted from an animal source to the child. This could have occurred by direct contact with animals or animal environment or through animal products. Its persistence however in the absence of colistin suggests that the mutations had minimal fitness costs and can be easily transmitted. The only other colistin resistant isolate reported in Kenya was isolated in Nairobi and had the transmissible *mcr-8* gene in a high risk ST15 *K. pneumoniae* strain but was carbapenem and third generation cephalosporin susceptible (Kyany’a and Musila, 2020) indicating presence of plasmid mediated colistin resistance in Kenya.

Eravacycline, cefiderocol, meropenem / vaborbactam and imipenem / relebactam are new drugs recently approved for treatment of difficult to treat infections and therefore the genetic basis of resistance is not fully understood. The drugs are not available in Kenya and therefore the basis for resistance is interesting. In the case of carbapenem / new β-lactamase inhibitors, resistance is expected as they are ineffective against MBLs, the predominant carbapenemase in my study. Plasmid encoded *tetX* conferred resistance to tigecycline and possibly eravacycline in isolates in China (Sun et al., 2019) while mutations in efflux pump, AdeABC resulted in eravacyline resistance in *A. baumannii* (Shi et al.,
TetX genes were not detected in any of the eravacycline resistant isolates in my study. There were other tetracycline resistance genes in eravacycline resistant isolates except in two K. pneumoniae isolates, while it is likely that these genes may have a role in eravacycline resistance, it is also possible that there are other tetracycline resistance genes that are yet to be discovered that may drive resistance to drugs within this class indicating the urgency in discovering them. Resistance to cefiderocol has been associated with mutations in siderophore receptors such as cirA of E. cloaca (Klein et al., 2021) and pirA of A. baumannii (Malik et al., 2020) which are involved in the uptake of the drug into the bacteria. Alterations in siderophore receptor genes as a possible explanation for cefiderocol resistance was not investigated for in this chapter. As the sequenced isolates have existing phenotypic resistance to these new drugs, it is likely that there may be additional unknown resistance mechanisms that are responsible, or the organisms are utilising already existing mechanism. It is therefore important to determine the resistance mechanism to avoid treatment failures in infected patients with little option for treatment.

WGS has shown that the carbapenem resistant bacteria in this study possess multiple resistance genes. This and other studies in Kenya show a dominance of blaNDM among resistant bacteria in contrast to the few studies in Uganda and Tanzania. Extensive studies in South Africa indicate that blaNDM and blaOXA-48 been identified (Osei Sekyere, 2016) while blaOXA-23 was the dominant gene in A. baumannii (Lowe et al., 2018). Kenya’s proximity and travel (medical and leisure) to the Indian subcontinent may also influence the frequency and diversity of carbapenemase genes in the country as blaNDM is endemic in India with outbreaks of strains with other carbapenemases (Logan and Weinstein, 2017). Nevertheless, more data is required to understand the prevalence and diversity of carbapenemases in Kenya.

The findings in this study suggests that the presence of a gene may not necessarily mean that that the bacteria will be resistant to the drug as shown in the case of amikacin, ciprofloxacin and cotrimoxazole. Even with the same sequence data, choice of bioinformatic pipeline, quality of sequence and interpretation of results could result in different interpretations of resistance affecting the drugs recommended for patient treatment (Doyle et al., 2020). Additionally, some genes might be similar to the reference gene used but may have variations in functional regions which affect the expression or activity of the product. A formal system of cataloguing all variants with direct correlation to the resistance phenotype for each will be needed before WGS can be fully implemented for clinical use.

The major limitation of the study is that few isolates were selected for WGS due to cost and time constraints and some of the findings such as those from A. baumannii suggests that the strains may be
related and could reveal possible hidden outbreaks. Also, the diversity of STs suggest that transmission of the resistant traits may be due to the plasmids rather than transmission of the organisms themselves. Similar plasmids were identified in Enterobacterales but the entire plasmid genome could not be reconstructed due to the use of short read sequencing technology. Future studies are required with extensive sequencing of carbapenem resistant and susceptible bacteria from various hospitals in the country with additional focus on long read plasmid sequencing. Further investigation is also required to reveal unknown resistance mechanisms that may confer resistance to new drugs reducing their potential against difficult to treat MDR infections. This will provide a clearer picture of the transmission dynamics of resistance genes especially in cases of linked resistant traits that will need to be factored in implementation of antimicrobial stewardship programmes.

In conclusion, \textit{bla}\textsubscript{NDM} was the dominant carbapenemases in \textit{Acinetobacter} and Enterobacterales with more than one carbapenemase gene identified in majority of the \textit{Acinetobacter} spp. \textit{Bla}\textsubscript{OXA-23} was the main oxacillinase in \textit{Acinetobacter}. \textit{Bla}\textsubscript{KPC}, \textit{bla}\textsubscript{VIM} and \textit{bla}\textsubscript{IMP} genes were not detected in any of the strains with the novel \textit{bla}\textsubscript{DIM-1} detected in \textit{P. aeruginosa}. The diverse STs and resistant traits suggest multiple introductions of resistant bacteria rather than a single successful clone. However more data particularly on plasmid sequencing is required to understand the plasmids associated with carbapenemases and determined linked resistant traits that might be selected for by other antibiotics.
4 Effect of multidrug resistance on biological fitness

Key points

- The biological fitness of carbapenem resistant Enterobacterales from clinical isolates was assessed using growth curve assays and in vitro pairwise competition assay.
- The findings show a general trend of carbapenem resistant bacteria being less fit than fully susceptible Enterobacterales, although this was not consistent for all strains.
- Biofilm production in all strains was tested using the microtiter plate method and, while K. pneumoniae and P. aeruginosa were found to be strong biofilm producers, there was no association between biofilm production and resistance profile or resistance genes.
- The findings suggest that while infection prevention control is important given that the strains were hospital acquired other concerted efforts such as drug stewardship as well as improved water, sanitation and hygiene is required to curb resistance in the country and no one effort should be implemented alone.

4.1 Introduction

While acquisition or development of antimicrobial resistance will confer selective advantage in the presence of the antimicrobial in question, such changes may have adverse effects on the basic physiological functions of the bacteria and, in a non-selective environment, resistance is no longer advantageous and resistant strains may be outcompeted by ‘wild type’ strains. This is the theoretical basis for supporting antimicrobial stewardship programmes, arguing that if misuse and improper use of antibiotics is controlled there will be a ‘reversion’ to a predominantly sensitive population as sensitive isolates outcompete resistant isolates. Indeed, measures to control improper use of some antibiotics in other sectors such animal and environment that have implications in the human sector has been documented to result in a reduction of some resistant bacteria in humans.

A notable example of this was in Canada where off-label in ovo use of ceftiofur, a 3GC, in broiler chickens to control E. coli infections resulted in increased ceftiofur resistant Salmonella enterica in both chickens and humans (Dutil et al., 2010). A withdrawal resulted in a significant drop in resistant bacteria which resulted in the ban of this off-label use by the Canadian government as well as other countries. Other notable successes include the ban of avoparcin (a glycopeptide) use as a growth promoter in farms in Europe that resulted in reduction in vancomycin resistant Enterococci (Klare et al., 1999) and the ban on use of colistin as a growth promoter in China reduced the carriage of colistin resistant E. coli
strains in both humans and animals (Wang et al., 2020). Nonetheless, this has not always been replicated, an example of which is the persistence of sulphonamide resistance over several years in the UK despite a reduction in drug prescription (Enne et al., 2001) or an increase in penicillin non-susceptible pneumococci in Iceland even with reduced use (Arason et al., 2002). The effect of withdrawal or reduction of antibiotics therefore seems to be specific to particular pathogen-drug combinations. Johnsen et al. described key factors affecting the reversal of drug resistance such as fitness cost, compensatory evolution mitigating the fitness cost, processes that counteract the reversal (that is selection of linked resistance determinants, re-acquisition of such traits and maintenance of mobile genetic elements) and drug re-introduction (Johnsen et al., 2009). The reversal of drug resistance is therefore not only dependent on the restriction of antibiotic use but is affected by a complex interaction of several factors. Multi-drug resistant bacteria are assumed to be less fit due to the energy requirement associated with replication of the additional DNA acquired by the organisms to evade antibiotics.

The fitness cost of resistance is highly variable with some studies reporting a cost associated with resistance while some studies reporting little or no cost if any and this variability may be attributed to a variety of factors (Vogwill and MacLean, 2015). Plasmid resistance carries a smaller cost compared to mutational resistance and this is thought to be due to the fact that plasmids have already undergone evolutionary selection to minimize costs compared to chromosomal mutations which reports de novo resistance and have not undergone adaptations to offset this cost (Vogwill and MacLean, 2015). The fitness cost of a resistant bacteria can be determined through competition assays both in vitro or in vivo in animal or insect models (DiRita et al., 2001, Hafza et al., 2018) by introducing competing bacteria in equal ratio and over time the changes in growth for both strains is compared. Alternatively, fitness can also be inferred through non-competitive growth curve assays. Bacteria are grown individually, and concentration at different time points estimated either through cell counting or optical density (OD). The resultant curves and growth kinetics are compared between organisms to estimate fitness (Wang et al., 2019a).

Bacterial virulence and resistance have evolved differently and while antimicrobial resistance helps a bacterium to evade antibiotic action during disease, virulence functions to evade host immune defences and cause disease. An inter-relationship between resistance and virulence has been described such as in S. aureus where methicillin resistance affected biofilm production and reduced virulence compared to
susceptible strains and hence is mainly associated with healthcare settings (Rudkin et al., 2012, Pozzi et al., 2012).

The complement system is a crucial innate and adaptive immune system, killing and clearing invading pathogens without previously being exposed to them. In the presence of pathogenic intruders, the system is activated through one of three methods: classical, alternative and lectin pathways whose final result is the formation of the membrane attack complex (MAC), a collection of proteins on the surface of pathogen cell membranes leading to lysis and cell death. Bacteria, in response, have developed various virulence mechanisms such as lipopolysaccharide and polysaccharide capsules in *Neisseria meningitidis* to resist the serum complement and establish an infection (Kugelberg et al., 2008). Serum sensitivity has been described as reason as to why *P. aeruginosa* in infected cystic fibrosis patients has not led to invasive disease unlike in other clinical indications (Speert et al., 1990, Thomassen and Demko, 1981). An MDR bacterium that also possess the mechanisms to evade the complement system is therefore a major health concern.

Biofilms, microbial cells enclosed in extracellular polymeric matrix composed of polysaccharides, proteins and extracellular DNA, provide protection against mechanical or chemical attack. These sessile sedentary communities form on natural and synthetic surfaces and are associated with chronic illnesses such as cystic fibrosis, periodontitis, osteomyelitis and otitis media. Biofilms are implicated in healthcare associated infections associated with medical devices, where it is more difficult to eradicate infection if the organisms form biofilms, or surgical site infections (Akyıldız et al., 2013, Muhammad et al., 2020, Percival et al., 2015). Bacteria that easily form biofilms are advantageous in that they tolerate or resist antibiotics (Patel, 2005) and in high touch areas are not easily eradicated by casual cleaning procedures becoming focal points for transmission to and between vulnerable patients in intensive care (Costa et al., 2019). This is therefore an important virulence trait for bacteria.

Despite the lack of carbapenem use in patient management at KCH, Kenya until 2020, my study has shown that carbapenem resistant bacteria have been isolated from patient samples several years prior to the introduction of carbapenems, with the majority of infections are hospital acquired either through presumed patient to patient transmission (where the donor patient has not been sampled) or acquisition from an environmental reservoir. Sequencing of selected isolates as described in chapter 3 of this thesis has revealed that the majority are unrelated as they are of different sequence types and represent sporadic introductions. It is hypothesized, therefore, that since KCH has not been using carbapenems, there may be a fitness cost attached to the carbapenem resistance and such bacteria will
not persist in the hospital. On the other hand, there is on-going ceftriaxone use in the hospital and thus providing the selective environment for proliferation of cephalosporin resistant and ESBL-producing bacteria.

I therefore sought to determine the effect of carbapenem resistance on the biological fitness of Enterobacterales in the absence of selective pressure. I also sought to understand the biofilm formation capabilities of the resistant bacteria to understand its ability to form biofilms on abiotic surfaces in the hospital and thus be reservoirs for hospital associated infections.

4.2 Methodology

4.2.1 Growth curve assay

This assay was used to monitor the growth and proliferation of clinical carbapenem sensitive and resistant *E. coli* and *K. pneumoniae* bacteria in standard LB broth media (salt concentration of 5 g/L or 0.5% w/v) (Sigma, USA) and in media with higher salt concentration as a biological stressor. To determine optimal conditions for comparison, salt concentrations of 2.5%, 5% and 7.5% w/v were tested on a subset of strains and based on the results, a concentration of 5% was selected for the final assay. Bacteria were cultured overnight at 35°C ± 2°C on blood agar plates and a few colonies suspended in normal saline to a turbidity of 0.5 McFarland as measured by a Sensititre™ nephelometer (ThermoFisher, USA). The mixture was diluted in fresh LB media (standard and 5% w/v salt concentration) and diluted 1:1000 in respective media and 150 µl added to a well in triplicate in 96 well flat-bottomed culture plate. The plate was incubated at 37°C in a microplate reader (Synergy H1, BioTek, USA) for 15 hours and the optical density measured at 600 nm (OD\textsubscript{600}) at 30-minute intervals for 15 hours with orbital shaking for five minutes prior to measurement (Wang et al., 2019a, Tsai et al., 2011). The assay was performed on three separate days and the average of absorbance values were used for analysis and bacterial growth determined by plotting the values against time using GraphPad Prism 9.2.0.

Each carbapenem resistant Enterobacterales (CRO) was matched to a non-ESBL producing carbapenem susceptible Enterobacterales (CSO) and an ESBL producing CSO (ESBL) from same species, sample type and time frame as described in chapter 2 (section 2.2.2). There were nine such matches, three for *E. coli* (M2, 6 and 8) and six for *K. pneumoniae* (M1, 3, 4, 5, 7 and 9).
4.2.2 In vitro mixed growth competition assay

The assay compared the *in vitro* competition of clinical carbapenem sensitive and resistant *E. coli* and *K. pneumoniae* in the same environment of standard LB broth media (Sigma, USA) and salt added LB media (5% w/v). Bacteria were cultured overnight on blood agar at 35°C ± 2°C and 2 – 3 colonies were suspended in 2 ml of standard and 5% salt LB media respectively for 4 – 6 hours. A 0.5 McFarland suspension was prepared from the growth suspension using a nephelometer and 10 µl each of matched carbapenem sensitive and resistant bacteria (as described in chapter 2, section 2.2.2) were added to 10 ml of fresh media and incubated at 37°C while shaking at 180 rpm. An aliquot of the mixture at 24 hours was serially diluted ten-fold and 50 µl of each dilution was plated on MHA plates without and with 0.25 mg/L meropenem. The plates were incubated at 35°C ± 2°C overnight and colonies from a dilution plate of between 30 and 200 colonies were counted and the concentration in colony forming units per milliliter (CFU/ml) of the original suspension was calculated. Carbapenem resistant CFU/ml from the original mixture was calculated from plates with meropenem while carbapenem susceptible CFU/ml was calculated from subtracting CFU/ml from plates with antibiotic from those without. The competition index (CI) was defined as the ratio between CFU/ml of carbapenem resistant strain over susceptible strain after 24 hours incubation. The CI values was calculated for each independent competition assay performed on three separate days and the mean CI determined (Barrick Jeffrey et al., 2007, Wang et al., 2019a, Hafza et al., 2018). Graphs were drawn and analysis performed using GraphPad Prism 9.2.0. Pairwise competition was not performed for isolates in M1 as the CRO was susceptible to meropenem and imipenem and resistant to ertapenem.

4.2.3 Serum bactericidal assay

Susceptibility of bacteria to normal human serum (NHS) was determined for carbapenem resistant and susceptible *E. coli* from blood and CSF samples of an adult meningitis patient (see case report, chapter 5, and for matched *K. pneumoniae* from blood samples of different patients (as described in chapter 2, section 2.2.2). Serum was obtained from four healthy adult volunteers (one male and three females), pooled and frozen at -70°C until use. Frozen bacteria were revived on blood agar at 35°C ± 2°C and 2 – 3 colonies cultured in 4 ml LB broth (salt concentration of 5 g/L) in similar conditions. The bacterial suspensions were standardized to 0.5 McFarland using a nephelometer and incubated at 35°C ± 2°C for 4 hours. A 1 ml aliquot of the suspension was pelleted at 10000 rpm for 10 minutes and washed twice with sterile 1X PBS and reconstituted in 1 ml of PBS of which 100 µl aliquot was incubated with 40% serum (in sterile 1X PBS). Serum concentrations of 20% and 40% were tested and 40% was found to be
effective. The mixture was incubated at 37°C for 3 hours at 180 rpm and a 50 µl aliquot was obtained every 45 minutes and serially diluted ten-fold and plated on MHA which was incubated overnight at 35°C ± 2°C. The colonies in a dilution plate with between 30 and 200 colonies, were counted and concentration (CFU/ml) in the original mixture at the different time points was determined. (Zlosnik et al., 2012, King et al., 2009, Dasgupta et al., 1994).

4.2.4 Biofilm formation assay
The biofilm formation assay was adapted from previously published literature (O'Toole, 2011, Nirwati et al., 2019, Naves et al., 2008, Wang et al., 2018). Frozen bacteria were revived on blood agar at 35°C ± 2°C overnight and a few colonies suspended in 4 ml of LB broth (Miller, 10 g/l salt concentration) (Sigma, USA) to a turbidity of 0.5 McFarland and grown overnight. The suspension was diluted 1:100 in fresh LB broth and 130 µl of the mixture plated in duplicate in a 96 well flat-bottomed culture plate (Corning, USA) and incubated at 35°C ± 2°C for 24 hours. The wells were washed thrice with phosphate buffered saline (PBS) and air dried completely. Biofilm mass was stained with 150 µl of 0.2% w/v crystal violet staining and incubated at room temperature for 30 minutes. The stain was discarded, and wells washed thrice with 1X PBS to remove excess stain and allowed to completely dry. Crystal violet stained biofilm mass were solubilized with 150 µl of 99.5% ethanol and 100 µl transferred into a fresh new plate and absorbance measured at 570 nm (OD₅₇₀). Negative controls consisting of media only were included in all plates. The assay was performed on three separate days. The strains were classified as non, weak, medium and strong biofilm producers based on the optical density cut-off (ODc), calculated as the average OD of negative controls plus three times the standard deviation of the control per plate. Non biofilm producing strains had an OD value ≤ ODc, weak producers were ODc < OD ≤ 2 × ODc, moderate producers were 2 × ODc < OD ≤ 4 × ODc and strong producers were OD > 4 × ODc (Nirwati et al., 2019).

4.3 Results
4.3.1 Carbapenem resistant strain growth rate was generally slower than susceptible bacteria
All carbapenem resistant and susceptible Enterobacterales were grown individually in standard LB and 5% salt LB media. In standard LB media, carbapenem susceptible, non-ESBL producing (CSO) growth was better than resistant (CRO) or ESBL producing strains in five matches (M1, 4, 5, 6, and 9)(Figure 4.2) during log phase (3 – 7 hours) with no differences in three matches (M3, 7 and 8). One E. coli CRO (M2) grew better than the susceptible strains.
Carbapenem resistant *K. pneumoniae* (M9), struggled to grow in standard LB media displaying a wide variability between wells and between days. A visual examination of the organism on blood agar plate depicted an unusual growth pattern to other clinical strains of *K. pneumoniae* usually isolated in the lab (Figure 4.1).

There was minimal difference in the log phase growth between CRO and ESBL strains except for pairs M6 and M7 where the carbapenem resistant isolate grew slightly better than the ESBL comparator strains and for pairs M1 and M9 where the ESBL comparator strains grew better.

All strains except for carbapenem resistant *K. pneumoniae* in match M9 displayed slower growth rates in 5% salt LB media compared to standard LB media. The carbapenem resistant strain (M9) growth rate was better with higher OD values in stationary phase. There was minimal difference between the sensitive and resistant strains in M2 in 5% salt. The carbapenem susceptible (CSO) strain grew better than CRO in 5% salt in five matches (M1, 4, 5, 6 and 7) but in one match (M8) the carbapenem susceptible, non-ESBL producing *E. coli* struggled to grow in the stressful environment.

There is minimal difference in growth between CRO and comparator ESBL strains in four matches (M3, 4, 5 and 7) in 5% salt with better growth in CRO compared to ESBL in M6 and M8 and, in matches M1 and M9, the ESBL comparator grew better than CRO.

![A](image1.png) ![B](image2.png)

Figure 4.1 Carbapenem resistant *K. pneumoniae* (A) with an altered growth appearance compared with carbapenem susceptible *K. pneumoniae* ATCC 13883 (B).

The carbapenem resistant *K. pneumoniae* had rough colonies unlike the comparison, fewer isolated colonies on agar media and took longer to achieve visible growth in broth media.
4.3.2 Carbapenem resistant bacteria were usually, but not always, less fit than susceptible bacteria in competition assays

Pairwise competition assays were performed for all matched pairs except in M1 where the carbapenem resistant *K. pneumoniae* was susceptible to meropenem. In standard LB media carbapenem resistant Enterobacterales (CRO) had a fitness advantage (CI >1) when competing with susceptible comparator bacteria both ESBL and CSO in M3 and 8 and against comparator CSO in M7 (Figure 4.3). In all other pairs, CRO was less fit (CI <1) except in M2 (ESBL) and M9 (CSO) where there was no difference (CI=1).

In 5% salt LB CRO were less fit compared to CSO in four matches (M3, 4, 5 and 6) and against ESBL in one match, M9 and against both CSO and ESBL in one match, M5. No fitness advantage was observed for the CRO strain in three matches (M4, 6 and 7) when compared to ESBL. There was a change from less fit in standard LB media to more fit in 5% salt media when CRO was compared to CSO comparator strains in matches M2 and to ESBL comparator strains in M4, 6 and 7 while the reverse (more fit to less fit) was observed only when comparing CRO to CSO in M3.
Figure 4.2 Growth curve analysis of carbapenem resistant organisms (CRO) (circle) compared to matched carbapenem susceptible ESBL (triangle) and non-ESBL producing strains (CSO) (square) in standard LB media (black) and LB media with added salt (5% w/v) (blue).

The optical density (OD$_{600}$ nm) of strains in M2, 6 and 8 (E. coli) and M1, 3, 4, 5, 7 and 9 (K. pneumoniae) (each match, M, consisted of CRO, non ESBL producing CSO & ESBL CSO) was measured every 30 minutes for 15 hours in a microtiter plate in a plate reader. The strains were grown in triplicate on three separate days with the mean plotted and error bars indicating standard deviation (SD).
Figure 4.3 In vitro pairwise competition of carbapenem resistant (CRO) versus carbapenem susceptible ESBL and non-ESBL producing strains (CSO) in standard LB media (black) and LB media with added salt (5% w/v) (grey).

The assay was performed on three separate days and the competition index (CI) for carbapenem resistant over susceptible strain calculated for each day. The mean CI and standard deviation were plotted for each competing pair and a CI=1 (dashed line) indicates no fitness advantage.
Table 4.1 In vitro pairwise competition of carbapenem resistant bacteria (CRO) versus susceptible bacteria (CSO or ESBL) in standard LB media and in 5% w/v salt concentration LB media

<table>
<thead>
<tr>
<th>Pair</th>
<th>Competition Index mean ± SD (LB)</th>
<th>Competition Index mean ± SD (5% salt LB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRO/CSO (M2: Eco)</td>
<td>0.11 ± 0.10</td>
<td>4.26 ± 1.11</td>
</tr>
<tr>
<td>CRO/ESBL (M2: Eco)</td>
<td>1.00 ± 0.18</td>
<td>1.63 ± 0.26</td>
</tr>
<tr>
<td>CRO/CSO (M3: Kpn)</td>
<td>3.62 ± 0.52</td>
<td>0.72 ± 0.14</td>
</tr>
<tr>
<td>CRO/ESBL (M3: Kpn)</td>
<td>5.50 ± 2.08</td>
<td>1.70 ± 0.50</td>
</tr>
<tr>
<td>CRO/CSO (M4: Kpn)</td>
<td>0.85 ± 0.07</td>
<td>0.59 ± 0.08</td>
</tr>
<tr>
<td>CRO/ESBL (M4: Kpn)</td>
<td>0.64 ± 0.12</td>
<td>1.06 ± 0.07</td>
</tr>
<tr>
<td>CRO/CSO (M5: Kpn)</td>
<td>0.57 ± 0.11</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>CRO/ESBL (M5: Kpn)</td>
<td>0.95 ± 0.06</td>
<td>0.85 ± 0.23</td>
</tr>
<tr>
<td>CRO/CSO (M6: Eco)</td>
<td>0.59 ± 0.15</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>CRO/ESBL (M6: Eco)</td>
<td>0.51 ± 0.03</td>
<td>1.02 ± 0.37</td>
</tr>
<tr>
<td>CRO/CSO (M7: Kpn)</td>
<td>2.52 ± 0.60</td>
<td>1.75 ± 0.37</td>
</tr>
<tr>
<td>CRO/ESBL (M7: Kpn)</td>
<td>0.27 ± 0.08</td>
<td>1.04 ± 0.22</td>
</tr>
<tr>
<td>CRO/CSO (M8: Eco)</td>
<td>1.48 ± 0.40</td>
<td>3.98 ± 2.05</td>
</tr>
<tr>
<td>CRO/ESBL (M8: Eco)</td>
<td>1.83 ± 0.07</td>
<td>2.08 ± 0.63</td>
</tr>
<tr>
<td>CRO/CSO (M9: Kpn)</td>
<td>1.02 ± 0.06</td>
<td>1.18 ± 0.15</td>
</tr>
<tr>
<td>CRO/ESBL (M9: Kpn)</td>
<td>0.58 ± 0.13</td>
<td>0.14 ± 0.01</td>
</tr>
</tbody>
</table>

SD, standard deviation
Overall, in the growth curve assay, 5/6 carbapenem resistant K. pneumoniae were less fit compared to the carbapenem susceptible, non-ESBL producing strain (CSO) when grown in LB media while 4/6 were less fit in salty media (Table 4.2). In pairwise competition on the other hand, 2/5 K. pneumoniae were less fit in LB media while 3/5 less fit in 5% salt media. The resistant strain in M4 and M5 was less fit in both growth curves and competition assays. On comparison of CRO and ESBL producing K. pneumoniae, 3/6 were less fit in both LB media and 5% salt LB media during growth curve assay while 4/5 and 2/5 were less fit in normal and LB media in competition assay respectively. The resistant strains in 4 and M9 were less fit in both assays.

Of the three matches in *E. coli*, only two resistant strains were less fit than CSO in normal LB media and one in salty environment in both assays with the resistant strain in M6 was less fit in both assays. On comparison of carbapenem resistant with ESBL strains however, only one strain (M6) was less fit during pairwise competition in standard LB media with the rest being more fit or no different than comparators (Table 4.2).
Table 4.2 Fitness cost of carbapenem resistant Enterobacterales when compared to carbapenem susceptible Enterobacterales using growth curves and in vitro pairwise competition

<table>
<thead>
<tr>
<th>Match</th>
<th>Growth curves (CSO)</th>
<th>Pairwise competition (CSO)</th>
<th>Growth curves (ESBL)</th>
<th>Pairwise competition (ESBL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard LB</td>
<td>5% salt LB</td>
<td>Standard LB</td>
<td>5% salt LB</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>Less fit</td>
<td>Less fit</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M3</td>
<td>Same</td>
<td>More fit</td>
<td>More fit</td>
<td>Less fit</td>
</tr>
<tr>
<td>M4</td>
<td>Less fit</td>
<td>Less fit</td>
<td>Less fit</td>
<td>Less fit</td>
</tr>
<tr>
<td>M5</td>
<td>Less fit</td>
<td>Less fit</td>
<td>Less fit</td>
<td>Less fit</td>
</tr>
<tr>
<td>M7</td>
<td>Less fit</td>
<td>Less fit</td>
<td>More fit</td>
<td>More fit</td>
</tr>
<tr>
<td>M9</td>
<td>Less fit</td>
<td>More fit</td>
<td>Same</td>
<td>Same</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>More fit</td>
<td>Same</td>
<td>Less fit</td>
<td>More fit</td>
</tr>
<tr>
<td>M6</td>
<td>Less fit</td>
<td>Less fit</td>
<td>Less fit</td>
<td>Less fit</td>
</tr>
<tr>
<td>M8</td>
<td>Less fit</td>
<td>More fit</td>
<td>More fit</td>
<td>More fit</td>
</tr>
</tbody>
</table>

ND – not done
4.3.3 Growth in human serum may be affected by carbapenem resistance

Resistance to killing by serum was tested on the isolates of greatest interest, namely two carbapenem resistant and one susceptible *E. coli* strains from a meningitis patient, as the both resistant and susceptible strains came from the same patient, and a carbapenem resistant *K. pneumoniae* bacteraemia isolate (isolate M9) chosen because it showed unusual phenotypic characteristics in the competition assay, growing poorly even on direct blood agar streaking and in standard LB broth. The carbapenem resistant *E. coli* from the meningitis patient was reduced by NHS from a starting concentration of log value 8 to 5 while the susceptible strain, from the same patient and acquired in the community, resisted serum action increasing in numbers from $2\times10^8$ to $5\times10^{10}$ (Figure 4.4A). On the other hand, resistant *K. pneumoniae* that had difficulty growing in standard LB media, was unaffected by NHS increasing two logs while ESBL producing *K. pneumoniae* reduced approximately one log in the first 45 minutes with an increase by end of the three hours. The non-ESBL producing susceptible *K. pneumoniae* increased by about two logs over the three hours but its ability to resist the complement system was significantly lower than the carbapenem resistant strain.

![Bacteria survival in human serum (E. coli)](image)
Figure 4.4 Survival of carbapenem resistant and susceptible Enterobacterales in serum. Strains were incubated in 40% pooled normal human serum for 3 hours. Aliquots were taken every 45 minutes and colonies enumerated by plating. Three separate experiments were performed for each isolate and the mean and standard deviation (error bars) plotted for each strain. Resistant E. coli from the meningitis patient (A) were killed by serum while resistant K. pneumoniae from blood (B) was not affected by the same. CFU, colony forming units.

4.3.4 *K. pneumoniae* and *P. aeruginosa* produce biofilms readily

Among *Acinetobacter* spp., 71% (15/21) of the non-*baumannii* were non-biofilm producers with the remainder being weak producers, on the other hand, 75% (15/20) of *A. baumannii* were capable of producing biofilms, 12 (60%) of which were weak, 2 (10%) moderate and 1 (5%) a strong producer (Table 4.2). All but one of the patients with a non-biofilm producing *A. baumannii* died in hospital while all with moderate and strong biofilm production were discharged alive. One moderate and one strong biofilm producing *A. baumannii* strains were isolated from the same patient and had identical AST profiles. The three moderate and strong biofilm producing strains all possessed *bla*<sub>OXA-23</sub> and were of two different STs 164 and 25 (Pasteur scheme).

All 10/10 *P. aeruginosa* isolates could form biofilms but only one, an ST2343 isolate from a burns patient and possessed *bla*<sub>DIM-1</sub>, was a weak producer. The isolate was unique in that it was the only one with raised MIC value for meropenem (4 µg/ml).
Four clinical carbapenem resistant, seven matched clinical carbapenem susceptible and 24 carbapenem resistant *E. coli* strains were tested for biofilm production. All *E. coli* isolates were non-producers except for a single carriage isolate which had the strongest biofilm production capacity of the entire collection. This strain was ST617 (Enterobase scheme) was positive for bla\textsubscript{NDM-like} by PCR however, this gene was not detected by WGS, although bla\textsubscript{CTX-M-15}, bla\textsubscript{OXA-1} and bla\textsubscript{CMY-42} were found. The detection of the gene by PCR and not WGS is thought to be possibly due to error in labelling or cross-contamination during the PCR process.

Of the seven clinical carbapenem resistant, twelve matched clinical carbapenem susceptible and six carbapenem resistant *K. pneumoniae* isolates, three were non producers of which one were carriage and two clinical carbapenem susceptible. Among the 22 (88%) biofilm producers, 3 (14%) clinical carbapenem resistant strains were weak producers.

Table 4.3 The biofilm formation capability of carbapenem non-susceptible clinical and carriage strains and carbapenem susceptible bacteria

<table>
<thead>
<tr>
<th>Clinical CRE isolates</th>
<th>Non producers</th>
<th>Weak producers</th>
<th>Moderate producers</th>
<th>Strong producers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. baumannii</em> (n=20)</td>
<td>5 (25)</td>
<td>12 (60)</td>
<td>2 (10)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Acinetobacter (non-<em>baumannii</em>) (n=21)</td>
<td>15 (71)</td>
<td>6 (29)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>E. coli</em> (n=4)</td>
<td>4 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (n=7)</td>
<td>0 (0)</td>
<td>3 (43)</td>
<td>3 (43)</td>
<td>1 (14)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (n=9)</td>
<td>0 (0)</td>
<td>1 (11)</td>
<td>2 (22)</td>
<td>6 (67)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical CSE isolates</th>
<th>Non producers</th>
<th>Weak producers</th>
<th>Moderate producers</th>
<th>Strong producers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (n=7)</td>
<td>7 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (n=12)</td>
<td>2 (17)</td>
<td>0 (0)</td>
<td>5 (42)</td>
<td>5 (42)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carriage isolates</th>
<th>Non producers</th>
<th>Weak producers</th>
<th>Moderate producers</th>
<th>Strong producers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (n=24)</td>
<td>23 (96)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (4)</td>
</tr>
<tr>
<td></td>
<td>1 (17)</td>
<td>0 (0)</td>
<td>4 (67)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>------------------</td>
<td>--------</td>
<td>-------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>K. pneumoniae (n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others* (n=2)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Others* include E. cloacae (1) and C. freundii (1)

4.4 Discussion
Overall, carbapenem resistant bacteria were less fit than carbapenem susceptible, non-ESBL producing strains when growing in normal environment as well as in stressful environment though in a small number of instances, the resistant bacteria appeared to be fitter than the susceptible strains. However, this was not the case when the carbapenem resistant strains were compared to ESBL producing strains with a fitness cost observed only in three K. pneumoniae strains with no difference or a fitness advantage observed in the case of E. coli. Carbapenem resistant Enterobacterales are multi-drug resistant strains that possess multiple plasmids which confer resistance to several antibiotic families. These multiple plasmids represent additional genetic material that require additional energy to replicate and maintain when compared to fully susceptible strains which have fewer plasmids. The fitness advantage or the minimal difference between ESBL producing and carbapenem resistant bacteria could be attributed to the fact that ESBL producing bacteria already possess multiple plasmids and the addition of carbapenem resistant gene cassette or plasmid to such may come with less fitness cost compared to its impact on a fully susceptible strain as the bacteria has already overcome the cost of adaptation.

In pairwise competition assay, there were fewer carbapenem resistant bacteria with a fitness disadvantage when compared to susceptible, non-ESBL producing strains with no difference or fitness advantage observed in some instances. These findings were different compared to growth curves analysis and was observed in both normal and stressful environment. Such differences may be attributed to active competition for the limited resources in the same environment in pairwise competition which is not a factor in growth curve analysis. Bacterial host genetics has a role in the fitness cost associated with a plasmid as it has been reported that the fitness cost of an organism possessing more than one plasmid is affected by host strain and even minor differences in host and plasmid genotypes has a substantial effect on fitness (Humphrey et al., 2012). The fitness cost of a plasmid is ameliorated by evolution of the plasmid with the host bacteria such that introduction of the evolved plasmid into the wild type strain will have no fitness cost compared to non-evolved plasmid
It can therefore be thought that some clinical strains have co-evolved with resistance plasmids for many generations and therefore the acquisition of these plasmids may have no accompanying fitness cost. However, the case may not be true for all clinical strains and may explain the differences observed.

All strains were stressed by the addition of 5% salt affecting the time taken to enter log phase, this was not true however for one carbapenem resistant *K. pneumoniae* (M9) which thrived in salty environment growing better than even the carbapenem susceptible non-ESBL producing strain. The isolate was unusual as it grew poorly in standard LB media and on blood agar suggesting a metabolic deficiency which is rectified upon addition of salt. It is likely that the strain may have acquired genes that enable it to thrive in a salty environment and which may result in changes in the morphology appearance. The isolate is ST530 and unlike other *K. pneumoniae* strains in the collection, it had no mutations in *ompK37* with a different mutation in *ompK36* than other strains and an *acrR* F204L mutation that confers resistance to tigecycline. In pairwise competition, the strain showed no difference compared to carbapenem susceptible, non-ESBL producing strains but was less fit than the ESBL strains in both growth curves and pairwise competition in normal and salt media.

An unusual case was observed in one instance where a significant fitness cost (CI=0.11) was observed when a carbapenem resistant *E. coli* (M2) competed with carbapenem susceptible, non-ESBL in a pairwise competition assay in standard media but was advantageous when the same occurred in salty media (CI=4.26). When compared to ESBL producing bacteria, the strain had no difference in standard media but was advantageous in a salty environment. The findings suggest a genetic defect in the strain that may be resolved by the addition of salt in the media. The isolate was the first carbapenem resistant bacteria identified from a patient sample admitted to the hospital (ST410) and was the only Enterobacterales with an IncC plasmid in addition to IncFIA/IncFIB. IncC plasmid is associated with dissemination of *bla*<sub>CMY</sub> and *bla*<sub>NDM</sub> among other resistance genes (Ambrose et al., 2018). Given its significance as the first *bla*<sub>NDM</sub> possessing isolate in the hospital, it is likely that the resistance gene or plasmid was recently acquired exerting a significant fitness cost on the bacteria, which may have prompted the bacteria to overcome through changes in chromosome which may have positively affected its ability to survive in salty environment.

Serum resistance in carbapenem resistant isolates was explored in two instances, *E. coli* from a meningitis patient and in resistant and susceptible *K. pneumoniae* isolates from different patients. In the meningitis patient, the carbapenem resistant bacteria were partially killed by the complement system in
the serum of the healthy human donors while the susceptible strain was unaffected. In this case, the multi-drug resistance strain is less virulent compared to the fully susceptible strain. The patient died in hospital as a result of the resistant bacteria which though less virulent acted as an opportunistic infection. This patient is discussed further in chapter five.

On the other hand, among the *K. pneumoniae* the carbapenem resistant strain was able to resist the activity of the complement system significantly more than the carbapenem susceptible strain. The resistant strain is more virulent than the susceptible strains. *K. pneumoniae* possess several virulence factors like the lipopolysaccharide and a capsule and this protects the organisms from complement action (Paczosa and Mecsas, 2016). This suggests that the bacteria ability to resist serum is not the only factor driving infection in humans and is not dependent on resistance of the bacteria.

In the biofilm formation assay, *P. aeruginosa* and *K. pneumoniae* were mainly moderate to strong producers while *Acinetobacter* species and *E. coli* were mostly weak or non-producers. No detectable pattern for biofilm production with resistance profile or carbapenem genes was found in this study. A study in Spain reported that 16% of carbapenem resistant *E. coli* were moderate to strong biofilm producers compared to 73% in *K. pneumoniae* (73%) suggesting that resistance was less important for biofilm formation in *E. coli* than in *K. pneumoniae* (Ramos-Vivas et al., 2019). Most *P. aeruginosa* are strong biofilm producers, similar to isolates in this study, and has at times been associated with virulence and or MDR (Cho et al., 2018, Rossi Gonçalves et al., 2017, Ratajczak et al., 2021, Gajdács et al., 2021, Awoke et al., 2019). Many of the *A. baumannii* in this study were weak biofilm producers a contrast to previous studies where mainly moderate to strong biofilm producers have been reported (Hazhirkamal et al., 2021, Al-Shamiri et al., 2021, Yang et al., 2019).

Existing in a biofilm may allow a resistant bacterium to survive as it is protected by other organisms and may share virulence and resistance genes with other organisms. Biofilms are also associated with virulence with more severe or persistent infections. In this study it appears that the biofilm production in *K. pneumoniae* and *P. aeruginosa* species functions to protect the organisms in an antibiotic and biocide hospital environment and enable transmission of the strains within the environment and between patients. The lack of biofilm production in *E. coli* and *Acinetobacter* species is interesting and while it is likely that the strains are poor biofilm producers it is also likely that the method used could be a contributing factor. In some studies *A. baumannii* were shown to have different biofilm producing capabilities depending on the assay methodology used (microtiter versus Congo red assay or tube assay) (Alamri et al., 2020, Avila-Novoa et al., 2019).
The microtiter plate assay is the most preferred method for detecting biofilms due to its ease and high reproducibility, though there are differences reported in literature in the type of media used, the addition of a stressor such as glucose and the proportion, incubation period and agent used for solubilization of crystal violet dye. Such differences limit the comparability of results, despite this however there are consistencies observed such as the ability of *K. pneumoniae* and *P. aeruginosa* to readily produce biofilms.

The major limitation is that the fitness cost of a resistance determinant is ideally studied by introducing the determinant into a laboratory strain and performing the assay using transformed strains and compared to wild type. Unfortunately, this could not be done due to the COVID-9 pandemic that prevented travel to collaborator’s lab in UK where the assays was to be performed. The fitness cost was determined instead using clinical carbapenem susceptible strains as comparators, however this did not factor the bias introduced by genetic differences (both host and plasmids) between the strains which may have implications for the findings. Nonetheless the findings show that that carbapenem resistant bacteria may not necessarily be less fit than carbapenem susceptible bacteria and that the evolution of the strains with the plasmids is an important factor to consider when designing control strategies to combat AMR. Secondly, an *in vivo* pathogenicity assay to determine the fitness and pathogenicity in a *Galleria mellonella* model could not be performed due to reasons described above. The findings from this experiment could have provided more knowledge on the relationship between fitness and pathogenicity during an infection and its implication. Currently highly resistant bacteria are considered superbugs associated with high mortality, and while this is attributed to the lack of treatment options, my studies to date show that the fitness and pathogenicity of such strains as well as the host immune response needs to be factored as well. Many studies have examined the association between resistance and patient outcome but what is lacking is the inclusion of fitness and pathogenicity in such studies which should be pursued in future studies in suitable models.

In conclusion, while there is a general trend of carbapenem resistant Enterobacterales being less fit than fully susceptible strains, this is not necessarily always the case. This mixed response between resistant and susceptible bacteria in growth, competition and sensitivity to serum indicates that the bacteria are in themselves unique and non-homogenous which has a Darwinian effect on their survivability and reproduction. Even though there are gaps in the understanding the effect of resistance in a bacteria especially from a clinical viewpoint, it can be concluded that the fitness and virulence of the pathogen is not only dependent on the chromosome, but also the number and type of plasmids carried which in turn
is influenced by external several factors such as the host immunity and as well as the environment and their selective pressures. A deeper understanding of this will influence the control measures put in place to combat antimicrobial resistance. These findings of this chapter suggest that antimicrobial stewardship and infection prevention and control particularly in hospitals to reduce spread nosocomial infections should be implemented together with other measures such as improved water sanitation and hygiene to reduce community infections and proper diagnostics to avoid misuse of antibiotics for unwarranted diseases should be implemented in national AMR prevention and containment measures.
5 Community and subsequent hospital acquired E. coli meningitis in an adult male

Key points

- A 58-year-old man presented with community acquired E. coli meningitis at admission in hospital and was successfully treated however, he had a relapse infection that proved fatal.
- Whole genome sequencing, antimicrobial susceptibility testing and in vitro fitness and virulence assays was performed on all strains isolated from the first and second episode.
- The findings show that the patient was infected with two unrelated strains of E. coli one of which a carbapenem susceptible strain, responsible for the initial episode while the second episode was due to a MDR strain.
- The MDR strain was found to be less fit and less virulent than the susceptible strain, and host factors may have a role in the outcome of the patient.

5.1 Introduction

The major causes of bacterial meningitis are the pathogens S. pneumoniae, N. meningitidis and H. influenzae. Gram negative bacilli causes are rare in adults and are mainly associated with neurosurgical procedures (hospital acquired), as a result of head trauma (Huang et al., 2001, van de Beek et al., 2004) or compromised immunity and concomitant infections in individuals (Bodilsen et al., 2018). E. coli meningitis is common in neonates but is extremely rare in immune competent adults (Zafar et al., 2020, Bichon et al., 2018, Kasimahanti et al., 2018). Spontaneous community acquired E. coli meningitis in adults tends to occur in the presence of a pre-existing risk factor such as advanced age, chronic alcoholism, cirrhosis, immune compromise state (HIV or immunosuppressive therapy) or diabetes mellitus and is secondary to a distant infection such as urinary tract infection or gastro-intestinal infection, with a high mortality in infected patients (Harder et al., 1999, Bichon et al., 2018, Bouadma et al., 2006, Bodilsen et al., 2018). Previously published case reports have been on community-acquired or hospital-acquired E. coli meningitis with no reports of cases with reinfection with a different strain.

5.2 Case presentation

A 58-year-old man presented to a peripheral facility in Kilifi County with history of headache, fever, diarrhoea and reduced consciousness. His initial clinical parameters at the peripheral facility were blood pressure (BP) 133/90 mmHg, heart rate 108 beats/minute, temperature 37.3°C, and saturation of
peripheral oxygen (SpO2) 97%. Initial blood testing showed a haemoglobin of 11 g/dl and was negative for malaria parasites. He was administered intravenous fluids (500 ml), ceftriaxone (1 g), gentamicin (160 mg) and diclofenac (150 mg). His temperature (36.2°C) and heart rate (82 beats/minute) reduced, however although his level of consciousness improved, he remained confused and was therefore referred to KCH for further treatment. On admission to KCH, he developed further diarrhoea and vomiting.

He was reported to have had progressive weight loss over two years due to loss of appetite and poor feeding habits. He consumed alcohol and smoked cigarettes regularly. He had no prior history of hospital admission, surgical procedures, blood transfusion or tuberculosis and was not a known diabetic or hypertensive. On physical examination at admission to KCH his clinical vitals were normal (BP 126/80mmHg, pulse 82 beats/min, temperature 36.2°C, respiratory rate 22 breaths/min, SpO2 97% and random blood sugar 8.3 mmol/l). His Glasgow Coma Score (GCS) was 14/15 (eye 4, motor 6 and verbal 4). The clinical team noted neck stiffness but with a negative Kernig’s test. Laboratory testing on admission to KCH revealed leukocytosis (whole blood cell (WBC) count of 17.9 x 10^3/µl), neutrophilia (84.4%) and lymphopenia (8.2%) (Table 5.1). A lumbar puncture yielded turbid CSF with protein level of 3.55 g/L, elevated WBC (>999/µl), low CSF glucose and Gram-negative rods on staining. The patient tested negative for HIV.

He was treated empirically with ceftriaxone (2 g twice daily) for Gram negative meningitis. Culture of the CSF was positive for *E. coli* which was susceptible to all the first line drugs tested by the laboratory (Table 5.2). Blood culture did not yield any growth. He was treated for 21 days with ceftriaxone. During that time, he was also treated for alcohol withdrawal with parenteral B vitamins and diazepam. He made a good recovery and was eligible for discharge but remained in the ward for a further three days (reason unknown). On the third day, after stopping the ceftriaxone, his level of consciousness began to decline, and he developed a further fever (38.1°C). He was also tachycardic (119 beats/min) with elevated BP (142/99 mmHg) and reduced oxygen levels (80%). He had a repeat blood culture and lumbar puncture performed.

Both were positive with *E. coli*, however, susceptibility testing on these isolates revealed the *E. coli* from both samples was resistant to all β-lactams. By this time he had been on ceftriaxone for three days, with no improvement. He was changed to intravenous chloramphenicol (1 g four times daily), but unfortunately, the patient deteriorated and died two days later.
Table 5.1 Haemogram results on first admission to hospital

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient result</th>
<th>Interpretation</th>
<th>normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (10^6/µl)</td>
<td>4.62</td>
<td>N</td>
<td>4.07 – 6.47</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>13.2</td>
<td>N</td>
<td>10.6 – 17.0</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>38.2</td>
<td>N</td>
<td>33.9 – 49.6</td>
</tr>
<tr>
<td>Platelets (10^3/µl)</td>
<td>164</td>
<td>N</td>
<td>139 – 398</td>
</tr>
<tr>
<td>White blood cells (10^3/µl)</td>
<td>17.9</td>
<td>H</td>
<td>3.0 – 10.1</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>84.4</td>
<td>H</td>
<td>24.7 – 68.2</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>8.2</td>
<td>L</td>
<td>22.0 – 58.0</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>5.8</td>
<td>N</td>
<td>5.3 – 13.8</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.8</td>
<td>L</td>
<td>1.0 – 22.1</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.8</td>
<td>N</td>
<td>0.2 – 1.1</td>
</tr>
</tbody>
</table>

Table 5.2 Initial antimicrobial susceptibility profile of *E. coli* strains from first and second episode of meningitis in adult patient

<table>
<thead>
<tr>
<th>Isolate episode</th>
<th>First <em>E. coli</em></th>
<th>Second <em>E. coli</em></th>
<th>Second <em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>Zone size</td>
<td>Zone size</td>
<td>Zone size</td>
</tr>
<tr>
<td>Drug</td>
<td>Interpretation</td>
<td>Interpretation</td>
<td>Interpretation</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>18 S</td>
<td>6 R</td>
<td>6 R</td>
</tr>
<tr>
<td>Augmentin</td>
<td>20 S</td>
<td>6 R</td>
<td>6 R</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>25 S</td>
<td>6 R</td>
<td>6 R</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>30 S</td>
<td>6 R</td>
<td>6 R</td>
</tr>
<tr>
<td>Ceftraxone</td>
<td>ND</td>
<td>6 R</td>
<td>6 R</td>
</tr>
<tr>
<td>Imipenem</td>
<td>26 S</td>
<td>17 R</td>
<td>15 R</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>21 S</td>
<td>23 S</td>
<td>22 S</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>22 S</td>
<td>18 S</td>
<td>22 S</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>30 S</td>
<td>6 R</td>
<td>6 R</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>19 S</td>
<td>21 S</td>
<td>20 S</td>
</tr>
<tr>
<td>Amikacin</td>
<td>19 S</td>
<td>22 S</td>
<td>22 S</td>
</tr>
<tr>
<td>ESBL</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

S – sensitive, R – resistant, ND – not done
5.3 Laboratory analysis

The strains were retrieved and additional laboratory analyses including antimicrobial susceptibility re-testing and testing for newer drug agents, whole genome sequencing (WGS) and in vitro assays on fitness and virulence were performed (as described in chapters two, three and four respectively). For the competition assay, comparison was made between *E. coli* strains from the CSF samples of the first and second episode while the second strain from blood was compared to strains from blood samples of other patients and matched as described in chapter 2.

5.4 Results

5.4.1 Community acquired susceptible strain is unrelated to nosocomial carbapenem resistant strain

The community acquired *E. coli* from CSF (on admission) was susceptible to all the drugs tested except to ampicillin and ampicillin clavulanate (augmentin) where it had reduced susceptibility (intermediate) on repeat testing (Table 5.3). The second pair of strains (from second episode) were resistant to nearly all the drugs tested, including to newer drugs, except aminoglycosides, chloramphenicol and colistin.

WGS showed that the carbapenem susceptible strain from CSF (CSE-CSF) was ST88 and serotype O8 H17 while the carbapenem resistant strains both from blood and CSF (CRE-blood and CRE-CSF) were ST167 (Enterobase) and serotype O101 H5 (Table 5.4). The CSE-CSF strain had two resistant genes *sul*2 and *tet*B detected by ResFinder as well as the efflux pump gene *mdf*A. While phenotypic susceptibility to tetracycline was not tested, the strain was susceptible to the drug sulfamethoxazole-trimethoprim (SXT) despite presence of *sul*2 gene which confers resistance to sulfamethoxazole.

The CRE-blood and CRE-CSF strains possessed several resistance traits that were not present in the susceptible strain including β-lactamases *bla*NDM-5, *bla*CTX-M-15 and *bla*OXA-1, aminoglycoside resistance genes *aad*A2, *aac(6’)-lb-cr* and *aac(3)*-Ila and genes conferring resistance to other antimicrobials and cleaning compounds *cat*B3, *dfr*A12, *sul*1, *tet*A, *mph*A, *mdf*A, *qac*E. In addition, point mutations in gyrase (*gyr*A S83L, D87N) and topoisomerase genes (*par*C S80I and *par*E S458A) conferring resistance to fluoroquinolones were also present in the resistant strains. Discordance between genotypic and phenotypic results were observed in the chloramphenicol and aminoglycosides results. For chloramphenicol, the resistant strains returned a hit for *cat*B3 gene, which codes for the enzyme chloramphenicol acetyltransferase that confers resistance to chloramphenicol, however, it had only 70% coverage to reference gene used in ResFinder and thus may not have yielded a fully functioning protein.
which may explain the genotype – phenotype discrepancy. However, in the case of aminoglycosides, the \textit{aadA2} encodes a protein that confers resistance to spectinomycin and streptomycin while the product of \textit{aac(3)-Ila} gene confers resistance to several aminoglycosides including gentamicin, tobramycin and sisomicin (Ramirez and Tolmasky, 2010) though the coverage of the gene was 80% to the reference and may lead to a non-functional protein. On the other hand, the presence of the \textit{aac(6')-Ib-cr} gene in Enterobacterales may not necessarily result in resistance to amikacin / tobramycin or ciprofloxacin as was the case in the resistant strains (Park et al., 2006).

There was a difference in the plasmids found in the susceptible and resistant strains with only one plasmid, Col8282, detected in the susceptible strain and four plasmids, Col(BS512), ColpVC, IncFIA and IncFII, detected in the resistant strain. This provides further evidence that the strains were unrelated with no transfer of plasmids between strains and that the resistant strain had several plasmids that carry multiple resistant determinants. There was a difference in the virulence traits carried by the strains. The CSE-CSF strain possessed additional virulence genes not found in the CRE-CSF and CRE-blood strains such as genes involved in iron transport and metabolism (\textit{iucC, iutA, fyuA, ireA, iroN, sitA}), increased serum survival (\textit{iss}, 3 copies), production of outer membrane vesicles (\textit{hlyF}), outer membrane protease (\textit{ompT}), fimbriae and pili (\textit{papC, papA\_F20, lpfA}) and microcin (\textit{cvaC}). This may have enabled the sensitive strain to cause spontaneous community acquired meningitis in the adult. The virulence genes present in CRE-CSF and CRE-blood strains were also present in CSE-CSF and included outer membrane protein complement resistance (\textit{traT}), heat resistant agglutinin (\textit{hra}) and increased serum survival (\textit{iss}, 1 copy).
Table 5.3 Extended antimicrobial susceptibility profile of *E. coli* strains from first and second episode of meningitis in adult patient

<table>
<thead>
<tr>
<th></th>
<th>AMP</th>
<th>AMC</th>
<th>FOX</th>
<th>CTX</th>
<th>CAZ</th>
<th>CRO</th>
<th>IMI</th>
<th>MEM</th>
<th>ETP</th>
<th>CN</th>
<th>AK</th>
<th>CIP</th>
<th>C</th>
<th>SXT</th>
<th>COL</th>
<th>ERV</th>
<th>FDC</th>
<th>FPS</th>
<th>IMR</th>
<th>MEV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>µg/ml</td>
<td>µg/ml</td>
<td>µg/ml</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>µg/ml</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
<td>mm</td>
</tr>
<tr>
<td>CSE- CSF</td>
<td>15</td>
<td>14</td>
<td>22</td>
<td>29</td>
<td>27</td>
<td>29</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>20</td>
<td>20</td>
<td>29</td>
<td>20</td>
<td>22</td>
<td>-</td>
<td>18</td>
<td>25</td>
<td>35</td>
<td>29</td>
<td>33</td>
</tr>
<tr>
<td>CRE- CSF</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>16</td>
<td>&gt;32</td>
<td>21</td>
<td>21</td>
<td>6</td>
<td>21</td>
<td>12</td>
<td>&lt;1</td>
<td>16</td>
<td>6</td>
<td>6</td>
<td>12</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>CRE- blood</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>32</td>
<td>&gt;32</td>
<td>21</td>
<td>22</td>
<td>6</td>
<td>22</td>
<td>6</td>
<td>&lt;1</td>
<td>15</td>
<td>6</td>
<td>6</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

All antimicrobial susceptibility determined by disc diffusion method except imipenem, meropenem, ertapenem and colistin which were determined by minimum inhibitory concentration.

(*) indicates not tested by MIC but by disc diffusion; (-) indicates not tested; red indicates resistance, orange indicates intermediate and green indicates sensitive to drug.

Antibiotics: ampicillin (AMP), ampicillin/clavulanate (AMC), cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), imipenem (IMI), meropenem (MEM), ertapenem (ETP), gentamicin (CN), amikacin (AK), ciprofloxacin (CIP), chloramphenicol (C), cotrimoxazole (SXT), colistin (COL), eravacycline (ERV), cefiderocol (FDC), cefepime/sulbactam (FPS), imipenem/relebactam (IMR), meropenem/vaborbactam (MEV)
Table 5.4 Sequence type, plasmid and resistance genes and resistance conferring mutations in *E. coli* strains

<table>
<thead>
<tr>
<th>MLST</th>
<th>Serotype</th>
<th>Plasmids</th>
<th>Carbapenemase</th>
<th>Other β-lactamases</th>
<th>Aminoglycoside resistance</th>
<th>Other resistance genes</th>
<th>Point mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSE- CSF</td>
<td>88</td>
<td>O8 H17</td>
<td>Col8282</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>sul2, tetB, mdfA, sitABCD</td>
</tr>
<tr>
<td>CRE- CSF</td>
<td>167</td>
<td>O101 H5</td>
<td>Col(BS512), ColpVC, IncFIA, IncFII</td>
<td>NDM-5</td>
<td>CTX-M-15, OXA-1</td>
<td>aadA2, aac(6’)-Ib-cr, aac(3)-Ila</td>
<td>catB3, dfrA12, sul1, tetA, mphA, mdfA, qacE, catB3, dfrA12, sul1, tetA, mphA, mdfA, qacE</td>
</tr>
<tr>
<td>CRE-blood</td>
<td>167</td>
<td>O101 H5</td>
<td>Col(BS512), ColpVC, IncFIA, IncFII</td>
<td>NDM-5</td>
<td>CTX-M-15, OXA-1</td>
<td>aadA2, aac(6’)-Ib-cr, aac(3)-Ila</td>
<td>gyrA: S83L, D87N; parC S80I and parE S458A</td>
</tr>
</tbody>
</table>

125
5.4.2 Carbapenem resistant strain is less fit and virulent than susceptible strain

In individual growth curves in normal LB broth the CSE-CSF strain grew slightly faster and peaked earlier than the resistant strains and this difference was amplified in media with 5% salt with detectable bacterial amplification detected at 5.5 hours in CSE-CSF strain and 8.5 hours in CRE-CSF and CRE-blood strains (Figure 5.1A).

Pairwise competition between CRE-CSF and CSE-CSF strains in standard LB broth media had a mean competition index (CI) of 0.695 (SD=0.25) which indicates that the resistant strain is less fit. However, when competed in 5% salt LB media the CI was 2.33 (SD=1.44) suggesting that the resistant strain was able to outcompete the susceptible strain in an *in vitro* stressful environment.

The CRE-blood strain was compared with carbapenem susceptible *E. coli* strains from other patients that were ESBL producing and non-producing (see chapter four, pairing M6). Against both comparator strains, the CRE-blood strain was less fit in LB media, while in 5% salt LB media it was less fit than the carbapenem susceptible, non-ESBL producing comparator strain, and there was no difference in fitness with the ESBL-producing *E. coli* (Table 5.5).

Table 5.5 *In vitro* pairwise competition of carbapenem resistant *E. coli* from blood (CRE-blood) of meningitis patient versus carbapenem susceptible bacteria (ESBL producing and non-producing) in standard LB media and in 5% w/v salt concentration LB media

<table>
<thead>
<tr>
<th></th>
<th>Standard LB media</th>
<th>5% salt LB media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pairwise competition of CRE-blood <em>E. coli</em> versus</strong></td>
<td>Competition index (SD)</td>
<td>Competition index (SD)</td>
</tr>
<tr>
<td>vs non-ESBL producing <em>E. coli</em></td>
<td>0.59 (0.15)</td>
<td>0.77 (0.09)</td>
</tr>
<tr>
<td>vs ESBL producing <em>E. coli</em></td>
<td>0.51 (0.03)</td>
<td>1.02 (0.37)</td>
</tr>
</tbody>
</table>

In the resistance to serum assay the CSE-CSF remained grew in the presence of the pooled serum (40%) while the CRE-CSF and CRE-blood strains were reduced (Figure 5.1B). At the beginning of the experiment (0 minutes) there were approximately equal number of bacteria (log$_{10}$ 8) but by 45 minutes the CRE-CSF and CRE-CSF *E. coli* strains had reduced by two logs while the CSE-CSF strain was unaffected. At the end of the experiment (3 hours) the susceptible strain had multiplied increasing by three logs while the resistant strain had reduced further.
Figure 5.1 In vitro assays of fitness and virulence of susceptible and resistant *E. coli* meningitis in adult patient.

A) Growth curve analysis of strains in standard LB media (black) and LB media with added salt (5% w/v) (blue). The strains were grown in triplicate on three separate days with the mean plotted and error bars indicating standard deviation (SD). B) Survival of strains in 40% pooled normal human serum every 45 minutes for 3 hours.

5.5 Discussion

Spontaneous community acquired *E. coli* meningitis in immune competent adults is rare, occurring as a result of pre-disposing risk factors and usually secondary to a distant infection. In Africa, the majority of the reported bacterial meningitis infections are due to meningococcus or pneumococcus are from
within the meningitis belt with 5% of the infections due to other less likely pathogens including *E. coli* (Mazamay et al., 2021). In Malawi, located outside the meningitis belt, *E. coli* and other bacteria (non-meningococcus and non-pneumococcus) accounted for 10% of all cases in adolescents and adults reported from 1990 to 2013 (Wall et al., 2013). This indicates that *E. coli* meningitis in African adults while rare can occur in some cases. Kilifi county in Kenya is located outside of the African meningitis belt. This case report describes a unique case of a spontaneous community acquired *E. coli* meningitis in an adult patient who later had a carbapenem resistant infection during hospitalization that proved fatal.

No distant source of infection (e.g. abdominal, urinary tract) was identified and the patient had not undergone any surgery. Initial blood culture on admission was negative although this may have been as a result of antibiotics administered in peripheral facility. He was HIV negative, however he had a history of excess alcohol consumption (identified through the development of withdrawal symptoms during his admission) which is a risk factor for adult *E. coli* meningitis. He was also a smoker and was found to be malnourished which may also have a negative effect on the immune function of an individual (Bourke et al., 2016, França et al., 2009). The primary source of infection is unknown however, given that the patient had complained of gastrointestinal infection prior to seeking medical attention, there may have been an intra-abdominal source which was not identified during his inpatient stay.

The initial isolated strain from CSF was susceptible to nearly all the antibiotics tested and was successfully cleared by ceftriaxone treatment as evidenced by improved outcome and recommendation for discharge. However, the patient remained in the wards for an additional three days, reasons for which are unclear, before he had a relapse. WGS revealed that the strains from the first and second episode were unrelated as they were of different sequence types and serotypes. Strains from the second episode were resistant not only to carbapenems and all β-lactams tested but also to newer drugs recently approved by the US FDA and Europe EMA such as cefiderocol and eravacycline. The mechanism of resistance to newer drugs is not yet fully understood but it is speculated to be due to existing resistance mechanisms or the mode of entry in the case of cefiderocol which utilises the iron transport channels in bacteria (Hackel et al., 2017, Sun et al., 2019). These differences between the strains obtained from the two episodes suggests that the second carbapenem resistant strain may have been acquired in the hospital during the patient’s stay.

An alternative explanation is that the patient may have had a mixed infection of carbapenem resistant and susceptible strains prior to admission to hospital. The resistant strain may have been present in low numbers and could have been missed during selection of a single colony for susceptibility testing, which
is the standard testing method in diagnostic labs. Administration of the broad-spectrum cephalosporin may have provided the selective pressure that allowed the resistant strain to survive while eliminating the susceptible strain. While possible, it is unlikely that the patient would have responded favourably to antibiotic treatment as the resistant strain would have quickly outcompeted the susceptible strain, in addition, infections with MDR strains are generally more likely to be hospital acquired.

*In vitro* bacteria analysis revealed the resistant strain to be less biologically fit than the susceptible strain in multiple assays: when comparing individual growth in normal and stressful environment; in pairwise competition in normal LB media; and in survival in human serum. It grew better than the susceptible strain in the 5% salt LB media only.

Despite this apparent reduced fitness, the strain was still able to infect the patient, with a lethal outcome. The results of the *in vitro* testing are consistent with this strain only having an advantage in the presence of an antimicrobial with activity against the susceptible strain – in this case, ceftriaxone. The treatment with ceftriaxone may therefore have allowed hospital-acquired colonization and subsequent invasive infection with the resistant strain. Alternatively, the patient may have been carrying the strain on admission, but it was not able to cause infection until the more fit, susceptible strain had been eradicated.

In either case, the second infection remains surprising given the fewer virulent genes and decreased survival in normal human serum of the resistant strain compared to susceptible strain. It seems likely that there was an undiagnosed underlying condition, such as an intra-abdominal infection, in addition to the excess alcohol consumption and malnutrition as risk factors for infection.

The only treatment option available for the individual upon microbiological investigations was chloramphenicol but despite treatment, the patient deteriorated and died two days after the chloramphenicol was started. Analysis of assembled genomes revealed the presence of the chloramphenicol acetyltransferase (*catB3*), but with 70% relative coverage compared to the reference gene. Disc diffusion indicated susceptibility to chloramphenicol and it is therefore likely that the less than 100% coverage may indicate that the complete gene is lacking and thus will not result in a fully functioning protein. It would therefore be expected that the chloramphenicol would have been effective *in vivo*, however, by this stage he had been on ineffective treatment for three days as he had only received ceftriaxone which had no activity against the resistant strain.
It is likely that host factors may have a role to play in the outcome of the patient as the resistant organism in this instance behaved as an opportunistic pathogen, as it had reduced fitness and was less virulent than the initial strain and it could be speculated that it might not cause infection in a fully health host. While recognizing that more than a third of spontaneous *E. coli* meningitis patients die (Bichon et al., 2018, Bodilsen et al., 2018), it is likely that the patient was at risk of death even with the initial treatment outcomes given his malnutrition status but it is clear the subsequent, initially untreated, infection with the resistant strain may have complicated his outcome.

The effect of AMR on mortality is not clearly understood as a result of limitation in previous study designs however, a recent study found no significant effect of AMR on mortality or length of stay in African bacteraemia patients of which KCH participated (Dramowski et al., 2021). The lack of difference was suggested to be due to appropriateness of early treatment in patients because of increased resistance in pathogens to the treatments recommended in guidelines. While this may influence the outcome of the patient the previous chapter has shown that resistant bacteria may not necessarily be less fit than susceptible bacteria and host factors including nutrition deficiencies may have a significant role in the patient outcome and should be considered in the treatment of patients especially in low and middle income settings.
6 Conclusions and future work

Antimicrobial resistance is currently one of the biggest threats to human health, threatening to usher in a post-antibiotic era that has the potential to reverse the many gains of modern medicine. Resistance, a natural phenomenon in microbes competing for nutrients within their habitat, has been amplified and extended by the use and misuse of antibiotics. The WHO advocates the research and development of new drugs, especially new antibiotic classes for which current bacterial resistance mechanisms would be ineffective, vaccines against bacteria with high resistance potential and new diagnostic tools that aid in reducing unnecessary antibiotic use in patients (WHO, 2015). It is important to note that in many LMICs where the threat of AMR is greatest, antibiotics are often used as a ‘quick fix’ substitute for poor health systems infrastructure, effective food production and poor hygiene and sanitation (Denyer Willis and Chandler, 2019) and therefore measures to control resistance in such countries need to factor in this context.

The SARS-CoV-2 virus that causes COVID-19 was first reported in China (Huang et al., 2020) and has since spread globally with new highly transmissible variants being reported (CDC, 2021). Some of the patients with viral lower tract respiratory illness such as influenza and respiratory syncytial virus (RSV) have been reported to have bacterial co-infections (Klein et al., 2016, Godefroy et al., 2020, Thorburn et al., 2006) and as such antibiotics have been prescribed as a prevention measure in such cases. Recent studies have shown that among hospitalised COVID-19 patients in high income countries, approximately 10% had a secondary bacterial infection, (Langford et al., 2020, Lansbury et al., 2020) nevertheless, up to 75% of these patients were administered antibiotics empirically especially among the elderly and those with severe disease (Langford et al., 2021).

In Bangladesh, nearly all patients (92%) hospitalised with suspected COVID-19 infections were given antibiotics empirically prior to testing for the virus or bacterial infections (Mah-E-Muneer et al., 2021). A rapid review of COVID-19 national treatment guidelines of ten African countries revealed that several broad spectrum antibiotics mainly from the Watch and Reserve list of the WHO AWaRe categorization of antibiotics were recommended for the empirical treatment of patients including recommendations for antibiotic use in mild cases in some guidelines (Adebisi et al., 2021). This widespread indiscriminate use of important antibiotics such as third generation cephalosporins, fluoroquinolones and macrolides despite the low risk of bacterial co-infection could potentially worsen the current antimicrobial resistance crisis and could have severe implications for the future.
On the other hand, containment measures to reduce the spread of COVID-19 disease (lockdowns, social distancing, travel bans, face masking and handwashing) could also result in reduced bacterial infections and therefore reduced antibiotic use and resistance (Clancy et al., 2020, Collignon and Beggs, 2020). The true impact of the pandemic on AMR will be determined in coming years as more data is obtained, however, it is important that the goals to reduce bacterial resistance are not forgotten but as existing pandemic control measures are put in place, they can also be expanded or exploited to include AMR control measures (active surveillance, proper sanitation and hygiene, antimicrobial stewardship, proper diagnostics and new drugs) and thus help tackle the ‘silent pandemic’.

Kenya launched its National Action Plan (NAP) on prevention and containment of AMR in 2017 in line with the WHO’s Global Action Plan (GOK, 2017) and set up the National Antimicrobial Stewardship Interagency Committee (NASIC). The AMR secretariat, based at the Ministry of Health, was set up to oversee the activities of the NAP and support the NASIC. The plan had five strategic objectives:

- Improve public awareness and understanding, and promote education and training of professionals
- Continuously monitor antimicrobial resistance and use of antimicrobials, and appropriately understand the trends and spread of antimicrobial resistance
- Prevent the spread of antimicrobial-resistant organisms by implementing appropriate infection prevention and control measures
- Promote appropriate use of antimicrobials in the fields of healthcare, livestock production, agriculture and aquaculture
- Promote research on antimicrobial resistance and foster research and development to secure the means to prevent, diagnose and treat the antimicrobial-resistant infections

My thesis fits largely within the second objective to strengthen the knowledge and evidence of AMR through surveillance and research. In addition, the findings of this thesis may also contribute to the knowledge guiding the activities of the third, fourth and fifth objectives. Kenya has limited data on the prevalence of AMR in the country with available published data obtained mainly from hospital-based studies of short duration or focused on specific illness or pathogen-drug combinations (Tadesse et al., 2017, Leopold et al., 2014). There are also limited data on the current situation of AMR in the country and this has been made apparent by the WHO Global Antimicrobial Resistance Surveillance System (GLASS) initiative. Though enrolled in the WHO Global Antimicrobial Resistance Surveillance System, Kenya is yet to provide AMR data due to low data quality of available data, inconsistent supply of
laboratory commodities, lack of microbiological testing capacity in many hospitals, poor diagnostic stewardship and limited technical capacity to detect and report AMR (WHO, 2020). However, recent survey on antibiotic use and resistance in several Kenyan hospitals indicate increased usage of ‘Watch’ antibiotics such as ceftriaxone and ‘Reserve’ antibiotics like carbapenems in tertiary public hospitals (Conference proceedings, Kenya National Antimicrobial Resistance Symposium 18th – 19th November 2021) due to increased reported or perceived resistance.

AMR Surveillance is therefore crucial in guiding the Ministry of Health in allocating necessary funds for control measures that are specific and targeted towards the AMR problem in the country. However, setting a nationwide active surveillance is costly and likely to be unachievable for countries with limited resources. Alternative methods of obtaining nationwide AMR estimates are therefore required such as equipping of sentinel sites with high quality facilities to perform microbiological diagnostics of patient samples or the use of a hub and spoke system for sending key clinical samples to a central laboratory for diagnosis and antimicrobial susceptibility testing. Such models could be employed by the AMR secretariat in Kenya to provide national estimates of AMR while maximising use of the minimal resources.

The aim of my thesis was to understand the mechanisms of resistance to carbapenems isolated from patient samples in a hospital setting with no carbapenem usage until recently. I utilised phenotypic and genotypic methods to investigate the resistance mechanisms in clinical and carriage strains and to determine the effect of multi-drug resistance on the fitness and virulence of the strains in the absence of a selective pressure.

The aim of Chapter 2 was to describe the antibiogram of carbapenem resistant bacteria to commonly known and newer drugs and to describe the β-lactamase activity possessed by the strains. I also described the clinical characteristics of patients infected with carbapenem resistant strains as well as those who had carriage of such organisms. Most of the clinical carbapenem resistant isolates were found mainly in young children and were acquired in hospital. However, these findings may also reflect the longer and well-established surveillance system for children in the hospital, who are more likely to have post-admission cultures taken during admission. Resistance to newer drugs such as cefiderocol and eravacycline was surprising as these drugs are, to the best of my knowledge, currently not available in Kenya. This seems to suggest that there are unidentified existing resistance mechanisms present in such organisms that could reduce the potential usefulness of the drugs in the country and could potentially be spread to other organisms if unidentified and not tackled. These resistance mechanisms could be
located in the same cassette as other known resistance traits or could be due to mechanisms that are not specific to the drugs such as efflux pumps or loss of porin.

There are minimal data on carbapenem resistance in the country and this could be due to limited use of the drugs in public healthcare facilities until recently (Maina et al., 2020). Nonetheless, such bacteria are present in the country, not only in infected patients (Musila et al., 2021), but carried in the gut of individuals as demonstrated by this study, suggesting that an increase in the usage of carbapenems could result in an increase in prevalence of carbapenem resistant bacteria. The findings of the chapter support the activities of the Kenya NAP such as to implement infection prevention and control (IPC) measures in hospitals and to establish a national healthcare associated infections surveillance system and to promote antimicrobial stewardship programs (ASP) in hospitals. The findings also suggest a need for implementing susceptibility testing to newer drugs for surveillance purposes as this could help guide the NAP’s objectives of introducing new antibiotics into the country that could address priority infections. In such cases, institutions with a biorepository of clinical isolates would be useful in evaluating the potential usefulness of such medications.

In chapter 3, I sought to understand the genomic epidemiology of the carbapenem resistant strains by using PCR to detect the presence of well-known carbapenemase genes and WGS to characterize the repertoire of AMR genes. I found that $\text{bla}_{\text{NDM}}$ was abundant in both carbapenem resistant clinical and carriage isolates with occurrence of more than one carbapenemase genes in majority of the \textit{Acinetobacter} species. Surprisingly, only one out of nine \textit{P. aeruginosa} strain possessed a carbapenemase gene, $\text{bla}_{\text{DIM}}$ a recently described resistance gene. None of the strains possessed $\text{bla}_{\text{KPC}}$, $\text{bla}_{\text{VIM}}$ and $\text{bla}_{\text{IMP}}$, however, except for $\text{bla}_{\text{VIM}}$ in \textit{P. aeruginosa}, none of the other genes have been reported in Kenya (Musila et al., 2021, Pitout et al., 2008). A wide array of resistant traits was present within the MDR organisms suggesting that incorporating WGS based surveillance would be valuable for determining resistant traits circulating in the country and identifying new unknown resistance determinants.

Most of the sequenced strains were unrelated as they were of different sequence types suggesting multiple introductory events of the resistant strains in the hospital rather than clonal expansion of a single successful clone, and there was minimal evidence for person-to-person transmission for the clinical isolates. Given that the strains are most likely hospital acquired, it raises questions on the source of the bacteria. One likely source of the strains could be through carriage by other patients as has been shown by carriage in young children in this thesis and further research is required in adult patients to
investigate this as well. A second likely source is an environmental reservoir in the hospital as has been shown in one case of *A. baumannii* and environmental sampling is required to scrutinise this. An alternative explanation would be that the resistance mechanisms are transmitted by plasmids between different strains and/or patients, rather than transmission of the organisms themselves and plasmid sequencing of patient and environmental bacteria can provide more insight on this. Finally, it is possible that the strains are prevalent in the community and patients are already colonised on admission to hospital, with subsequent use of broad-spectrum antimicrobials creating a niche for the resistant organisms to cause clinical infection.

The finding that the majority of CRE infections were hospital acquired supports implementation of IPC measures in hospital to prevent transmission of MDR bacteria to admitted patients who are vulnerable to such infections, but understanding the mechanism of acquisition through further studies will be valuable in understanding where to focus IPC measures and the relative contribution of antimicrobial use.

The aim of Chapter 4 was to understand the effect of multiple resistant traits on the fitness and virulence of resistant bacteria. The hypothesis was that carbapenem resistant bacteria are less fit than susceptible bacteria and therefore implementing an antimicrobial stewardship programme that would control the consumption of carbapenems would prevent a rise in carbapenem resistance in the country. Through individual growth curves and *in vitro* pairwise competition, I demonstrated that while overall carbapenem resistant Enterobacterales were less fit compared to fully susceptible strains, there were instances where the resistant strain had equal or better *in vitro* fitness than the susceptible strain. On comparison of carbapenem resistant strains with ESBL producing carbapenem susceptible strains, I found that many of the carbapenem resistant strains were either more fit or equal in fitness to ESBL strains suggesting that the addition of a carbapenemase bearing cassette or plasmid to an already ESBL resistant strain may have minimal effect on fitness. This is concerning as it may mean that, even in the absence of carbapenem use, an ESBL-producing strain which acquires carbapenem resistance may be sustained by cephalosporin selection pressure. Control of cephalosporins would therefore also be necessary to minimise emergence and transmission of carbapenem resistance.

While bias introduced by genetic differences between the strains may have an effect on the study findings as clinical isolates rather than similar laboratory strains were used, the overall findings support the hypothesis that carbapenem resistant bacteria are less fit than fully susceptible bacteria. However, given that exceptions were observed in some of the strains and comparisons, it implies that
antimicrobial stewardship measures alone will not be effective in controlling carbapenem resistance and additional measures such as IPC and improved water, sanitation and hygiene and new drugs should also be incorporated in efforts to control AMR in the country.

In chapter 5, I illustrate a clinical case showing the negative impact of resistance from a patient perspective. While the patient was treated with antibiotics for the fully susceptible strain, he was later infected with a highly resistant strain, which was less fit and less virulent in vitro, but which proved fatal suggesting that host specific factors may have a significant role to play in the clinical outcome of an individual. These findings outline the importance of robust IPC and antimicrobial stewardship in all hospitals in the country in preventing infections with MDR organisms in patients with underlying conditions that may lead to poor outcomes.

My findings and those from a recently published paper by Musila and colleagues (Musila et al., 2021) demonstrate that multi-drug resistance and in particular carbapenem resistance is present in several regions of the country and is carried in the gut of some individuals (this thesis). While the limited data available from hospital-based surveillance suggests a low prevalence of carbapenem resistance in Kenya, this situation will change when carbapenem use increases significantly as more pathogens become resistant to third generation cephalosporins. Active surveillance, both phenotypic and genotypic, is therefore required to monitor MDR and prevent the occurrence of difficult to treat infections with worse outcomes and high economic burden.

Given the limitations in resources available in implementing the Kenya NAP on containment and prevention of AMR, several options such as use of key hospital sentinel sites across the country that are equipped with fully functioning microbiological labs and trained staff could be used to generate nationwide estimates of AMR. Alternatively, a hub and spoke model where samples collected from patient hospitalised with the major illness can be sent to central laboratories to perform microbiological diagnosis while providing AMR estimates for key bug-drug combinations.

Granted, implementing a national genomic AMR surveillance in LMICs is challenging given the limited technological expertise in conducting WGS (both laboratory and bioinformatics), the associated high cost of setting up the laboratory infrastructure and the challenges of a reliable supply chain of consumables (NIHR-GHRU, 2020, Okeke et al., 2020). This may be overcome through several measures such as partnering with donors and the government to set up central laboratories to perform WGS in-country while partnering with institutions in HICs to build capacity for performing laboratory and
bioinformatic analysis. In Kenya, to maximise on limited resources, WGS can be performed on selected organisms based on antibiogram results and potentially scalable molecular methods such the Oxford Nanopore sequencing apparatus that do not require complex laboratory infrastructure can be used to provide genomic data on AMR in the country. Challenges in bioinformatic analysis could be overcome by use of cloud-based bacterial genomic analysis tools to obtain relevant data.

The main challenge of the study was the small number of resistant Enterobacterales obtained from clinical samples in Kilifi which limited the diversity of strains. This may be due to limitations in systematic sampling post admission especially in adults that may affect the number of hospital-acquired infections detected. I attempted to increase the diversity of Enterobacterales by collaborating with a tertiary private hospital in Nairobi where carbapenems are in use for comparison with Kilifi strains. However, this was hampered by delays in communication and agreements on a working collaboration due to institutional requirements and this option was not pursued further. A second major challenge was the COVID-19 pandemic which prevented my scheduled travel to Queen Mary University of London, the UK collaborating institution, where the in vitro fitness and virulence assays as well as the in vivo pathogenicity in a Galleria mellonella model was to be conducted with comparison with clinical carbapenem resistant strains from UK. This was overcome by conducting the in vitro assays at KWTRP using Kenyan clinical strains only which may have introduced genetic bias in the results obtained for the effect of MDR on fitness but did not affect the overall ability to answer the research question. Nevertheless the in vivo assay could not be performed whose findings would have provided more insight on how multi-drug resistant affects the pathogenicity of the bacteria in vivo as it tackles the host immune response. A third challenge was timely and constant access to laboratory consumables due to importation procurement challenges at port of entry into the country. Access to the KWTRP laboratories was restricted for several months during the start of the pandemic and this affected my timelines for laboratory analysis. This meant that assays had to be designed with available substitutes in the laboratory, however this did not affect the ability to answer the research question. The procurement challenge was experienced before the pandemic due to ongoing difficulties with importing laboratory supplies into the country but was made worse by the pandemic. Finally, WGS which was to be performed at MicrobesNG in the UK was not completed due to illness on the part of the collaborator, however I was able to sequence a selection of the isolates at KWTRP though with time constraints due to delays in reagent procurement. The delay limited my ability to perform further bioinformatic analysis, particularly phylogenetic analysis of my strains with other strains from the country or the region with similar sequence types.
Overall, WGS revealed a diversity in the sequence types of carbapenem resistant bacteria suggesting more than one introductory event of resistant bacteria at KCH. Further work is required to determine if certain resistant traits are associated with particular plasmids (and other mobile genetic elements such as insertion sequence and transposons) and which resistance traits are carried on the same plasmid and could be selected for even in the absence of particular antibiotics. A previous WGS study of *K. pneumoniae* at KCH detected the presence of a pNDM-mar-like plasmid backbone in several previous isolates of different STs however the *blaNDM* gene was not detected (Henson et al., 2017). The authors hypothesized that the plasmid may have been introduced into the county from neighbouring regions, but the resultant gene was lost as a result of lack of carbapenem usage, however, the overall plasmid was probably maintained due to cephalosporin resistance. Only one isolate in my collection had the pNDM-mar-like plasmid though several other plasmids were identified. It is likely that there is a probable link between ESBL genes and *blaNDM* however this would need to be determined by plasmid sequencing using long read technology and thus future work is required to understand this.

Resistance to eravacycline and cefiderocol was detected in both carbapenem susceptible and resistant bacteria and further analysis of the sequence data is required to determine the genetic basis of this resistance as mutations is highly unlikely. Future work is needed to investigate the *in vitro* pathogenicity of the resistant strains in the wax moth caterpillar model while also investigating the efficacy of the newer drugs. These findings could then be compared with the *in vitro* fitness results provide more evidence on the virulence potential of resistant bacteria and help refine AMR control measures in the country.

My thesis has demonstrated the presence of carbapenem resistant bacteria in Kenya capable of causing clinical disease. In many cases the organisms were resistant to all drugs routinely available in Kenyan public hospitals. While I have demonstrated that this resistance may come at a fitness cost, exploiting this cost will only be effective if the use of carbapenems is carefully controlled, and robust, effective antimicrobial stewardship programmes are urgently needed. Additional control efforts, such as infection prevention control and improved water sanitation and hygiene implemented in tandem would also be beneficial.
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Novel Chromosomal Integrations in Extensively Drug-Resistant Pseudomonas aeruginosa.


**Pseudomonas** spp. and **Acinetobacter** spp. *Journal of Clinical Microbiology*, 40, 3798-3801.


Appendices

8.1 Appendix 1: Ethical approval

KENYA MEDICAL RESEARCH INSTITUTE
P.O. Box 54640-00208, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-295901, 0733-400003, Fax: (254) (020) 2720038
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KEMRI/RES/7/3/1

November 29, 2018

TO: ANNE AMULELE,
PRINCIPAL INVESTIGATOR

THROUGH: THE DIRECTOR, CGMR-C,
KILIFI

Dear Madam,

RE: KEMRI/SERU/CGMR-C/139/3748 (RESUBMISSION 2 OF INITIAL SUBMISSION): THE EFFECT OF MULTI-DRUG RESISTANCE ON THE FITNESS AND PATHOGENICITY OF CARBAPENEM RESISTANT ORGANISMS (CORs) IN THE ABSENCE OF SELECTIVE PRESSURE.

Reference is made to your letter dated November 22, 2018. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on November 23, 2018.

This is to inform you that the issues raised during the 279th Committee C meeting of the KEMRI Scientific Ethics Review Unit (SERU) held on September 27, 2018, have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, November 29, 2018 for a period of one year. Please note that authorization to conduct this study will automatically expire on November 28, 2019. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by October 17, 2019.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
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