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Triangulating the Molecular Epidemiology of Carbapenem-Resistant Enterobacterales from Humans, Food Animals, and the Environment

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A thesis submitted to the School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal (UKZN) for the degree of Doctor of Philosophy in Medicine.

This thesis consists of chapters which are written as a set of research manuscripts submitted or intended for submission to internationally recognized peer-reviewed journals with an overall introduction and final summary.

This is to certify that the content of this thesis is the original research work of Dr Yogandree Ramsamy, carried out under our supervision at the Department of Medical Microbiology, College of Health Sciences, School of Laboratory Medicine and Medical Sciences, Antimicrobial Research Unit (ARU), University of KwaZulu-Natal (UKZN), Durban, South Africa.

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DECLARATION

I, Dr. **Yogandree Ramsamy,** declare that

1. The research undertaken, reports and manuscripts in this thesis, is my original work, except where otherwise indicated.

2. This thesis has not been submitted for consideration for any degree or examination at any other university.

3. This thesis does not contain other person's writing, work, data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

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Signed:

Date: 20 July 2021

DEDICATION

To my dearest sister

Shamendri Ramsamy

My dearest Sham, words cannot express the loss I feel not having you near, but I do take solace in the fact that you have been one of my greatest supporters. I have no doubt that you have watched over me and guided me through this trying process in my life. My deepest gratitude to God for allowing me the opportunity to care for and love someone such as yourself. I know that you are proud of me as you

WALK in Heaven beside angels such as yourself.

Love always, Yogan

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LIST OF MANUSCRIPTS INCLUDED IN THIS THESIS

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2. **Ramsamy, Y**., Mlisana, KP., Amoako, DG., Allam, M., Ismail, A., Singh, R., Abia, ALK and Essack SY. Pathogenomic Analysis of a Novel Extensively Drug-Resistant *Citrobacter freundii* Isolate Carrying a bla_{NDM-1} Carbapenemase in South Africa. *Pathogens*. 2020;9(2):89. Published 2020 Jan 31. doi:10.3390/pathogens9020089

3. **Ramsamy, Y**., Mlisana, K.P., Amoako, D.G., Allam M, Ismail A, Singh R, Abia, ALK and Essack, SY. Comparative Pathogenomics of *Aeromonas veronii* from Pigs South Africa: Dominance of the Novel ST657 Clone. *Microorganisms*. 2020;8(12):2008. Published 2020 Dec 16. doi:10.3390/microorganisms8122008

LIST OF PEER-REVIEWED PUBLICATIONS RELATED TO THIS THESIS (2016 – 2021)

The following publications related to this study were carried out at a central, specialized level 4 healthcare facility in KwaZulu-Natal. The related study undertook genomic analysis of carbapenem-resistant Enterobacterales (CRE) obtained from rectal swabs of colonized patients and from blood cultures of patients with invasive carbapenem-resistant infections admitted to the intensive care unit (ICU). Amongst the key findings was that of a novel ST 3136 *Klebsiella pneumoniae* strain. The publications appear in the appendices of this thesis.

1. **Ramsamy, Y**., Mlisana, KP., Allam, M., Ismail, A., Singh, R., Amoako, DG and Essack, SY. 2018. Whole-Genome Sequence of a Novel Sequence Type 3136 Carbapenem-Resistant *Klebsiella pneumoniae* strain, Isolated from a Hospitalized Patient in Durban, South Africa. *Microbiology Resource Announcement*, 7, 1-2.

2. **Ramsamy, Y**., Mlisana, K.P., Allam, M., Amoako, D.G., Abia, A.L.K., Ismail, A., Singh, R., Kisten, T., Swe Han, K.S., Muckart, D.J.J., Hardcastle, T., Suleman, M and Essack, S.Y. 2020. Genomic Analysis of Carbapenemase-Producing Extensively Drug-Resistant *Klebsiella pneumoniae* Isolates Reveals the Horizontal Spread of p18-43_01 Plasmid Encoding $bla_{\text{NDM-1}}$ in South Africa. *Microorganisms*, 8, 1-16.

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1. **Ramsamy, Y**., Muckart, DJJ and Mlisana, KP. 2016. Antimicrobial Stewardship in South Africa: A Fruitful Endeavour. *Lancet Infectious Diseases,* 16, 1324.

2. **Ramsamy, Y**., Muckart, DJJ., Han, KSS., Hardcastle, TC., Bruce, JL and Mlisana, KP. 2016. The Effect of Prior Antimicrobial Therapy for Community Acquired Infections on Bacterial Isolates from Early and Late Ventilator Acquired Pneumonia in a level I Trauma Intensive Care Unit. *South African Journal of Infectious Diseases,* 32, 91-95.

3. **Ramsamy, Y**., Hardcastle, TC and Muckart, DJJ. 2017. Surviving Sepsis in the Intensive Care Unit: The Challenge of Antimicrobial Resistance and the Trauma Patient. World Journal of Surgery, 41, 1165-1169.

4. **Ramsamy, Y**., Essack, SY., Sartorius, B., Patel, M and Mlisana, KP. 2018. Antibiotic Resistance trends of ESKAPE Pathogens in KwaZulu-Natal, South Africa: A Five-year Retrospective Analysis. *African Journal of Laboratory Medicine,* 7, 1-8.

5. Chetty, S., Reddy, M., **Ramsamy, Y**., Naidoo, Y and Sabiha, Essack. 2019. Antimicrobial stewardship in South Africa: a scoping review of the published literature, *JAC-Antimicrobial Resistance*, 1, 1-16.

6. Nansook, HB., Parbhoo, N., Steele, G., Mhlongo, T., Naidoo, N., Ramjee, RJ., Reddy, M and **Ramsamy, Y**. 2019. Antimicrobial stewardship at Prince Mshiyeni Memorial Hospital in KwaZulu-Natal – a pharmacist's perspective on this multidisciplinary strategy, *South African Pharmaceutical Journal*, 86, 7-11.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABC	ATP-binding cassette
ACT	AmpC-type β-lactamase
AIM	Australian imipenemase
ABR	Antibiotic resistance
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ARG	Antibiotic resistance gene
BEL-1	Belgium extended spectrum ß-lactamase
BES-1	Brazilian extended spectrum ß-lactamase
CDC	Centers for Disease Control and Prevention
CIM	Carbapenem inactivation method
CRE	Carbapenem-resistant Enterobacterales
CPE	Carbapenemase-producing Enterobacterales
CP-CRE	Carbapenemase-producing Carbapenem-resistant Enterobacterales
non-CP-CRE	Non-carbapenemase-producing Carbapenem-resistant Enterobacterales
CLSI	Clinical Laboratory Standards Institute
CMY	Cephamycin-hydrolyzing β-lactamase
DIM	Dutch imipenemase
DHP-I	Dehydropeptidase-I
DNA	Deoxy-ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FIM	Florence imipenemase
GIM	Guiana Imipenemase
GES	Guiana extended spectrum β-lactamase
IBC	Integron-borne cephalosporinase
IBM	Inhibitor-based method
IMI	Imipenem-hydrolyzing β-lactamase
KHM	Kyorin Health Science metallo-β-lactamase

KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MATE	Multidrug and toxic compound extrusion
MBL	Metallo-β-lactamase
MIC	Minimum inhibitory concentration
MDR	Multidrug resistant
MDRO	Multidrug-resistant organism
MFS	Major facilitator superfamily
MGE	Mobile genetic element
MHT	Modified Hodge Test
MRSA	Methicillin-resistant *Staphylococcus aureus*
NDM	New Delhi metallo-β-lactamase
NICD	National Institute for Communicable Diseases
NMC	Non-metallo carbapenemase
OM	Outer membrane
OMP	Outer membrane protein
OXA	Oxacillinase
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PER	Pseudomonas extended resistance
QS	Quorum sensing
RCT	Randomized control trial
RND	Resistance-nodulation-division
SFO-1	*Serratia fonticola*
SIM	Seoul Imipenemase
SMB	*Serratia marcescens* MBL
SMR	Small multidrug resistance
SPM	Sao-Paolo metallo-β-lactamase
ST	Sequence type
TLA	Tlahuicas
TMB	Tripoli metallo-β-lactamase
UV	Ultraviolet
VEB	Vietnam extended spectrum ß-lactamase
VIM	Verona integron-encoded metallo- β-lactamase
WGS	Whole genome sequencing

WHO	World Health Organization
WWTP	Wastewater treatment plant
XDR	Extremely drug resistant

ABSTRACT

Introduction

Antimicrobial resistance (AMR) is largely a consequence of selection pressure, from indiscriminate antimicrobial use in humans and animals, however release of other resistance-driving chemicals such as metals and biocides also play a role in development of AMR. Disposal of these drivers of AMR into the environment, requires One Health approach towards its understanding and containment.

The ongoing dissemination of carbapenem-resistant Enterobacterales (CRE), particularly carbapenemase-producing Enterobacterales (CPE), represents a significant public health issue threatening the lives of millions globally. Carbapenems, in human health, are antibiotics of last resort, and conserving them for the future is of utmost importance. Therefore, it is critical to conduct surveillance of CRE and CPE in a One Health context using molecular techniques to determine a representative picture of the overall problem of CRE, its evolution and dissemination. This point prevalence study ascertained the carriage of CPE in humans, livestock animals (pigs), and environmental sources within the same geographical area of the uMgungundlovu district, KwaZulu-Natal, South Africa and triangulated the molecular epidemiology of CPE in humans, food animals, and the environment.

Methodology

The point prevalence study involved collecting rectal swabs from pigs and humans along with environmental water samples collected from a wastewater treatment plant that received water from both the hospital and abattoir. All samples were processed at the accredited National Health Laboratory Service (NHLS) Public Health Laboratory in KwaZulu-Natal. Selective chromogenic agar was used to isolate CPE from all samples obtained across the three sectors. Microbiological processing and analysis of samples were undertaken as per standard operating procedures of the NHLS. Bacterial identification and antibiotic susceptibility testing were performed using the VITEK® 2 automated system. Pure isolates were then subjected to whole genome sequencing (WGS), and generated sequence data were analysed using different bioinformatic tools, to determine the resistomes, virulomes, mobilomes, clonality, and phylogenomics of these isolates.

Results

Of 587 rectal swab samples screened for CPE, 230 (39.1%) were from humans, 345 (58,7%) were from pigs with 12 (2%) water samples. A total of 19/587 (3.2%) isolates i.e., 15 from humans and four from the environment, were CRE. All the environmental isolates (4) and 12/15 human isolates were carbapenemase producers. The three non-carbapenemase producing human isolates were resistant to ertapenem but susceptible to meropenem and imipenem. No CPE were isolated from the pig samples. Sixteen of the nineteen isolates were CPE. The most common CPE was *Klebsiella pneumoniae* 9/16 (56%), followed by *Enterobacter hormaechei* 3/16 (19%), *Klebsiella quasipneumoniae* 2/16 (13%), a novel ST498 *Citrobacter freundii* 1/16 (6%), and *Serratia marcescens* 1/16 (6%). Carbapenem

resistance was attributed to plasmid-mediated carbapenemase encoding genes: *bla*$_{\text{OXA-181}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-484}}$, *bla*$_{\text{NDM-1}}$, and *bla*$_{\text{GES-5}}$. Notably *bla*$_{\text{OXA-181}}$ and *bla*$_{\text{NDM-1}}$ were found in both human and environmental isolates. Common MGEs were found in different bacterial species/clones across humans and the environment. The IncFIB(K) plasmid replicon was found in all isolates of *K. quasipneumoniae* (2) from the environment and the majority of the *K. pneumoniae* strains (7/9) from humans. The majority of the *K. pneumoniae* isolates were OXA-181 (5/9) producers. The vast majority of β–lactamase encoding genes were associated with class 1 integrons *IntI*1, insertion sequences (IS) (*IS*91, *IS*5075, *IS*30, *IS*3000, *IS*3, *IS*19, *ISKpn*19, *IS*5075) and transposons (*Tn*3). The Col440I plasmid replicon the most common and identified in 11 (26.82%) isolates, mostly *E. hormaechei* (n = 6). The IncL/M(pMU407) and IncL/M(pOXA48) plasmid replicons were found exclusively in *Klebsiella pneumoniae*, with all but one of these isolates being OXA-181 producers. Virulence determinants were predicted for the eleven *Klebsiella spp.* as the most common species isolated where a total of 80 virulence genes were delineated. Phylogenomic analysis with other South African carbapenemase-producing *K. pneumoniae*, *E. hormaechei*, *S. marcescens,* and *C. freundii* from different sources (animals, environmental sources, and humans) revealed that some species from this study clustered with clinical isolates, some clustered according to sequence type and other species belonged to the same clonal node as other clinical isolates. Phylogenetics linked with metadata revealed that some isolates clustered according to the source. Notably, five *Aeromonas* spp. isolates, part of a novel sequence type – ST657, and habouring the *bla*$_{\text{CPHA-3}}$ and *bla*$_{\text{OXA-12}}$ genes were obtained from pigs during the screening process of this One Health point prevalence study. Although these isolates were resistant to imipenem, they were not CPE. Two ARGs were noted, *bla*$_{\text{CPHA3}}$ and *bla*$_{\text{OXA-12,}}$ conferring the resistance to imipenem and penicillin (ampicillin and amoxicillin). No MGEs were identified in these isolates.

Conclusion

This One Health Study delineated the resistome, mobilome, virulome, and phylogeny of CPE in human and the environment sectors, highlighting the potential propagation of carbapenemase antibiotic resistance genes via diverse MGEs across the sectors. Such genomic fluidity highlights the need for comprehensive, integrated genomic surveillance in a One Health context to address AMR successfully.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0. Introduction

The exponential escalation of drug-resistant infections has become a major public health concern. Initially, a phenomenon of health care institutions and especially intensive care units, bacterial resistance is now common in community-acquired infections, and not only critical care physicians but all health care personnel are confronted by this therapeutic dilemma (CDC, 2013). Bacteria have evolved several mechanisms to combat antimicrobials such as a) enzymatic inactivation, b) interruption of cell wall synthesis, c) inhibition of protein synthesis via interaction with ribosomal subunits, d) disruption of nucleic acid synthesis, e) impairing metabolic pathways, and f) disruption of the cell membrane (Tooke et al., 2019).

The estimated number of cases of drug-resistant infections in the United States is more than 2,8 million, with a predicted death rate of more than 35,000 patients (CDC, 2019). The annual fiscal burden of treating resistant infections in the United States amounts to $20 million and an estimated 8 million health care days, which translates into an additional 21,000 years. In Europe, the estimate is an additional €1.6 million and 2.5 million additional days or 6,800 health care years (Fair and Tor, 2014, Boucher et al., 2009). Although there is a dearth of similar statistics on deaths attributable to drug-resistant infections in Africa, antibiotic resistance is not well-documented on the continent. A study conducted in South Africa reviewing neonatal sepsis caused by multidrug-resistant Enterobacterales revealed an increase in carbapenem-resistant Enterobacterales (CRE) from 2.6% to 8.9% ($p = 0.06$) over 2 years (2013-2015). *Klebsiella pneumoniae* was the most common isolate recovered (66.2%) (Ballot et al., 2019). Mathew and colleagues (2019) reported increasing resistance to multiple classes of antibiotics in many African counties. High overall resistance rates were observed amongst Gram-negative isolates to commonly used antibiotics such as amoxicillin (92.8%), ampicillin (94%), trimethoprim-sulfamethoxazole (85.7%), ceftriaxone (58.3%), and gentamicin (54.8%) from various African countries including Ethiopia, Tanzania, and Benin (Mathew et al., 2019). As reported in a review by Osei Sekyere et al. (2016), there has been an increase in resistance to carbapenems amongst Gram-negative bacteria in South Africa mediated predominantly by NDM-1 or OXA-48 carbapenemases amongst isolates of *K. pneumoniae, Acinetobacter baumannii, Enterobacter cloacae, Serratia marcescens* and other Gram-negative bacteria. The review by Osei Sekyere et al. (2016) highlighted that the following between January 2000 and May 2016, NDM (n = 860), OXA-48 (n = 584), 131 VIM (n = 131), and 45 IMP (n = 45) carbapenemases were isolated from *K. pneumoniae* (*n* = 1138), *A. baumannii* (*n* = 332), *Enterobacter cloacae* (*n* = 201), and *S. marcescens* (*n* = 108). Carbapenems are considered antibiotics of last resort globally, and resistance to these antibiotics negatively impacts the therapeutic

management of life-threatening bacterial infections at healthcare institutions. Due to the high attributable mortality associated with carbapenem-resistant infections and their implications on public health, this global phenomenon cannot be ignored. The substantive increase in broad-spectrum cephalosporin, aminoglycoside, and quinolone resistance observed in Gram-negative bacteria is forcing increased reliance on carbapenems (Livermore, 2009). CRE, especially those containing transferable carbapenemase-encoding genes, are spreading rapidly, leaving agents such as colistin and tigecycline as the last line of defense (Karaiskos et al., 2019, Meletis, 2016, Fair and Tor, 2014).

The threat of a post-antibiotic era within the 21st century prompted the World Health Organisation (WHO) to launch a priority pathogen list for the research and development of new antibiotics for drug-resistant bacteria while urging equal attention to infection prevention and control and antimicrobial stewardship. This list contains three tiers, viz., critical, high, and medium priorities. Priority one and of critical concern are the Gram-negative carbapenem-resistant *A. baumanni*, *Pseudomonas aeruginosa*, and Enterobacterales. High priority species include vancomycin-resistant *Enterococcus faecium*, methicillin and vancomycin-resistant *Staphylococcus aureus*, clarithromycin-resistant *Helicobacte*r spp., and quinolone-resistant *Campylobacter* spp., *Salmonella* spp., and *Neisseria gonorrhoeae*. Of lower and medium priority are penicillin-resistant *Streptococcus pneumoniae*, ampicillin-resistant *Haemophilus influenzae*, and quinolone-resistant *Shigella* spp. (WHO, 2017).

Although indiscriminate prescribing of antibiotics by clinicians accounts for much of the problem, non-therapeutic use in the food industry for prophylaxis, metaphylaxis, or growth promotion has also contributed substantively. The volume of antibiotics used for non-therapeutic reasons has been estimated to be 12 times greater than that for therapy. When used therapeutically, the consumption may amount to more than eight times that in human practice. In addition, the use of quinolones, cephalosporins, and glycopeptides in animal husbandry has proven to be associated with the development of resistance. This misuse is not limited to livestock as aminoglycosides and tetracyclines are also used on fruit such as apples and pears (Fair and Tor, 2014).

The development of resistance challenges the development of new antibiotic agents to combat multidrug-resistant pathogens due to the normal evolutionary process of mutation resulting in natural selection conferring a survival benefit (Munita and Arias, 2016). Furthermore, although antimicrobials account for 30% of pharmaceutical budgets in the United States, less than 2% is allocated to research into new drugs. Although several reasons account for this, fiscal considerations are paramount. The cost of developing a single new drug is estimated at an excess of $1.7 billion. However, antibiotics are generally prescribed in short courses in contrast to chronic long-term prescriptions making them less profitable (Fair and Tor, 2014).

1.1. AMR - A One Health Perspective

Antimicrobial resistance (AMR) is a global issue that extends beyond the realms of human health and is thought to be 'quintessentially a One Health issue' (Robinson et al., 2016). The One Health approach is defined as '...the collaborative effort of multiple disciplines – working locally, nationally, and globally – to attain optimal health for people, animals, and our environment…', (King et al., 2008), highlighting the critical fact that human health is connected to animal and environmental health. Antibiotics are used in humans and animals in the treatment of infectious diseases. In the animal population, antibiotics are also used for prophylaxis, metaphylaxis, and growth promoters to meet the increasing global food demand and security (Rushton et al., 2014). The overuse and misuse of antibiotics increase selection pressure, accelerating antibiotic resistance (ABR) development. Therefore, the large-scale use of antibiotics in animals is thought to be a significant contributor to ABR in animals, with evidence highlighting the correlation between antibiotic consumption and the development of ABR antibiotic resistance (Bennani et al., 2020).

ABR development is a natural evolutionary process as bacteria can produce and use antibiotics against other bacteria, leading to a low level of natural selection for resistance to antibiotics requiring no human action. In some circumstances, bacteria are naturally/inherently resistant to certain antibiotics. Bacteria can also acquire resistance via 1) by a genetic mutation or 2) by acquiring resistance from another bacterium (Munita and Arias, 2016). Spontaneous genetic mutations in the chromosomal DNA can often prevent a drug from binding to its target site; bacteria may acquire enzymes that hydrolyse antibiotics and express efflux pumps that extrude antibiotics from the bacterial cell. In most cases, genes encoding these mechanisms are located on mobile genetic elements (MGEs) such as transposons, integrons, and plasmids that can be acquired by horizontal gene transfer (HGT), allowing the sharing of antibiotic resistance genes (ARGs) between similar or different species of bacteria, thereby facilitating the dissemination of ABR (Fletcher, 2015).

Naturally occurring resistance in bacteria found in soil can be amplified because of human activities such as adding manure to the soil. While persistent antibiotics in an environment induce selective pressure for the development of ABR, ARGs discharged into the environment (genes found in manure, sewage, hospital waste) have contributed to the ABR in the environment. Discharges of antibiotics from the pharmaceutical industry, health care facilities, animal facilities, and wastewater treatment plants (WWTP) can select for ABR in rivers, estuaries, and other running surface water bodies, providing a means for the evolution and dissemination of ABR. WWTPs, which, in many cases, cannot completely remove antibiotics, antibiotic-resistant bacteria, or ARGs, despite the implementation of sanitation protocols, dispose effluents into rivers which in turn affects the environment by changing the biodiversity and enhancing the selection of antibiotic-resistant bacteria (Eduardo-Correia et al., 2019).

To date, environmental sampling is an often-neglected aspect in current AMR surveillance systems, despite the information available on environmental influences on AMR. The role played by the environment as an AMR reservoir maintaining AMR genes must be acknowledged and included in AMR surveillance. Therefore, it is important to establish "One Health" surveillance systems incorporating AMR surveillance in humans, animals, and the environment (Thakur and Gray, 2019).

The movement of ARGs between humans, animals, and the environment can occur directly or indirectly via food or environmental sources. Bacteria harboring ARGs can be transferred from animals to humans via the food chain through food contamination during the handling process and the subsequent consumption of raw or undercooked food, or indirectly via the environment (Bennani et al., 2020). Antibiotic-resistant bacteria (ARB) and ARGs are discharged via drainage, solid waste, and wastewater from animal farms into receiving environments. Humans are likely to encounter these bacteria via ingestion (food/water) and occupational exposure (e.g., ingestion and inhalation) (He et al. 2020). During farming, the application of manure containing ARGs is likely to introduce ARB and ARGS in the environment. The contamination of the environment with ARBs ARGs presents a serious threat to human health, the natural environment, and animals. ARB containing ARGs, shed by animals, contaminate agricultural sites where there may be transported through run-off into surface water to result in contamination water pollution (Laxminarayan et al., 2013). River water polluted by agriculture and aquaculture activities propagates the spread of ABR, with bacteria spreading to more than one food chain (Bonardi and Pitino, 2019). A recent study conducted by Swift et al. further suggests that AMR and multidrug resistance (MDR) prevalence in wildlife extends beyond anthropogenic factors (Swift et al. 2019). This fluidity and transmission of ARGs between different environments propagate the spread of ABR, threatening all three sectors (Bonardi and Pitino, 2019.

The selection, transmission, and persistence of AMR in humans, animals, and the environment are complex problems requiring collaboration and multisectoral partnerships in addressing this challenge. As outlined by the UN General Assembly and the tripartite alliance of the WHO, the Food and Agriculture Organization, and the World Organization for Animal Health, a One Health approach is required to curb AMR (Essack, 2018). The One Health approach to addressing AMR in humans, animals, and the environment requires joint efforts across all three sectors on surveillance, research, education, awareness, promoting good practices in food supply, the implementation of infection prevention and control measures (such as hand hygiene and immunization) and stewardship practices. This involves close working relations between governmental, non-governmental, and private sector representatives from the human, animal, and environmental health sectors, enabling information sharing on implementing interventions addressing AMR holistically and measuring its outcomes (Bright-Ponte et al., 2019).

2.0. Literature review

The background and literature review explores antibiotics, relevant antibiotic resistance mechanisms emphasising carbapenems and carbapenem resistance. First, a broad overview of the β-lactam class of antibiotics is provided, followed by a discussion of the carbapenems. Next, a brief general discussion on the various mechanisms of resistance to β-lactams is followed by an in-depth look at carbapenem resistance focusing on distinct carbapenemases of global, local, and clinical relevance**.** Finally, the epidemiology of carbapenem resistance is discussed, emphasising carbapenemases and relevant carbapenemase-conferring genes in humans, animals, and the environment forming a case for the One Health study.

2.1. β-Lactam antibiotics

Antibiotics, considered the mainstay of treatment for bacterial infections, have saved many lives for several years. The β-lactam class is the most widely used antibiotic class for both community and hospital-acquired infections due to its proven efficacy and safety profile (Fritsche et al., 2005). For example, before penicillin, the mortality rate from streptococcal pneumonia was 50% after little more than seven days, whereas this fell to around 10% in the penicillin era. The success of penicillin prompted the search and development of additional derivatives (Drawz and Bonomo, 2010). This paved the way for the development of other β-lactam antibiotics that are in clinical use today. Since the discovery of penicillin by Alexander Fleming in 1928, several β-lactam antibiotics have been produced, including penicillins, narrow and broad-spectrum cephalosporins, cephamycins, monobactams, and carbapenems (Babic et al., 2006).

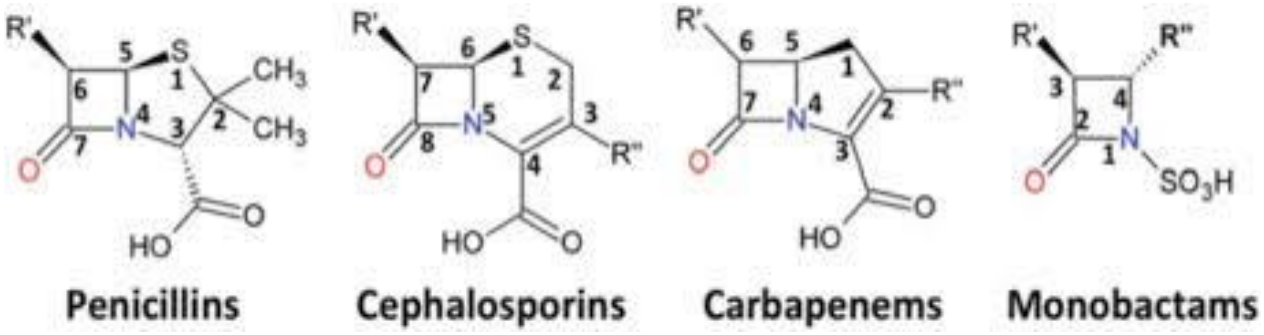

Figure. 1 Chemical structure of β-lactam antibiotic classes that are in current clinical use King et al., 2017)

2.2. Mechanism of action of β-lactams

β-Lactams are bactericidal antibiotics that interfere with the formation of the bacterial cell wall by covalent binding to penicillin-binding proteins (PBPs) (Öztürk et al., 2015). The peptidoglycan found

in bacterial cell walls is composed of long sugar polymers. Transglycosidases facilitate cross-linking of the glycan strands within the peptidoglycan layer, enabling the peptide chains to extend from the sugars in the polymers and form cross-links from one peptide to another. For example, within this layer, the D-alanyl-alanine portion of the peptide chain is cross-linked by glycine residues in the presence of PBPs (Kapoor et al., 2017, Kahne et al., 2005, Reynolds, 1989).

PBPs are enzymes involved in the terminal steps of the cross-linking of peptidoglycan synthesis in bacteria. They are unique to each bacterial species and vary in number per organism. In bacterial cells, PBPs 1a, 1b, 2, and 3 are essential for growth and survival. PBPs 1a and 1b are thought to be dual transpeptidases-transglycosylases, catalyzing glycan chain elongation and peptidoglycan cross-links. PBP 2 and PBP 3 function as transpeptidases, with PBP 3 being important in forming the bacterial septum during cell division. PBP 2 is required for lateral cell wall elongation and the maintenance shape. Around 70% of peptidoglycan synthesis and thus the shape of the bacteria is attributable to the activity of PBP2. PBPs 4, 5, 6a, 6b, and 7 are generally dispensable, as inactivity of these PBPs does not impact the vitality of the cells. Non-essential PBPs 4, 5, and 6 function as DD-carboxypeptidases, accounting for around 50% of the penicillin-binding capacity in bacteria which essentially degrade pentapeptide side chains to tetrapeptide in the peptidoglycan (Pfeifle et al., 2000). PBPs are categorized into low and high molecular mass PBPs, with the low molecular mass PBPs being mono-functional. The high molecular mass PBPs are divided into two categories, i.e., class A and class B. Class A consists of bifunctional enzymes, including a transpeptidase plus a transglycosylase domain, and class B consists of D-ala-D-ala carboxypeptidases. High molecular mass PBPs 1a and 1b are found in Gram-negative bacteria involved in cell lysis. The arrest of cell division is accomplished by inhibiting PBP2 with an associated formation of spherical cells. The inhibition of PBP3 arrests cell division, resulting in filamentation (Goffin and Ghuysen, 1998, Bush and Bradford, 2016). Essentially cell death is accomplished through the inhibition of one or more of these PBPs.

β-lactam antibiotics contain the highly reactive four-membered β-lactam ring that mimics the D-alanyl D-alanine portion of the peptide chain that PBP normally binds. The interaction between PBP and the β-lactam ring prevents the synthesis of new peptidoglycan. This disruption of the peptidoglycan results in the lysis of the bacterium (Kapoor et al., 2017). Simultaneous blocking of transpeptidases and activating autolysins lead to the halting of cell wall synthesis resulting in cell lysis and destruction of the bacterium (Rahman et al., 2018). β-lactams exhibit time-dependent killing where bactericidal activity continues as long as the plasma concentration is more than the (MIC) minimum bactericidal concentration (Lodise et al. 2006).

The various sub-classes of β-lactam antibiotics will be briefly highlighted in the discussion below, emphasizing the carbapenems and their development.

2.2.1 Penicillin

Penicillins are categorized into naturally-occurring penicillins, isoxazolyl penicillins, aminopenicillins, ureidopenicillins, carboxypenicillins, amidinopenicillins, and the non-classical 6-α-methoxypenicillin. Benzylpenicillin has activity against non-penicillinase and non-oxacillinase-producing Gram-positive cocci, small Gram-positive bacilli including *Listeria*, non-penicillinase producing Gram-negative cocci, and most anaerobes excluding *Bacteroides.* Examples of isoxazolyl penicillins which include oxacillin, cloxacillin, dicloxacillin, and nafcillin, are structurally different from penicillins due to the incorporation of the additional side chain to the 6 APA nucleus common to all penicillins (Essack, 2001, Herman and Gerding, 1991). Although these agents have activity against penicillinase-producing staphylococci, they display no activity against oxacillin-resistant staphylococci (Mulligan et al., 1993). Broad-spectrum aminopenicillins such as ampicillin display appreciable activity against non-β lactamase-producing strains of *Proteus mirabilis*, *E. coli*, *H. influenzae*, *Salmonella* spp. and *Shigella* spp. due to the additional amino group in the α-position of the side chain. (Ieven et al., 2018). Ureidopenicillins, including piperacillin, piperazine, azlocillin, and mezlocillin, have heterocyclic groups substituted on the α-amino group, which increases its activity against organisms such as *Klebsiella* spp. Carboxypenicillins such as carbenicillin and ticarcillin are effective against *P. aeruginosa*, *Enterobacter* spp. and indole positive *Proteus* spp. due to the addition of a carboxylic, sulfamic, or sulphonic acid on the carbon atom of the β-lactam side chain (Essack, 2001). Piperacillin has greater activity against Enterobacterales and *P. aeruginosa* than carbenicillin and ticarcillin (Bush and Johnson, 2000).

2.2.2 β-Lactamase inhibitors

β-lactamase production by bacteria is a growing problem and has compromised the use of penicillins as monotherapy for various clinical conditions. To address the problem, β-lactam/β-lactamase inhibitor combinations have been introduced, allowing ampicillin, amoxicillin, piperacillin, and ticarcillin to be useful due to their combination with appropriate β-lactamase inhibitors (Bush and Bradford, 2016). These include the first-generation β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam), which are essentially derivatives of β-lactams that inactivate class A and some class C serine β-lactamases. The newer generation of β-lactamase inhibitors includes avibactam, relebactam, and vaborbactam based on non-β-lactam structures combined with β-lactams such as ceftazidime, imipenem/cilastatin, and meropenem, respectively. This generation of lactamase inhibitors has an extended spectrum of inhibition to the class A carbapenemase, KPC (*K. pneumoniae* carbapenemase). The majority of β-lactamases in class D and virtually all important class B β-lactamases are resistant to the current FDA-approved β-lactam/β-lactamase inhibitor combinations (Tehrani and Martin., 2018).

2.2.3 Cephalosporins

Cephalosporins contain a side chain derived from D-α-aminoadipic acid coupled with a dihydrothiazine β-lactam ring system (Essack, 2001). They are classified into five generations based on their spectrum of activity against various bacterial pathogens. Cefazolin, a first-generation cephalosporin, is active against Gram-positive cocci except for oxacillin-resistant staphylococci, enterococci, and penicillin-resistant pneumococci (Mulligan et al., 1993, Friedland and McCracken, 1994). First-generation cephalosporins also display activity against *E. coli, P. mirabilis*, and *K. pneumoniae*. Second-generation cephalosporins such as cefamandole and cefuroxime display activity against *H. influenzae* and *Moraxella catarrhalis* (Bohnen et al., 1992). This generation of cephalosporins has appreciable activity against some Enterobacterales compared to first-generation cephalosporins (Essack, 2001). Third-generation parenteral cephalosporins display less activity against most Gram-positive organisms than first-generation agents; however, they are highly effective against *H. influenzae, Neisseria* spp. and Enterobacterales. Third-generation cephalosporins such as ceftriaxone and cefotaxime can penetrate the cerebrospinal fluid and are stable against common β-lactamases found in Gram-negative bacteria. Ceftazidime has potent activity against *P. aeruginosa* (Klein and Cunha, 1995). Fourth-generation cephalosporins display a similar spectrum of activity to third-generation cephalosporins. Agents such as cefepime have activity against *Pseudomonas aeruginosa* and enteric Gram-negative bacilli with inducible chromosomal β-lactamases (Sanders et al., 1996). Ceftaroline is a fifth-generation cephalosporin with activity against Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), β-haemolytic streptococci, and penicillin-resistant pneumococci. Additionally, ceftaroline displays activity against Gram-negative bacteria such as *H. influenzae*, Enterobacterales excluding extended-spectrum β-lactamases (ESBLs), and AmpC producers (File et al., 2012).

2.2.4 Cephamycins

Cephamycins such as cefoxitin, cefotetan, ceftametazole are structurally like cephalosporins but are a distinct class of β-lactams that contain a methoxy group at the 7-alpha position of the β-lactam ring of 7-aminocephalosporanic acid. These compounds are inducers of β-lactamases that are chromosomally mediated, resulting in the selection of derepressed mutants found in organisms such as *E. cloacae* and *C. freundii* (Essack.2001). Unlike cephalosporins, cephamycins are active against anaerobes such *Bacteroides* (Bohnen et al., 1992).

2.2.5 Monobactams

Monobactams such as aztreonam consist of a monocyclic β-lactam ring with activity against Gram-negative rods. Aztreonam represents the only member available for clinical use in its class (Ramsey and

MacGowan, 2016). These agents are resistant to the action of β-lactamases and display no activity against anaerobes and Gram-positive bacteria (Fernandes et al., 2013).

2.2.6 Carbapenems

Carbapenems were developed in the 1980s and are widely accepted as the most potent category of antibiotics within the β-lactam class. Carbapenems possess a five-membered β-lactam ring which is different from other β-lactams. In addition, carbapenems have a carbon instead of a sulphur atom in the four positions of the thiazolidine moiety of the β-lactam ring resulting in a double bond between C-2 and C-3, creating a molecular structure that is unique and confers stability against the majority of β-lactamases (Elshamy and Aboshanab, 2020, Jeon et al., 2015, Bonfiglio et al., 2002).

In addition to being rapidly bactericidal, carbapenems have a broad spectrum of activity against Gram-positive and Gram-negative aerobic and anaerobic bacteria (Kattan et al., 2008, Shah, 2008). This broad spectrum of activity may be attributed to the following factors: (i) carbapenems are smaller molecules that are zwitterionic when compared to cephalosporins (i.e., they have both positive and negative charges in solution) that facilitates rapid penetration across the Gram-negative outer membrane (Hellinger and Brewer, 1999), (ii) carbapenems have a great affinity for PBP-1b, PBP-2, PBP-3, and PBP-4, (iii) this group of β-lactams is resistant to the action of β-lactamases including ESBLs and AmpC cephalosporinases thus making carbapenems an attractive and in some cases the only option to treat infections due to bacteria producing these particular enzymes (Bush, 2001), (iv) carbapenems are widely distributed across various body compartments, tissues, and fluids. Unlike other β-lactam agents, all carbapenems require intravenous administration due to low oral bioavailability; however, some agents such as ertapenem and imipenem can also be administered via intramuscular injection (Matteo et al., 2009, Papp-Wallace et al., 2011, Goa and Noble, 2003, Kattan et al., 2008, Shah, 2008). Like other members of the β-lactam group, carbapenems are excreted via the renal system.

Carbapenems are derivatives of a compound called thienamycin, a natural product produced by *Streptomyces* spp. This discovery was first made in the Merck, Sharp, and Dohme Laboratories (NJ, USA), from a *Streptomyces cattleya* culture, isolated by Compañia Española de la Penicillinia y Antibioticos. Initially, the compound was volatile, but later a more stable compound was developed. The first carbapenem licensed for clinical use in patients was imipenem, followed by meropenem ten years later in 1995 (Spratt et al., 1977, Shah, 2008). Biapenem and panipenem have been available for use in South Korea and Japan since 1993. Ertapenem was licensed after that in the early 2000s, followed by doripenem in 2007 (Walsh, 2007).

Carbapenems have been classified into three groups based on their spectrum of activity and the results of clinical trials (Shah and Isaacs, 2003). Group 1 carbapenems include ertapenem, panipenem, and tebipenem, which are suitable for community-acquired infections. These agents are not recommended for nosocomial infections due to limited activity against non-lactose fermenting Gram-negative pathogens. Agents in group 2 include biapenem, meropenem, imipenem, and doripenem. In addition to the spectrum of activity for group 1 agents, group 2 agents also display activity against non-fermentative gram-negative pathogens such as *P. aeruginosa* and *A. baumannii*, making them suitable agents for treating nosocomial sepsis. Group 3 agents such as topopenem (also known as CS-023) and razupenem (also known as SMP-601, PTZ601, PZ-601, or SM-216601) have a similar spectrum of activity as group 2 agents with additional activity against MRSA. Topopenem and razupenem are not approved for clinical use following increase side effects from phase 2 trials (Shah, 2008) (Bassetti et al., 2013).

The first carbapenem approved for clinical use was imipenem administered intravenously with cilastatin (Clissold et al., 1987). Imipenem is still widely used, although it is not FDA approved for meningitis and central nervous system infections due to its propensity to cause seizures (Kattan et al., 2008). Imipenem is prone to deactivation by dehydropeptidase I (DHP-I), located in the human renal brush border (Graham et al., 1987, Hikida et al., 1992, Kropp et al., 1982); therefore, co-administration with an inhibitor such as cilastatin (compound 6) or betamipron (compound 7) was recommended (Norrby et al., 1983). Imipenem has broad-spectrum antibacterial activity against Gram-negative, Gram-positive aerobic, and anaerobic bacteria making it an appropriate agent in treating infections caused by multidrug-resistant bacterial species producing ESBLs and AmpC β-lactamases. Imipenem in combination with equal doses of cilastatin is generally well tolerated. However, postmarketing surveys have demonstrated that imipenem/cilastatin can be associated with seizures more frequently in patients who received imipenem/cilastatin at higher than recommended doses with underlying renal insufficiency and structural damage to the central nervous system (Kattan et al., 2008, Kabbara et al., 2015, Calandra et al., 1988, Brotherton and Kelber, 1984). Imipenem and panipenem have greater antibiotic activity against Gram-positive organisms (Goa and Noble, 2003, Queenan et al., 2010, Rodloff et al., 2006). Doripenem, ertapenem, meropenem and biapenem have enhanced activity against Gram-negative bacteria (Bassetti et al., 2009, Nix et al., 2004, Perry and Ibbotson, 2002).

Panipenem, released in 1983, because of its broad-spectrum activity and meropenem and imipenem, was recommended to treat nosocomial infections.

Meropenem was introduced in 1995. The spectrum of action of meropenem is like that of imipenem which includes but is not limited to *P. aeruginosa* and *Acinetobacter* spp. However, unlike imipenem, meropenem is not susceptible to hydrolysis by the enzyme DHP-I and thus does not require the co-administration of a DHP-I inhibitor such as cilastatin. Additionally, meropenem can be safely

administered for central nervous system infections such as meningitis (Fukasawa et al., 1992, Kattan et al., 2008).

In 2001, ertapenem was released. Ertapenem is highly bound to protein, and its prolonged half-life permits once-daily dosing, making it an attractive option for treating infections in people residing in the community. However, ertapenem has no activity against *Acinetobacter* spp. *Pseudomonas* spp. and *Enterococcus* spp. making the drug less likely to be utilized for hospital-acquired infections (Livermore et al., 2003, Zhanel et al., 2007, Burkhardt et al., 2007). Nevertheless, due to the once-daily dosing and a prolonged elimination half-life, ertapenem is recommended to treat community-acquired infections due to ESBL producing pathogens (Kattan et al., 2008).

Doripenem entered the market in 2007 with comparable activity to meropenem against Enterobacterales and greater activity against *P. aeruginosa*. Doripenem has the most potent antipseudomonal cover in comparison to other carbapenems. Compared to imipenem and meropenem, doripenem is twice as active by minimum inhibitory concentrations (MIC) MIC90 against *Acinetobacter* spp. (Pillar et al., 2008). MIC90 refers to the lowest concentration of an antibiotic required to inhibit the growth of 90% of the bacterial population.

The four carbapenems approved for use in the United States include meropenem, imipenem, doripenem, and ertapenem. There are also two carbapenem/beta-lactamase inhibitor combinations, meropenem/vaborbactam and imipenem/cilastatin/relebactam, approved and available for clinical use in the United States (Lee and Bradley, 2019). In addition, Panipenem and biapenem are registered for use in Japan (Doi and Chambers, 2015, Yamazhan et al., 2005). Finally, carbapenems available for use in South Africa include meropenem, imipenem, doripenem, and ertapenem (Department of Health – Republic of South Africa, 2020). Initially, nearly all Enterobacterales were vulnerable to the action of carbapenems; however, like many other anti-infective agents, resistance has emerged (Shah, 2008, Papp-Wallace et al., 2011, Perez and Van Duin, 2013).

2.2.7 Novel carbapenems

Novel carbapenems include, among others, carbapenems with antipseudomonal cover, anti-MRSA carbapenems, orally available carbapenems, trinem carbapenems, and others. In addition to activity against aerobic bacteria, razupenem (PZ601), a novel carbapenem, exhibits antibacterial activity against anaerobic bacteria with enhanced activity against MRSA. The anti-MRSA activity is attributed to the high affinity of razupenem for the PBP2a receptors found in MRSA (Papp-Wallace et al., 2011). In vitro studies have shown that despite razupenem's effectiveness against ESBL producers, the antibiotic agent's activity is compromised by the production of carbapenemases and AmpC β-lactamases. Studies

have demonstrated razupenem as highly effective and comparable if not superior to other antibiotics against *Prevotella* spp., *Porphyromonas* spp., and *Fusobacterium* spp. These pathogens cause abdominal, head, and neck space infections and pleuropulmonary infections (MacGowan et al., 2011, El-Gamal et al., 2017). Despite its broad-spectrum antibiotic activity, especially against MRSA, further development of razupenem ended during Phase II trials due to increased adverse effects (Shahid et al., 2009, Gajdács, 2019).

Tebipenem, an antibiotic agent with broad-spectrum activity, formulated as an ester tebipenem pivoxil (compound 15) and regarded as the world's first oral carbapenem (El-Gamal et al., 2017). Tebipenem was approved for pediatric use in Japan in 2009 (Jain et al., 2018). To facilitate intestinal absorption of the carbapenem, these agents are administered as prodrugs, which are then activated by host enzymes located in the liver or intestinal wall (Kattan et al., 2008, Mammeri et al., 2010, Mainardi et al., 1997). Tebipenem exhibits *in vitro* and *in vivo* activity with favorable pharmacokinetics and high bioavailability. The tablet formulation has proven more effective than the granule formulation due to its higher bioavailability (Yao et al., 2016). In addition to activity against Enterobacterales, tebipenem pivoxil also has appreciable activity against MDR *S. pneumoniae*, *N. gonorrhea*, β-lactamase-non-producing, ampicillin-resistant *H. influenzae*, and other Gram-positive bacteria. On the other hand, Tebipenem demonstrates no antibacterial activity against MRSA and *P. aeruginosa* (Papp-Wallace et al., 2011, Isoda et al., 2006, El-Gamal et al., 2017, Jain et al., 2018).

SM-295291 and SM-369926 are parenteral carbapenems in development. Both agents demonstrate antibiotic activity against *Streptococcus pyogenes*, methicillin-susceptible *S. aureus*, penicillin-susceptible and penicillin-resistant *S. pneumoniae*, *M catarrhalis*, *Enterococcus faecalis*, *K. pneumoniae*, and *H. influenzae* (including β-lactamase-negative ampicillin-resistant strains), making them ideal agents for community-acquired infections. There has been special interest in their ability to treat infections caused by *Neisseria gonorrhea* in the context of widespread resistance to current therapeutic regimens. SM-295291 and SM-369926 are 2-aryl carbapenems stable against the action of human DHP-I exhibiting pharmacokinetics like those of meropenem. Intravenous and oral formulations have been developed to allow for switching from parenteral to oral treatment. Both SM-295291 and SM-369926 are still in development, with ongoing preclinical investigations as potential therapeutic agents for community-acquired and gonococcal infections (Fujimoto et al., 2013, Lewis, 2019).

Trinem carbapenems are tricyclic β-lactams with a carbapenem backbone. Sanfetrinem (compound 16) was the first carbapenem trinem formulated with the ability to enter phagocytes and potentially kill intracellular pathogens (Papp-Wallace et al., 2011, Cuffini et al., 1998). The development of this compound was short-lived, as in 2009, sanfetrinem-cilexetil development ended after phase II clinical trials citing selection of de-repressed mutants from inducible populations as a reason. This contrasts

imipenem and meropenem, which are stable to AmpC enzymes that retain full activity against derepressed mutants and so do not select this mode of resistance (Babini et al., 1998).

2.3. Mechanisms of resistance in β-lactams

There are four primary mechanisms of bacterial resistance to β-lactam antibiotics (Babic et al., 2006).

a. Inactivation by β-lactamases,
b. Modification of the target.
c. Decreased uptake by outer membrane proteins (OMPs) alterations, and
d. Efflux

2.3.1 Inactivation by β-lactamases

The production of β-lactamases is the most common mechanism of acquired resistance to β-lactams amongst bacteria (Rossolini et al., 2017, Suay-García and Pérez-Gracia, 2019). β-lactamases are enzymes that degrade the β-lactam ring rendering the antibiotic ineffective. The development and emergence of these inactivating enzymes followed the introduction and use of the β-lactams. Since the introduction of penicillin, the number and types of β-lactamases found in bacteria have steadily risen. As the spectrum of β-lactams widened, so too did the activity of the β-lactam inactivating enzymes. Cephalosporinases followed simple penicillinases, then ESBLs, and most recently, carbapenemases (Codjoe and Donkor, 2018, Bush, 2018).

Genetic basis for β-lactamases production

β-lactamases are generally located in the periplasmic space in Gram-negative bacteria and excreted into the extracellular space in Gram-positive bacteria (Miller and Gilligan, 2012). *Bacillus coli* was the first isolate reported with an enzyme displaying β-lactamase activity published in 1940, which is now considered an AmpC cephalosporinase producer (Bush, 2018).

β-lactamases are encoded either chromosomally or via extrachromosomal elements such as plasmids and transposons, with the latter having the potential to move between bacterial populations (Dhillon and Clark, 2012, Rahman et al., 2018). Molecular analyses have calculated the age of serine β-lactamases to be over 2 billion years old, and β-lactamases that are plasmid-encoded are thought to have been in existence for millions of years (Bush, 2018). Penicillinases that were plasmid-encoded were first reported in Staphylococci**.** Despite being plasmid-encoded in Gram-positives, transfer to other bacterial species was not common other than a few reported isolates of *Enterococcus* species (Murray and Mederski-Samaroj, 1983). This contrasts with the high numbers of MGEs encoding for many β-

lactamases found in Gram-negative organisms. This acquisition of β-lactamases is made possible through the horizontal transfer of genetic material.

Classification of β-lactamases

There are currently two classifications schemes used to classify β-lactamases. The functional classification scheme (Bush, Jacoby, and Medeiros) classifies β-lactamases according to substrate preference, functional properties, and response to inhibitors (Bush and Jacoby, 2010) into three groups: group 1 (class C), cephalosporinases; group 2 (classes A and D), broad-spectrum, inhibitor-resistant, (ESBLs), and serine carbapenemases; and group 3 (class B), metallo-β-lactamases (MBLs) (Rahman et al., 2018). The molecular classification (Ambler), the most widely used classification scheme for β-lactamases, classifies β-lactamases according to amino acid sequences into four molecular classes: A, B, C, D (Ambler, 1980, Silveira et al., 2018). Biochemically these four classes can be divided into two broad divisions based on how they perform hydrolysis. Hydrolysis can be accomplished either by forming an acyl-enzyme with an active-site serine or using one or two essential zinc ions in the active sites of MBLs (Bush, 2018). Carbapenemases, found in classes A, B, and D, are β-lactamases that have reduced the utility of carbapenems in the treatment of infections caused by multidrug-resistant organisms (MDROs) (Codjoe and Donkor, 2018). Carbapenemases in Ambler class A and D contain a serine active site, and class B MBLs have an active zinc site. (Queenan and Bush, 2007, Ambler, 1980, Walsh et al., 2005). The Ambler class C β-lactamase in combination with outer membrane porins (OMPs) has been associated with imipenem hydrolysis (Kim et al., 2006). Enterobacterales have demonstrated increased resistance to carbapenems following acquisition carbapenemases (Papp-Wallace et al., 2011) constituting a major public health concern (Livermore, 2012). CREs are subdivided into 2 categories carbapenemase-producing CRE (CP-CRE) and non-carbapenemase-producing CRE (non-CP-CRE) (Lutgring and Limbago, 2016). The widespread transmission of genes encoding for carbapenem resistance has been widely described (Diene and Rolain, 2014). Mobilization of these genes has created the perfect scenario for the acquisition and spread of CRE (Walsh, 2008, Walsh, 2010, Da Silva and Domingues, 2016, Bonomo et al., 2017, Potron et al., 2014). The various classes of β-lactamases will be discussed below, followed by a description of the carbapenemases relevant to that class.

Class A β-lactamases

Class A β-lactamases include serine penicillinases (TEM, SHV, and CTX-M ESBLs), carbapenemases (KPC, GES - Guiana extended-spectrum), NMC-A/IMI (non-metallo carbapenemase)/(imipenem-hydrolyzing β-lactamase), and SME (S. *marcescens* enzyme) β-lactamases. Class A β-lactamases are generally plasmid-mediated; however, they may also be located on bacteria's chromosomes. These enzymes are inactivated by β-lactamase inhibitors such as clavulanate, tazobactam, sulbactam, and

avibactam (Bonomo, 2017). Discovered in the 1980s, ESBLs were initially point mutations of the TEM and SHV β-lactamases and variants of SHV or TEM (Bush, 2018, Knothe et al., 1983, Kliebe et al., 1985). In particular, members of the Enterobacterales family, *E. coli,* and *K. pneumoniae* are known producers of ESBLs (Falagas and Karageorgopoulos, 2009). ESBLs are also produced by non-fermenting Gram-negative organisms such as *P. aeruginosa* and *A. baumannii* (Jacoby and Munoz-Price, 2005, Dhillon and Clark, 2012). ESBL-producing isolates are generally resistant to penicillins and many cephalosporins; however, they may be susceptible to β-lactamase inhibitor combinations such as clavulanic acid, tazobactam, or sulbactam (Zhou et al., 1994). Over time the selective use of combinations such as amoxicillin-clavulanic acid and piperacillin-tazobactam for ESBL producing isolates has resulted in "inhibitor-resistant" TEM and SHV enzymes (Prinarakis et al., 1997, Bush, 2018). Over the past decade, the CTX-M family of ESBLs have replaced the previously dominating TEM, IRT, and SHV variants as a major contributor to the multidrug-resistant isolates amongst Gram-negative bacteria. Extensive distribution of CTX-M β-lactamases, specifically CTX-M-14 and CTX-M-15, are responsible for the resistance observed to the more advanced generation cephalosporins (Barrios et al., 2017). In addition to TEM, SHV, and CTX-M types, other clinically significant ESBLs include VEB (Vietnam Extended-spectrum ß-lactamase), PER (Pseudomonas Extended Resistance), BEL-1 (Belgium Extended-spectrum ß-lactamase), BES-1 (Brazilian Extended-Spectrum ß-lactamase), SFO-1 (Serratia Fonticola), TLA (Tlahuicas), and IBC (Integron-borne cephalosporinase). From a clinical perspective, prolonged hospitalization in patients with invasive medical devices such central venous lines, endotracheal tubes and urinary catheters are risk factors for developing infections caused by ESBL-producing bacteria. Other studies have also found a relationship between the acquisition of an ESBL-producing strain and the use of third-generation cephalosporins. Prolonged antibiotic use and exposure is also a known risk factor for infection caused by ESBL producers (Pena et al., 1997, Ariffin et al., 2000, Lautenbach et al., 2001). Exposure to antibiotics such as quinolones, penicillins, and cephalosporins predisposes to colonization and infection with CTX-M producers (Paterson and Bonomo, 2005).

Class A carbapenemases

There are three main families of carbapenemases found in Class A: NMC/IMI, SME, and KPC. Class A carbapenemases have been detected in various Gram-negative Enterobacterales, including *Klebsiella spp., E. cloacae,* and *S. marcescens.* NMC and IMI were identified in *E. cloacae* in France, the United States, and Argentina (Nordmann et al., 2012a, Pottumarthy et al., 2003, Radice et al., 2004, Rasmussen et al., 1996). SME was first detected in the United Kingdom in 1982, and KPC was initially identified via surveillance in a *K. pneumoniae* isolate from North Carolina in 1996 (Yigit et al., 2001, Naas et al., 1994, Yang et al., 1990, Queenan and Bush, 2007).

The GES (Guiana extended-spectrum) enzyme, previously regarded as an ESBL, could be considered the fourth family of this class (Giakkoupi et al., 2000). The IBC (integron-borne cephalosporinase) group of β-lactamases are grouped as variants of GES and are not as frequently isolated as the other β-lactamases in Class A (Jacoby, 2006). KPC and GES are plasmid-encoded enzymes, which explains the widespread dissemination of KPC enzymes and their variants soon after their initial discovery within and across the borders of the United States. In 1996 on the east coast of the United States, the first KPC producer in *K. pneumoniae* (KPC-2) was identified, and since its discovery, there have been many variants identified. In just a few years, there was widespread dissemination of KPC producers globally identified in numerous Gram-negative species. However, KPC remains the most identified in *K. pneumoniae.* Due to their plasmid-borne location, the KPC group of enzymes have one of the greatest propensity of spread (Bratu et al., 2005, Woodford et al., 2004). Transposon, Tn4401, a MGE associated with the global spread of KPC genes within the Enterobacterales family (Cuzon et al., 2010), has been identified in other bacteria like *E. cloacae and Salmonella enterica* (Miriagou et al., 2003, Hossain et al., 2004). SME, IMI, and NMC are chromosomally encoded enzymes and have not been implicated in widespread outbreaks. Imipenem and cefoxitin are known inducers of these chromosomally encoded enzymes (Queenan and Bush, 2007).

Class B β-lactamases

Class B β-lactamases hydrolyze β-lactam antibiotics in the presence of zinc as these enzymes are Zn^{2+}-dependent and are referred to as metallo-β-lactamases (MBLs) (Bush, 2018). Organisms harboring class B β-lactamases are resistant to penicillins, cephalosporins, carbapenems, and known β-lactamase inhibitor combinations (Bonomo, 2017). In addition, environmental and opportunistic bacteria such as *Stenotrophomonas maltophilia*, some *Aeromonas* spp., and *Bacillus cereus* expressing chromosomal MBLs are inherently resistant to carbapenems (Franco et al., 2010, Iaconis and Sanders, 1990, Saino et al., 1982, Kuwabara and Abraham, 1967, Queenan and Bush, 2007).

Class B carbapenemases - Metallo-β-lactamases (MBLs)

Class B carbapenemases are metallo-β-lactamases (MBLs). Class B carbapenemases, contrary to serine-based carbapenemases (Class A and D), hydrolyse carbapenems in the presence of zinc and are inactivated by metal chelators such as EDTA (Palzkill T, 2013). While these enzymes can hydrolyse penicillins, cephalosporins, and carbapenems, they cannot hydrolyse aztreonam. The Class B MBLs consist of IMP (active against imipenem), the first metallo-carbapenemase enzyme described in the Enterobacterales family. The IMP MBL, first reported in 1990 from a clinical isolate in *S. marcescens* from Japan, has since made an appearance in various *P. aeruginosa* and *A. baumannii* isolates and other Enterobacterales globally (Osano et al., 1994, Cornaglia et al., 2011). VIM (Verona integron-encoded metallo-β-lactamase) was discovered in Verona, Italy, in 1997 and originally found in clinical isolates

of *P. aeruginosa* located on class 1 integrons (Lauretti et al., 1999), GIM (German imipenemase), which shares a 29% homology to SPM (Sao Paulo metallo-β-lactamase), 30% homology to VIM and 43% homology to the IMPs was discovered in Germany in 2002. SIM (Seoul imipenemase) was isolated in Korea during a study screening imipenem-resistant *Pseudomonas* spp. and *Acinetobacter* spp. isolates. SPM was initially discovered from a *P. aeruginosa* strain in Sao Paolo, Brazil. SPM-1-containing clones of *P. aeruginosa* have been implicated in numerous outbreaks in Brazilian hospitals associated with high mortality (Marra et al., 2006, Poirel et al., 2004b, Zavascki et al., 2005). In 2008, Jun-ichiro Sekiguchi et al. reported the KHM-1(Kyorin Health Science MBL) enzyme isolated from a *C. freundii* clinical isolate recovered in 1997 (Nordmann and Poirel, 2014). NDM (New-Delhi metallo-β-lactamase), since the first report of many clinical isolates from India, Pakistan, and the UK in 2010, has rapidly traversed continents (Kumarasamy et al., 2010) and spread globally. NDM, IMP, and VIM are generally plasmid-mediated and have a global distribution (Bonomo, 2017). SPM-1, GIM-1, SIM-1, KHM-1, AIM-1 (Australian imipenemase), DIM-1 (Dutch imipenemase), SMB-1 (*S. marcescens* MBL), TMB-1 (Tripoli MBL), and FIM-1 (Florence imipenemase) are geographically restricted MBLs (Jabalameli et al., 2018).

Class C β-lactamases

Class C enzymes consist of AmpC β-lactamases, which are generally encoded by *bla* genes located on the chromosome of bacteria; however, plasmid-borne AmpC β-lactamases have become increasingly prevalent in recent times (Bonomo, 2017). AmpC β-lactamase production that is chromosomally mediated occurs mainly via the expression of the AmpC gene, which can either be constitutive or inducible. In the majority of the Enterobacterales family, AmpC β-lactamases are inducible. Plasmid-mediated AmpC enzymes are generally expressed constitutively, and the expression of these enzymes at increased levels occurs because of multiple gene copies. The distinction between the chromosomal and plasmid-mediated enzymes can only be performed using genotypic confirmatory tests (Ashok et al., 2016). AmpC β-lactamase-producing bacteria are resistant to penicillins, β-lactamase inhibitors (clavulanate and tazobactam). Although enzymes can hydrolyse cephalosporins such as cefoxitin, cefotetan, ceftriaxone, and cefotaxime, they poorly hydrolyse other cephalosporins such as cefepime. Carbapenems are stable to hydrolysis by AmpC β-lactamases; however, the action of AmpC β-lactamases is induced by certain antibiotics. Some agents such as benzylpenicillin, ampicillin, amoxicillin, and cephalosporins such as cefazolin and cephalothin are strong inducers of AmpC β-lactamases (Jacoby, 2009). Despite being strong inducers of AmpC β-lactamases other agents such as cefoxitin and imipenem are also stable to hydrolysis by AmpC enzymes. In wild-type bacteria, the expression of AmpC β -lactamases is low and can be induced during β-lactam therapy, often leading to clinical resistance to β-lactams that are strong inducers, as noted above. In cases of spontaneous mutations, stable derepression of the *ampC* gene occurs, resulting in a constantly high-level expression

of AmpC, leading to clinical resistance to all β-lactams excluding carbapenems and at times cefepime (Kohlmann et al., 2018).

Several "Class C carbapenemases" have been identified in Enterobacterales such as ACT-1 (AmpC-type β-lactamase), DHA-1 (Dhahran Hospital in Saudi Arabia β-lactamase), CMY-2 (cephamycin-hydrolyzing β-lactamase), and CMY-10. They are plasmid-encoded, displaying hydrolytic activity against imipenem. CMY-10 is a plasmid-encoded class C β-lactamase with a broad spectrum of substrate hydrolysis, including imipenem. This wide spectrum of substrate hydrolysis and imipenem, in particular, is based on the structural change of the CMY-10 class C β-lactamase. A three-amino-acid deletion in the R2-loop appears to be responsible for the extended-spectrum activity of CMY-10. Therefore, deletion mutations into the R2-loop can extend the substrate spectrum (which includes imipenem amongst others) of class C non-extended spectrum β-lactamases (Kim et al., 2006; Jeon et al., 2015). Although ACT-1, CMY-2, and CMY-10 have been described as carbapenemases, their producers usually display decreased susceptibility to carbapenems because of low enzyme catalytic efficiency and a permeability defect. Therefore, they are not considered true carbapenemases (Meletis, 2016, Branka and Sanda, 2018, Codjoe and Donkor, 2018).

Class D β-lactamases

Class D β-lactamases were originally known as "oxacillinases" due to their ability to hydrolyze oxacillin compared to penicillin. It has been shown that various Gram-negative bacteria naturally possess genes in encoding for OXAs (Oxacillanses), which are considered resident enzymes. Like class A and C β-lactamases, class D β-lactamases are active-serine-site enzymes. OXAs possess either a narrow or expanded spectrum of hydrolysis. Different enzymes in this diverse class confer resistance to penicillins, cephalosporins, i.e., acquired narrow-spectrum class β-lactamases such as OXA-1, OXA-2, OXA-10, extended-spectrum (OXA-type ESBLs) such as OXA-15, which is a point mutation of OXA-2, and carbapenemases (OXA-type carbapenemases) such as OXA-48 (Eftekhar and Naseh, 2015, Poirel et al., 2009). Carbapenem-hydrolysing class D β-lactamases have been isolated predominantly in *Acinetobacter* spp. However, there have been increasing reports of class D β-lactamases, specifically, OXA-48 in Enterobacterales (Bonomo, 2017).

Class D carbapenemases

The first "OXA β-lactamase with carbapenemase activity" was reported by Paton et al. in 1993 (Paton et al., 1993). Subsequently, in 1995, the OXA β-lactamase was isolated from an *A. baumannii* strain from a patient in Scotland (Zahedi Bialvaei et al., 2015). OXA -23, -24, -40, and OXA-58-like β-lactamases have since been identified in *A. baumannii* and amongst the Enterobacterales. Genomic surveillance programs have shown that the OXA-48-like enzymes are the 2nd most common carbapenemases amongst Enterobacterales globally. KPC is the most common, 55%, followed by OXA-

48-like carbapenemases – 27% and NDMs - 26%. The OXA-48-like group of enzymes hydrolyze the aminopenicillins, carboxypenicillins, ureidopenicillins, narrow-spectrum cephalosporins, and weakly hydrolyze the carbapenems. Class D β-lactamases are not inhibited by clavulanic acid, tazobactam, and sulbactam, however may be inhibited in vitro by sodium chloride (NaCl). OXA-48-like group of carbapenemases comprise of OXA-48, OXA-181, OXA-232, OXA-204, OXA-162, and OXA-244 in order of most frequently isolated. Molecular epidemiology and surveillance have shown that Enterobacterales harboring OXA-48-like carbapenemases are endemic to some geographical regions. These enzymes are also found in non-endemic areas where they are introduced and often associated with nosocomial outbreaks. OXA-436, OXA-245, OXA-484, and OXA-519 are other OXA-48-like carbapenemases that are less frequently isolated and reported globally. OXA-48 enzymes are endemic to regions such as the middle east and North Africa, with documented hospital outbreaks occurring in Europe, Australia, Mexico, China, and South Africa. They are commonly found in *K. pneumoniae* ST15, ST11, ST147, ST101, *Escherichia coli* ST38, *E. cloacae*, and *S. marcescens.* The spread of the *blaOXA*-48 gene is generally via the composite transposon Tn*1999* situated on pOXA-48a-like IncL conjugative plasmids. OXA-181 carbapenemases are endemic to countries of the sub-continent such as India, Bangladesh, Pakistan, and Sri Lanka. Hospital outbreaks of OXA-181 carbapenemases have occurred in Nigeria, South Africa, Angola, and United Arab Emirates. The OXA-181 carbapenemases are most often found in *K. pneumoniae* ST147, ST14, ST307, ST34 and ST43, *E. coli* ST410, *E. cloacae* and *C. freundii*. The $bla_{\text{OXA-181}}$ gene is harbored mainly on plasmid types that belong to the ColE2, IncX3, IncN1, and IncT replicon types. OXA-232 is also endemic to India, with reports of hospital outbreaks in Mexico, China, and the United States. Dissemination of *bla*OXA-232 is associated with IS*Ecp1* situated on the ColE2-type pOXA-232 plasmid. The geographic distribution of OXA-162 is mainly in Turkey, Germany, Hungary, and Greece, with OXA-244 occurring mainly in Spain, Germany, Russia, France, Netherlands, United Kingdom. OXA-484 and OX-245 are also found in the United Kingdom, with OXA-591 reported in Belgium. OXA-436 is mainly found in Denmark (Jatan et al., 2020, Poirel et al., 2009; Nordmann and Poirel, 2014, Pitout et al., 2019). Locally, Lowe et al. (2019) described the dissemination of a carbapenemase-producing (OXA-181) clone, *K. pneumoniae* ST307 containing an IncX3 plasmid with $bla_{\text{OXA-181}}$, across 23 cities and towns within six South African provinces over two years. Significantly, the study noted that the plasmid (p72_X3_OXA181) was identical to other IncX3 plasmids carrying $bla_{\text{OXA-181}}$ as previously reported from China and Angola (Lowe et al., 2019). This reaffirms that Enterobacterales with OXA-48-like-carbapenemases, particularly OXA-48 and OXA-181, are emerging in different parts of the world, and there should be an awareness of the looming threat they may pose (Pitout et al., 2019).

2.3.2. Modification of drug target

PBPs play a significant role in developing the bacterial cell wall and are pivotal in incorporating peptidoglycan into the bacterial cell wall (Hamilton and Lawrence, 1975, Vashist et al., 2011). Alteration or changes of PBPs is a well-recognised mechanism of resistance amongst Gram-positive bacteria such as *S. pneumoniae* (Tomasz, 1997) and *S. aureus.* In *S. aureus*, methicillin and oxacillin resistance are associated with incorporating a MGE, "staphylococcal cassette chromosome mec," into the chromosome of *S. aureus* containing the mec A gene (Kapoor et al., 2017, Alekshun and Levy, 2007). *mec*A encodes for PBP2a (penicillin-binding protein 2a), and PBP2a confers resistance to β-lactams by reducing its affinity for β-lactam antibiotics (Laible et al., 1991). In some Gram-negative pathogens such as *N. gonorrhoeae*, chromosomally mediated β-lactam resistance due to alterations of PBPs has been attributed to the introduction of multiple amino acid substitutions within the transpeptidase domain of the PBPs (Spratt and Cromie, 1988). Alterations in PBP coupled with porin loss and the expression of efflux pumps result in high-level carbapenem resistance observed in *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* (Vashist et al., 2011, Rodriguez-Martinez et al., 2009, Mena et al., 2006, Limansky et al., 2002). In many instances, mutations leading to an alteration in the function and expression of PBPs have resulted in carbapenem resistance. It must be emphasized that the mechanisms of carbapenem resistance in Gram-negative rods and Gram-positive cocci differ. Acquiring new carbapenem-resistant PBPs or substitution in the sequence of amino acids found in PBPs often results in carbapenem resistance in Gram-positive cocci (Katayama et al., 2004, Koga et al., 2009). In Gram-negative rods, the production of β-lactamases and the expression of efflux pumps combined with porin loss plus an alteration in PBPs has resulted in resistance to carbapenems (Nordmann et al., 2011b, Pearson et al., 1999, Wareham and Bean, 2006, Papp-Wallace et al., 2011). This observation was made in *P. aeruginosa*, where decreased transcription of *pbp 2* and *pbp3* resulted in decreased PBP2 and PBP3 respectively and when combined with porin loss and increased expression of efflux pumps resulted in carbapenem resistance (Giske et al., 2008).

2.3.3. Decreased uptake of drug and Outer Membrane Porins (OMPs)

The lipopolysaccharide (LPS) layer found in the outer membrane (OM) of Gram-negative bacteria provides protection acting as a selective barrier against the antibiotic, offering an innate resistance mechanism. OMPs facilitate the permeability of the OM of bacteria. There are four large groups of porins: gated porins, general/non-specific porins, substrate-specific porins, and efflux porins (Hancock and Brinkman, 2002, Martinez-Martinez, 2008). Further to the classification, Choi and Lee in 2019 reported that porins are categorized into three subgroups according to their roles in the transport of antibiotics and membrane integrity: antibiotic transport-related specific porins (LamB, YddB), membrane integrity-related non-specific porin (OmpA), and non-specific porins involved in both antibiotic transport and membrane integrity (OmpC and OmpF) (Choi and Lee, 2019). *OmpR* acts as a

porin gene promoter regulating OmpF and OmpC expression (Slauch et al., 1988). Changes in the permeability of the OM can prevent the accumulation of antibiotics within bacteria. This is accomplished by decreasing the number of porin channels, altering porin selection and size, leading to the decreased entry of β-lactams into the bacterial cell resulting in resistance to β-lactams. As a group, Enterobacterales reduce porin number as a mechanism for resistance to carbapenems. Mutations resulting in porin changes have resulted in imipenem resistance observed in *E. aerogenes* (Reygaert, 2018, Kapoor et al., 2017, Kumar and Schweizer, 2005). The term "intrinsic antibiotic resistance of a bacterial species" refers to the innate ability of an organism to effectively reduce the efficacy of an antibiotic via inherent structural or functional characteristics. *P. aeruginosa* has a high intrinsic antibiotic resistance to most antibiotics through decreased OM permeability, efflux pumps, and the production of β-lactamases (Pang et al., 2019). The deletion or decrease of OMP proteins leads to antibiotic resistance, and in the case of carbapenems, a reduction in OMPs coupled with the production of AmpC enzymes and ESBLs potentiates carbapenem resistance (Codjoe and Donkor, 2018).

Mutations observed in *ompC* and *ompF* have been associated with MDROs, while mutations in *ompR* have been specifically associated with carbapenem resistance (Kong et al., 2018). Organisms such as *K. pneumoniae*, *Enterobacter* spp., and *E. coli* exhibit carbapenem resistance based on OMP loss coupled with other mechanisms. Imipenem resistance and reduced susceptibility to meropenem in non-fermenters such as *Ps. aeruginosa* is associated with the loss of OprD (Hopkins and Towner, 1990, Jacoby et al., 2004, Livermore, 2001, Nikaido, 1994, Oteo et al., 2008, Studemeister and Quinn, 1988). The loss of CarO OMP in clinically significant MDR *A. baumannii* isolates has been associated with imipenem and meropenem resistance (Mussi et al., 2005, Poirel and Nordmann, 2006). It must be emphasized that the disruption or decreased functioning of porin proteins alone does not always produce a resistant phenotype.

2.3.4 Efflux

Efflux pumps are proteins located in the plasma membrane of pathogenic and commensal bacteria and can export a wide range of substrates from the periplasm to the surrounding environment (Codjoe and Donkor, 2018). They function as a protective mechanism extruding noxious chemicals before them, reaching the intended site of action, resulting in resistance, including MDR phenotype (Nikaido, 2001, Poole, 2004, Gootz, 2006, Amaral et al., 2014). As antibiotics enter the bacterial cell, they are simultaneously removed using efflux mechanisms before reaching their target. Efflux pumps can be antibiotic-specific or multidrug transporters that pump a wide range of unrelated antibiotics out of the bacterial cell (Wise, 1999, Kapoor et al., 2017).

Efflux pumps exert both intrinsic and acquired resistance to a variety of antibiotics in similar or different classes. Genes encoding for efflux pumps can be located either on the chromosome or plasmids (Markham and Neyfakh, 2001). Five families of efflux transporters have been described: 1. adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, 2. resistance-nodulation-division (RND) family, 3. small multidrug resistance (SMR) family, 4. major facilitator superfamily (MFS) and 5. multidrug and toxic compound extrusion (MATE) family. Family groupings are based on the similarity of their sequence, the specificity of the substrate, single or multiple components, the number of regions that span the bacterial membrane, and the source of energy. ATP hydrolysis is used to export substrates by the ABC family, while the other families use proton motive force as a source of energy. All families except the RND family of efflux pumps are widely distributed in Gram-positive and Gram-negative bacteria. The RND superfamily of efflux pumps is specific to Gram-negative bacteria, with members of this family forming part of a tripartite complex spanning across the two membranes of Gram-negatives. The MFS family is the most relevant efflux pump group amongst the Gram-positives (Blanco et al., 2016, Singh et al., 2017). Examples of pathogenic organisms with recognized efflux mechanisms against imipenem include *P. aeruginosa, E. aerogenes,* and *Klebsiella* spp. (Walsh, 2000). OprD porin loss and overexpression of efflux pumps are the commonest mechanism of carbapenem resistance in *P. aeruginosa*, especially in the case of imipenem (Walsh, 2000). The upregulation of efflux pumps found in *A. baumannii* (AdeABC, an RND-type efflux pump) can enhance the carbapenem resistance mediated by a β-lactamase that has poor activity (e.g., OXA-23) (Heritier et al., 2005, Perez et al., 2007).

2.4. Epidemiology of Carbapenem-Resistant Enterobacterales

Carbapenem resistance observed in Gram-negative bacteria is attributable primarily to two primary mechanisms: i) carbapenemase production, or ii) the combination of β-lactamases (ESBLs, AmpC) with a structural mutation (porin loss, change in OMPs, efflux pumps). The worldwide spread of CRE due to the acquisition of carbapenemases by Enterobacterales has become a great global concern (Guerra et al., 2014). Most β-lactamases, including carbapenemases, are encoded by genes that are located on MGEs. Efficient MGEs such as insertion sequences, integrons, transposons are easily transmissible between various bacterial species contributing to their rapid spread between continents due to international travel and via health care institutions (Nordmann et al., 2012b). As previously described, chromosomally-encoded carbapenemase genes were first identified in Gram-positive bacilli with carbapenemases, using zinc at their active site during the 1980s. After that, another set of carbapenemases using a serine active site was described in Enterobacterales., plasmid-encoded carbapenemases appeared in different species later in the 1990s, contrary to the chromosomally encoded and species-specific carbapenemases initially described. Data sources on the global distribution and epidemiology of carbapenemases exist, and these provide a snapshot of carbapenemases worldwide (Logan and Weinstein, 2017, Queenan and Bush, 2007, Patel and Bonomo, 2013, Poirel et al., 2012, Nordmann et al., 2011a, Carrer et al., 2010, ECDC.,

2016, Glasner et al., 2013, Munoz-Price et al., 2013, Canton et al., 2012, Castanheira et al., 2011, Adler et al., 2011, Giakkoupi et al., 2000, Giakkoupi et al., 2011, Pitout., 2015, Badal R, 2014).

KPC is the most transmissible carbapenemase in class A. Apart from being isolated from various nosocomial *K. pneumoniae* isolates, KPC has been found in numerous other Enterobacterales such as *Enterobacter* spp., *E. coli, Serratia* spp. , *Citrobacter* spp., *and Proteus* spp.(Patel and Bonomo, 2013, Queenan and Bush, 2007, Yang et al., 1990, Nordmann et al., 2009). KPC-producing Enterobacterales have been described as being the causative agent of "one of the most successful MDRO pandemics in the history of Gram-negative bacilli" (Logan and Weinstein, 2017). In particular, the ST258 multidrug-resistant strain of *K. pneumoniae* has traversed borders and has since been implicated in numerous outbreaks globally (Munoz-Price et al., 2013, Patel and Bonomo, 2013). Since the first discovery of the KPC-producing strain of *K. pneumoniae* in 1996 in North Carolina, many cases in the northeastern areas of the United States were reported by 2001. In New York, the hospital prevalence rate was 50.8% (Rhomberg et al., 2007, Deshpande et al., 2006). Between 2004-2007, Israel was the second country to report outbreaks of KPC-producing *K. pneumoniae* infections. Interventions were implemented nationally in Israel to curb the monthly increase in nosocomial infection cases due to KPC-producing *K. pneumoniae* from 55.5 to 11.7 per 100 000 patient days (Schwaber et al., 2011). KPC-producing strains of *K. pneumoniae* were reported from a group of patients in Columbia during the late 2000s. Following this, an outbreak of infections due to KPC-producing *K. pneumoniae* was reported, and it was noted that the index patient had recently travelled to Israel (Mojica et al., 2012, Maya et al., 2013). Columbia, in 2006, was the first Latin American country to identify KPC in *P. aeruginosa*. Other Latin American countries like Mexico, Chile, and Argentina have since reported KPC in various Enterobacterales with Brazil, accounting for most KPC-producing isolates outside Columbia (Villegas et al., 2007). With one of the highest carbapenem resistance rates globally, Greece has experienced an exponential increase in carbapenem resistance within ten years. Carbapenem resistance rates escalated from < 1 % to 30 % in wards and up to 60 % in ICUs between 2001 and 2008 (Souli et al., 2010, Logan and Weinstein, 2017). Surveillance highlights the predominance of KPC-producing *K. pneumoniae*, specifically the ST 258 clone in Greece, which has also spread globally. Greece, Latin America, Israel, and the US are well-known KPC endemic areas (Giakkoupi et al., 2011, Munoz-Price et al., 2013).

The first report of the IMP-1 carbapenemase was in 1991 from a *S. marcescens* isolate in Osezaki, Japan. This was followed by outbreaks reported in seven hospitals in Japan and reports of dissemination of Enterobacterales harboring $bla_{\text{IMP-1}}$ in other parts of Japan and Taiwan (Ito et al., 1995). IMP type MBLs have since been discovered in other Enterobacterales such as *E. cloacae* harboring a $bla_{\text{IMP-2}}$ variant (Yan et al., 2002). During 1996 and 1997, VIM-type MBLs were isolated in *P. aeruginosa* from Verona (VIM-1) and Marseilles (VIM-2). Soon after that, Enterobacterales harboring VIM type MBLs were identified globally, and Greece was known as the "epicenter of VIM type MBLs in Enterobacteriaceae."

In addition, VIM type MBLs found in *K. pneumoniae*, *E. coli*, and other Enterobacterales have been implicated in outbreaks globally (Walsh et al., 2005, Glasner et al., 2013, Nordmann et al., 2011a, Vatopoulos, 2008, Djahmi et al., 2014).

MBL gene, $bla_{\text{NDM-1}}$, encoding for the NDM-1 carbapenemase, was first isolated in *K. pneumoniae* from a patient in Sweden who was hospitalized in New Delhi before returning home to Sweden in 2008 (Yong et al., 2009). There have been suggestions that $bla_{\text{NDM-1}}$ strains have been in circulation within the population and environment in India since 2006 (Castanheira et al., 2011). Endemic areas such the Balkans and the Gulf region are described as "secondary but significant reservoirs" of NDM-producers, with the South Asian countries, India, and Pakistan, being primary reservoirs (Zowawi et al., 2013, Meletis et al., 2014, Kumarasamy et al., 2010). Since the discovery, NDM-producing organisms have traversed continents and reported globally, including North and South America (Rozales et al., 2014). Thus, NDMs have disseminated rapidly globally and have been associated with infections caused by strains found in community and healthcare settings (Doi and Paterson, 2015). Over 50% of *K. pneumoniae* harboring NDM in India are ST11 or ST147 (Sivalingam et al., 2019).

Initially discovered in 2001, the $bla_{\text{OXA-48}}$ gene was first identified in a *K. pneumoniae* strain in Turkey. In addition, OXA β-lactamases have been found in *Acinetobacter* spp. and OXA-48 variants in Enterobacterales (Carrer et al., 2010). The $bla_{\text{OXA-48}}$ gene is plasmid-encoded, facilitating spread from one bacterial species to another, and is most often found in *K. pneumoniae* (Doi and Paterson, 2015). Outbreaks of OXA-48 producing *K. pneumoniae* strains have been reported in various regions, including North Africa, the Gulf, India, Egypt, Libya, South Africa, China, South America, and Turkey (endemic) (Poirel et al., 2004a, Van Duin and Doi, 2017) with the clonal type, ST101 *K. pneumoniae*, accounting for the sporadic spread of OXA-48 in developed nations (Pitart et al., 2011, Seiffert et al., 2014). As noted by Sivalingam et al., KPC, OXA-48, and NDM genes, in a significant way, account for the worldwide distribution and spread of CRE (Sivalingam et al., 2019).

The epidemiology of carbapenemase-producing bacteria has been well described, in particular, for continents and counties outside Africa. Compared to Europe, Asia, North America, South America, and Australia, data outlining the epidemiology, clinical relevance, and dissemination of CRE within Africa is minimal. As per Manenzhe et al. (2015), OXA-48 carbapenemases are the most common carbapenemases reported among Enterobacteriales in Africa, particularly in Northern Africa, and have been found in various Enterobacterales, including *K. pneumoniae*, *Providencia stuartii*, *E. coli*, *C. freundii*, and *E. cloacae*. The OXA-48-producing *K. pneumoniae* clone found in France, Turkey, and the Netherlands has been circulating in countries North of Africa (Nordmann et al., 2011a, Potron et al., 2011a, Canton et al., 2012, Barguigua et al., 2012, Pitart et al., 2011). KPC-2 producing strains of *K. pneumoniae* have been found in Egypt, South Africa, and Tanzania (Metwally et al., 2013). As in other parts of the world, the emergence of MBLs and, in particular, NDMs is of great concern with NDM,

DIM, IMP, and VIM type MBLs reported in Africa. NDMs have been found in *Acinetobacter spp.* and Enterobacterales isolates from Egypt, Algeria, Libya, and South Africa (Mesli et al., 2013, Lafeuille et al., 2013, Hrabak et al., 2012, Manenzhe et al., 2015, Brink et al., 2012) while reports of other carbapenemases such as SIM, SME, NMC and GIM, have been minimal in Africa (Sekyere et al., 2016).

In 2016, Osei brought to light the growing problem of CRE in South Africa, having reviewed CRE infections reported between 2000 and 2016. Data were extracted from published literature, including the National Institute of Communicable Diseases (NICD) communiqués, and highlighted the increasing number of CRE cases from 2000 to 2016. An estimated 2315 carbapenem-resistant cases/infections occurred during this period, with the largest number of cases reported from the Gauteng province, with 1220 cases, followed by KwaZulu-Natal with 515 cases. *K. pneumoniae* (1138) was the predominant organism that was carbapenem-resistant, followed by *A. baumannii* (332), *E. cloacae* (201), and *S. marcescens* (108). The study highlighted the significant dissemination of carbapenem resistance, mediated predominately by NDM-1 or OXA-48 carbapenemases. This was observed in isolates of *K. pneumoniae, A. baumannii, E. cloacae,* and *S. marcescens*. Since 2011 there has been an increase in reports of carbapenem resistance mediated by NDM and OXA-48-like carbapenemases in South Africa from provinces such as Gauteng, KwaZulu-Natal, Eastern, and the Western Cape. Interestingly, in most cases, there were no reports of travel outside South Africa, suggesting that carbapenem resistance in these isolates was selected from increased carbapenem use within the country with local dissemination (Osei Sekyere, 2016). Antimicrobial resistance surveillance in the South African public healthcare sector performed by Perovic et al. in 2018 also identified NDM-1 and OXA-48 as the predominant carbapenemases in South Africa with the highest incidences of CPE in *K. pneumoniae* (Perovic et al., 2018).

2.5. Carbapenem-resistant Enterobacterales in animals

In our efforts to address AMR, emphasis has been placed on the One Health concept reviewing the role of humans, animals, and the environment in the dissemination of antibiotic-resistant bacteria, including CRE (Cahill et al., 2019). It must be noted that there is a paucity of AMR data and in particular data related to carbapenem resistance in the animal and environment sectors when compared to the human sector. Due to the lack of a large body of evidence related to AMR, there is a large gap in the literature pertaining to AMR being reported from the animal and environment sectors. Reservoirs for carbapenem-resistant organisms are slowly increasing and are more frequently described in household pets, livestock animals, and animals in the wild. In 2012, Fischer *et al*. published the first report of an *E. coli* isolate harboring a carbapenemase-encoding gene from a livestock farm (Fischer et al., 2012). The origin of this isolate remains unknown. VIM-1-producing *Salmonella* spp. and *E. coli* and OXA-23 and NDM-1-producing *Acinetobacter* spp. have also been isolated from farm animals such as cattle, swine, and

poultry. Carbapenemase-producing organisms such as OXA-48 and NDM-1-producing *E. coli*, OXA-48 producing *K. pneumoniae,* and OXA-23 producing *Acinetobacter* spp. have been isolated from dogs, cats, and horses categorized as "companion animals. In 2012, *Salmonella infantis* harbouring $bla_{\text{ACC-1}}$ and $bla_{\text{VIM-1}}$ gene was the first carbapenemase-producing Non-Typhoidal *Salmonella* isolated from animals in Germany with NDM-1-producing *S. enterica* subsp. *enterica serovar corvallis* isolated from a wild bird thereafter (Guerra et al., 2014, Fernández et al., 2018). OXA-48-producing *S. marcescens* isolated from surface water is concerning as substandard sanitation practices and dubious water supplies could result in their spread (Potron et al., 2011b). In the United States, the isolation of carbapenemase-producing organisms has been reported from the feces of dairy cattle (Webb et al., 2016). Environmental samples obtained during a study conducted by Dixie *et al*. of sow-farrowing farms identified bacteria with MBL genes such as $bla_{\text{IMP-27}}$ on IncQ1 plasmids. Interestingly, no carbapenemase encoding genes were identified in sow feces during the same study (Mollenkopf et al., 2017). In 2017, a report of carbapenem-resistant *Salmonella indiana* harbouring $bla_{\text{NDM-1}}$ isolated from a slaughtered chicken carcass in China was released (Wang et al., 2017). A systematic review of 68 publications on the PubMed database between 1980-2017 of CRE isolated from samples obtained from wildlife, companion animals, and food-producing animals was published by Kock et al. (2018) VIM, KPC, NDM, OXA and IMP were the predominant carbapenemases isolated, mainly from *E. coli* and *Klebsiella* spp. Two studies reported that 33-67% of exposed humans from poultry farms carried CP-CRE resembling isolates from the farm environment. Following selective screening for CRE, 27 studies demonstrated a CRE prevalence of <1% among livestock and companion animals in Europe, 2-26% in Africa, and 1-15% in Asia. Gulls from Europe and Australia, reflective of wildlife, carried CRE in 16-19% (Kock et al., 2018).

To the best of our knowledge, there have been no studies investigating CRE and their associated carbapenemases in animals on the African continent (Kempf et al., 2012). The detection of carbapenemase-producing organisms in various animals such as horses, cattle, swine, and dogs in the Western parts of Europe has prompted the need for active surveillance to detect carbapenem-resistant bacteria in food-producing animals (Manenzhe et al., 2015, Fischer et al., 2013a). The European Food Safety Authority (EFSA) has highlighted the need for combined monitoring to detect antimicrobial resistance in humans, food-producing animals, and the environment (EFSA, 2018).

2.6. Carbapenem-resistant Enterobacterales in the environment

The existence of antibiotic-resistant bacteria and ARGs in environmental water sources is of great concern due to the implications on public health, the aquatic environment, and animals. Water is commonly used for consumption, hygiene, provision of food (fish), irrigation, and various recreational activities (Manyi-Loh et al., 2018). Several studies demonstrate the isolation of antibiotic-resistant

bacteria from wastewater, highlighting the potential role of wastewater in the transmission of antibiotic-resistant bacteria into the environment (Hocquet et al., 2016, White et al., 2016, Galler et al., 2014). A recently published study in Belgium demonstrated carbapenemase genes such as bla$_{KPC}$, *bla*$_{NDM,}$ and *bla*$_{OXA-48}$ in the Zenne river due to continuous discharge from WWTPs (Proia et al., 2018). In Japan, Azuma *et al.* (2019) demonstrated the distribution of antibiotic-resistant bacteria to surface waters (Azuma et al., 2019). KPC, VIM-1, and IMI-2 have been isolated from river ecosystems in Spain. Water samples from the rivers in Portugal have reported the isolation of KPC-producing Enterobacterales. A report of the Danube, Europe's second-longest river, revealed the isolation of Enterobacterales harbouring NDM-1 and KPC-2. In South Korea, *Klebsiella variicola* harbouring NDM-9 was isolated from an urban river source (Sivalingam et al., 2019). It has been noted that the antibiotic-resistant bacteria and ARGs isolated from environmental sources are closely related to those isolated from clinical environments (Sivalingam et al., 2019). During a study investigating *E. coli* strains and their resistance determinants from hospital effluents in central India, coexisting ARGs, including TEM, CTX-M, and OXA 48, were identified in addition to an isolate harboring NDM-1(Chandran et al., 2014). The *bla*$_{NDM-1}$ gene has been isolated from sewage and water used for human consumption in Delhi (Walsh et al., 2011). The contamination of drinking water by *bla*$_{NDM}$ producers was explained by contamination of water pipes with sewage following heavy rains. NDM genes have also been identified from sediment samples collected from the upper Ganges River consistent with poor hygiene practices, unsanitary disposal of waste, and mass bathing during pilgrimage (Ahammad et al., 2014). In addition to the Ganges River, *bla*$_{CMY-42}$ has been isolated from the Yamuna River (India) (Bajaj et al., 2015). Different ST types among *E. coli* and *K. pneumoniae* have been isolated from rivers in Algeria harboring *bla*$_{OXA-48}$. The six ST types in *E. coli* included ST559, ST38, ST212, ST3541, ST1972, and ST2142, with the 3 ST types in *K. pneumoniae* being ST133, ST2055, and ST2192 (Tafoukt et al., 2017). *K. pneumoniae* harbouring KPC-2 associated with ST437 has been isolated from rivers in Brazil (Oliveira et al., 2014).

Studies have demonstrated common carbapenemases, specifically MBLs and corresponding MGEs, in two sectors of the One Health triad. In 2017, Mollenkopf et al. isolated plasmid-borne carbapenemase genes in Enterobacterales from environmental samples and fecal samples obtained from swine farrowing and nursery barns in the US. *E. coli*, *Proteus* spp., *Morganella* spp., *Providencia* spp. *Citrobacter* spp. and *Klebsiella* spp. were amongst the Enterobacterales bearing *bla*$_{IMP-27}$ that were isolated. He et al. (2017) similarly reported for the first time *K. pneumoniae* bearing the *bla*$_{NDM-5}$ carbapenemase from milk and fecal samples of dairy cows in China. By this time, *bla*$_{NDM-5}$ was extensively described in humans from different parts of the world. It is interesting to note that all isolates had a similar genetic environment and that all carbapenemases were found on a self-transmissible IncX3 pNDM-MGR194-like plasmid. Furthermore, the IncX3 plasmid was the same in the *K. pneumoniae* from human designated pNDM-MGR194 described in India. Additionally, these *bla*NDM-5-carrying plasmids were also found in human *E. coli* isolates in China (He et al., 2017) and a report of one isolate of *bla*$_{NDM-5}$

carrying *E. coli* from a dog in Algeria (Yousfi et al.,2015). Drawing from the above, evidence suggests the transmission of IncX3 plasmids from humans to animal . In China, the $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-5}}$ genes located on IncX3 plasmids among several species of Enterobacterales, including *E. coli*, *K. pneumoniae*, *C. freundii*, and *E. cloacae*, have been reported suggesting that IncX3 plasmids are efficient carriers for dissemination of bla_{NDM} genes within bacterial species (Ho et al., 2012, Chen et al., 2016). In Algeria, a similar occurrence of the $bla_{\text{NDM-5}}$ gene on IncX3 plasmid in isolates of *E. coli* from teats and milk of dairy cows further strengthens the case made regarding the fluidity of antibiotic resistance determinants between sectors of the One Health triad (Bonardi and Pinto, 2019). The constant movement of humans, animals, and food products across different geographical locations enables the spread of CRE hosted with them (Sivalingam et al., 2019). Dissemination of these antibiotic-resistant bacteria has now become one of the most challenging health concerns globally. Therefore, mitigation of AMR and its spread requires a "One Health" approach.

2.7. Detection of carbapenem-resistant Enterobacterales

There are various phenotypic and genotypic methods available to detect carbapenem resistance amongst Enterobacterales, with some limitations.

Generally, reduced susceptibility to a carbapenem with or without concomitant susceptibility to aztreonam and inhibition by a beta-lactamase inhibitor combination such as amoxicillin/clavulanate, piperacillin/tazobactam or ampicillin/sulbactam in Enterobacterales, *P. aeruginosa*, and *A. baumannii* ought to be tested for carbapenem resistance, including carbapenemase production. Carbapenem resistance amongst these organisms may be due to the up-regulation of efflux pumps and porin loss and not necessarily due to carbapenemase production, necessitating further investigations to identify the correct mechanism of carbapenem resistance (Osei Sekyere et al., 2015, Rasmussen et al., 1996, Queenan et al., 2006). Initial screening of breakpoint values to carbapenems during antimicrobial susceptibility testing may indicate potential carbapenemase production (Nordmann et al., 2012c, Nordmann et al., 2011c). As per 2019 CLSI guidelines, any Enterobacterales isolate with a zone diameter of </= 19mm or MIC >/= 4µg/ml for imipenem, meropenem, or doripenem indicates reduced carbapenem susceptibility. Additionally, any Enterobacterales isolate with a zone diameter of </= 18mm or MIC >/= 2µg/ml for ertapenem has reduced carbapenem susceptibility (CLSI, 2019).

There is no universal medium for detecting carbapenemase-producers; however, various chromogenic agars are available with varying sensitivity and specificity depending on the type of carbapenemases produced (Adler et al., 2011, Osei Sekyere et al., 2015). Of note is that initial screening requires further testing to confirm carbapenemase production (Nordmann et al., 2012c, Ambretti et al., 2019).

ChromID CARBA (bioMérieux) is a chromogenic screening agar used for the detection of CPE. Previous evaluations performed comparing ChromID CARBA (bioMérieux) chromogenic screening agar to other methods in the detection of CPE showed a comparable performance. These included, amongst other, an evaluation of 200 rectal swabs, 133 presumptive CPE with 92 confirmed by phenotypic and genotypic tests, were screened using ChromID CARBA (bioMérieux) chromogenic media. Plating of rectal swabs onto ChromID CARBA agar was carried out in parallel with four other methods employed to screen for CPE. These methods involved (a) plating onto a McConkey agar plate following overnight tryptic soya broth enrichment with a 10-µg ertapenem disk, (b) plating onto ChromID ESBL chromogenic media following a short incubation in brain–heart infusion broth plus a 10-µg ertapenem disk. (c) a direct plate out of the rectal swab on ChromID ESBL chromogenic agar; and (d) on a McConkey plate supplemented with 1 mg/L of meropenem. ChromID CARBA (bioMérieux) chromogenic media was shown to be superior to the other methods with a sensitivity (92.4%) and specificity (96.9%) for screening of CPEs when compared to other methods. All the media mentioned above, including ChromID CARBA (bioMérieux) chromogenic media, had a low sensitivity for detecting OXA-48 producers. A reason for this is that OXA-48 producing strains are generally sensitive to cephalosporins and their resistance to carbapenems is often of a low-level (Vrioni et al., 2012, Hrabák et al., 2014). Girlich et al. (2013) stated that although chromID CARBA (bioMérieux) chromogenic media fared poorly in the detection of OXA-48 producers, it is "a powerful tool for detection of all other classes of CPE" (Girlich et al., 2013, Zarakolu et al., 2015). Shortly after that, a bi-plate chromID Carba Smart chromogenic media (bioMérieux) was produced. This bi-plate is superior to its predecessor as it screens for carbapenemase-producing Enterobacterales and OXA-48-producing Enterobacterales. The bi-plate contains a selective OXA mixture for selecting OXA-48-type CPE and the selective CARB mixture to select KPC and metallo-carbapenemase-type CPE, hence, can detect more CPE compared to chromID CARBA (bioMérieux) (Perry, 2017). Therefore, the bi-plate with this combination of media can screen for the most common CPEs encountered globally (Zarakolu et al., 2015). The advantages of plating samples on McConkey media containing a meropenem disk include low cost, ease of evaluation of suspected colonies, and assessing the quality of specimens (poor quality if no bacterial growth is noted). The major disadvantage of this method is the poor sensitivity in detecting CPEs with MICs to the carbapenems around the breakpoint range. Selective chromogenic media offer the advantage of easy incorporation in laboratory workflow during inoculation, assessment of bacterial growth, and good specificity and sensitivity. It is important to note that while only a few selective chromogenic media detect OXA-48, the vast majority were designed to detect KPC-producing isolates. Additionally, unlike the McConkey media containing a meropenem disc, selective chromogenic cannot assess the quality of a rectal swab (Papadimitriou-Olivgeris et al., 2014, Ambretti et al., 2019).

The CLSI has recommended the Modified Hodge Test (MHT) to detect carbapenemase-producers. The test involves using an imipenem-impregnated disc on Mueller-Hinton agar with imipenem susceptible

Enterobacterales strains spread on them. The tested clinical isolate is streaked from the plate's end towards the disc placed in the plate's centre. Growth of the susceptible strain and clinical isolate through the streak line towards the disc indicates a possible carbapenemase-producing organism. However, the MHT is time-consuming, subjective to interpreter variability, and may produce false-positive results. The test is also not sensitive and specific as MBL producers are not detected. Additionally, the MHT detects high-level AmpC producers (Osei Sekyere et al., 2015, Wayne, 2019, Castanheira et al., 2011). Despite these shortcomings, the MHT can detect KPC and OXA-48 producers.

Other tests use inhibitors or chelators such as EDTA or thiol-based compounds with imipenem discs for detecting MBLs. The chelators work by inhibiting the zinc co-factor needed for MBL activity in one disc while the other disc is free from chelators. The E-test-EDTA test is based on a similar principle for detecting the MICs of highly resistant MBL producers, while the ability to detect less resistant MBL producers is deficient. These tests are costly and require trained personnel. The Carba NP test (developed by Nordmann and colleagues) for detecting all carbapenemase-producing organisms is increasingly being used in many clinical laboratories due to its lower cost, efficiency, and shorter turnaround time (Nordmann et al., 2012d, Osei Sekyere et al., 2015). Spectrophotometry-based assays used for detecting carbapenemase-producers based on their carbapenem hydrolysis activity are time-consuming and require well-trained personnel compared to tests such as the Carba NP test. Also, the need for additional equipment such as a UV spectrophotometer and sonicator for the UV spectrophotometric assay tends to be costlier.

The carbapenem inactivation method (CIM) is a phenotypic method used for the detection of carbapenemase production. The CIM was first described in 2015 (Pierce et al., 2017) and is based on the following principle: a 10-µg meropenem disk is subjected to 2 h incubation in an aqueous suspension with a carbapenemase-producing organism, and the carbapenemase produced by the microorganism degrades the meropenem in the disk. If the test microorganism does not produce carbapenemase, the antibacterial activity of meropenem will be retained, which will be confirmed when the very same meropenem disc is tested for antibacterial activity against a carbapenem susceptible isolate. This is accomplished by removing the same meropenem disk from suspension and placing it onto a plate, in this case, Mueller-Hinton agar lawned with a carbapenem-susceptible organism. This is followed by incubation overnight, and the zone of inhibition is determined to asses if meropenem has undergone the process of hydrolysis (indicator organism growing within proximity of the meropenem disk) or an active disc (wide zone of inhibition around the meropenem disk) (Wayne, 2019).

Although a multiplex PCR for detecting specific carbapenemases within 4.5 hours has been developed, the PCR cannot detect novel or unknown carbapenemase resistance genes (Osei Sekyere et al., 2015, Dortet et al., 2014). Additionally, while PCR-based assays have a good level of sensitivity and

specificity, reliable detection of multiple genes and the extraction process pose a challenge for applying nucleic acid amplification assays on rectal swab and stool specimens. Despite these challenges, there has been significant development of various in-house, commercial, and rapid commercial molecular assays to detect CRE/CPE(Ambretti et al., 2019, Smiljanic et al., 2017). Most reference laboratories have adopted Molecular-based technology such as whole genome sequencing (WGS) globally to detect carbapenemase genes (Nordmann et al., 2011c). Slowly but surely, WGS is being adopted as the "gold standard" in the typing of bacterial isolates. This methodology can provide high discriminatory power in the characterization of bacterial strains. These techniques are becoming more accessible, advanced, and rapid to assist our understanding of the dissemination of bacteria. In addition to research, these technologies impact clinical care and assist in outbreak situations (Rimoldi et al., 2017). The rapid identification of pathogens, their strain types, antibiotic resistance genes, and MGEs are critical to identifying and controlling hospital-acquired infections during an outbreak. WGS can identify antibiotic resistance genes harbored within bacterial chromosomes and MGEs (Pecora et al., 2015). The major drawback of molecular techniques is their cost, the infrastructure required, and the need for trained scientists, microbiologists, and bioinformaticians.

2.8. Clinical manifestations and Treatment of Infections caused by carbapenem-resistant Enterobacterales

Infections caused by ESBL-producing Gram-negative organisms have posed a tremendous therapeutic challenge to clinicians with heavy reliance on carbapenems (Dhillon and Clark, 2012). Carbapenems have become the best, and in most cases, the last option in treating these infections accompanied by increased carbapenem resistance observed in Enterobacterales. Infections caused by CRE have emerged in both developed and developing countries, with the dissemination of these MDR pathogens being a problem in the clinical care of patients and public health (Ballot et al., 2019). CRE have been implicated in ventilator-associated pneumonia, bacteremia, complicated urinary tract infection, acute pyelonephritis, hospital-acquired pneumonia, skin and soft tissue infections, and sterile body fluids infection (SBFI), including meningitis (Guangzui et al., 2020 Pang et al., 2018).

Regarded as "antibiotics of last resort," carbapenem resistance has increased substantially globally over the last decade, and with the high burden of carbapenem resistance observed in Enterobacterales, optimal therapeutic options for these organisms are minimal. Thus, CRE and other MDROs are in the critical group of the WHO list of antibiotic-resistant "priority pathogens," and there is a great need for research and development of new antibiotics to treat infections caused by this category of bacteria. Options for the treatment of CRE include tigecycline, fosfomycin, polymyxins, and aminoglycosides; however, there is still an urgent need to develop new antibiotics to combat infections against these pathogens in particular (Tacconelli et al., 2018). Ceftazidime-avibactam, a combination of a third-generation

cephalosporin (ceftazidime) and a non-β-lactam β-lactamase inhibitor (avibactam), has potent activity against KPC and OXA-48 producing Enterobacterales. This drug, however, has no activity against CRE that are MBL-producing isolates (Shirley, 2018). A recent study performed by Alraddadi et al. (2019) on a small cohort of patients with infections caused by CRE (OXA-48 type) showed ceftazidime-avibactam to be a promising antibiotic in such a cohort of patients, however, concluded that larger prospective studies are needed (Alraddadi et al., 2019). Taniborbactam is a new cyclic boronate β-lactamase inhibitor in clinical development in combination with cefepime to treat infections caused by CRE and carbapenem-resistant *P. aeruginosa*. Taniborbactam is the first β-lactamase inhibitor with direct inhibitory activity against serine- and metallo-β-lactamases. Cefepime-taniborbactam is currently in phase 3 trials (Hamrick et al., 2020).

Various other strategies have been adopted to treat such infections, including 1) the use of combination antibiotic therapy, 2) the use of tigecycline in high doses, 3) high-dose carbapenem therapy coupled with a prolonged infusion, and 3) the use of dual carbapenem therapy. Paul et al. recently published the first randomized control trial (RCT) comparing colistin monotherapy versus combination therapy with colistin plus meropenem for severe infections caused by carbapenem-resistant Gram-negative bacteria. The study outcomes revealed that colistin combination therapy was not superior to colistin monotherapy; however, it must be noted that most of the infections amongst study patients were caused by *A. baumannii*. Also, there seemed to be a lower rate of clinical failure in patients who received combination therapy (46% vs. 68%, $P = 0.185$), and lower 28-day mortality (21% vs. 35%, $P = 0.235$) was observed in the combination therapy arm. Another RCT(NCT01597973) comparing colistin monotherapy versus combination therapy with a carbapenem in patients with pneumonia or bacteremia due to Gram-negative pathogens is ongoing until 2021. Lower rates of mortality with carbapenem-based combination therapy have been observed in several other retrospective studies. However, due to the dearth of quality data surrounding combination therapy, it is not easy to make convincing recommendations for or against it. However, there is increasing evidence that combination therapy may find its place among critically ill patients with CRE infections (Paul et al., 2018, Sheu et al., 2019).

2.9. Triangulating the Molecular Epidemiology of Carbapenem-Resistant Enterobacterales from Humans, Food Animals, and the Environment.

Infections caused by carbapenemase-producing bacteria have mainly been nosocomial in origin rather than community-based. The increasing prevalence of carbapenemases in animal and environmental samples and the colonization of CREs in the normal intestinal flora could prompt a change in the epidemiology of CREs. The prevalence of carbapenem-resistant bacteria in healthcare institutions, food-producing animals, and the environment is unknown, and no comparisons nor triangulations between strains from the different sectors has been made. There is a need to perform research drawing

comparisons of the genomic epidemiology of MDROs, particularly CREs isolated from humans, animals, and the environment in Africa. The comparison of CRE at the genome level provides valuable information and a better understanding of the molecular mechanisms and spread of CRE between these three sectors. Understanding the molecular epidemiology and identifying clonal relationships and resistance mechanisms amongst resistant strains is important in establishing effective infection prevention and control programs and providing information for developing new antibiotic targets. Early detection and infection prevention and control strategies are key in limiting dissemination and addressing CRE within all three sectors. Molecular epidemiology also provides valuable insight into the evolution, dissemination, and containment of CRE. (Sekyere et al., 2016, Nordmann et al., 2011c, Poirel et al., 2011, Poirel et al., 2012, Kock et al., 2018). With these important aspects brought to light, this study aimed to investigate AMR in the One Health context drawing attention to carbapenem resistance mediated by carbapenemase-enoding genes, focusing on the molecular epidemiology, and triangulating them in humans, animals, and the environment in KwaZulu-Natal, South Africa.

The human health component consisted of rectal screens for CPEs from patients admitted to 3 public healthcare facilities. The animal health component consisted of rectal screens from pigs at a slaughterhouse, while the environmental component consisted of influent, effluent, and upstream and downstream surface water within the same geographical area of uMgungundlovu, KwaZulu-Natal, South Africa.

3.0 Aim and Objectives

3.1. Aim

This project aimed to triangulate the molecular epidemiology of CRE in hospitalized patients (humans), pigs at slaughter (food-producing animals), and water samples from WWTP (influent and effluent), and its receiving surface water body (upstream and downstream from the WWTP discharge point) taking a "One Health" approach in the uMgungundlovu district, KwaZulu-Natal, South Africa.

3.2. Objectives

- To isolate and putatively identify CPE carriage from rectal swabs of in-patients using ChromID CARBA chromogenic agar medium (bioMérieux).
- To isolate and putatively identify CPE carriage from rectal swabs of pigs at slaughter using ChromID CARBA chromogenic agar medium (bioMérieux).
- To isolate and putatively identify CPE from samples obtained from the environment (WWTP - effluent, influent, upstream and downstream wastewater plant samples) using ChromID CARBA chromogenic agar medium (bioMérieux).

- To determine the antibiotic resistance profiles of CPE using the automated Vitek 2 platform (bioMérieux).
- To determine the multilocus sequence types, phylogeny, and novel mutations using WGS.
- Identify antibiotic resistance genes using bacterial analysis pipelines and a comprehensive antibiotic resistance database.
- Identify genes encoding for carbapenemases along with their flanking sequences.
- Identify and delineate MGEs such as plasmids, transposons, insertion sequences, and integrons associated with resistance and virulence.
- To triangulate the results from human, animal & environmental sectors in a One Health context.

4.0 Synopsis of methodology

4.1. Ethical consideration

Human: This study was approved by the Biomedical Research Ethics Committee (approval no: **BE599/16**, Sub-study of **BCA444/16**), College of Health Sciences, University of KwaZulu-Natal.

Animal: This study was approved by the Animal Research Ethics Committee (approval no: **AREC/079/018D**), University of KwaZulu-Natal. Permission for animal and environmental sampling was obtained from the Department of Agriculture, Forestry and Fisheries, Republic of South Africa (approval no**: Ref 12/11/15**).

Environmental: Permission was sought and received from uMgeni Water in KwaZulu-Natal.

4.2. General Methodology

4.2.1. Study design

The study describes the molecular epidemiology of CRE in humans, animals, and the environment in uMgungundlovu, KwaZulu-Natal, South Africa. This point prevalence study involved participation from patients admitted to three public sector hospitals, an abattoir, and a wastewater treatment plant located within the same geographical area of uMgungundlovu, KwaZulu-Natal, South Africa (Figure 2). A point prevalence study was undertaken as it represented the circulating strains of CRE across all three sectors at a certain point in time within the same geographical area. This was considered a snapshot of the current epidemiology of CRE in a One Health context. Following isolation, a comparison and triangulation of the molecular epidemiology of CRE from the three sectors reflect the possible dissemination of bacterial clones, resistance, and virulence genes and their associated MGEs between the three sectors. Three different levels of healthcare are represented in the study ranging from a level 1 district healthcare facility to a level 3 provincial tertiary healthcare facility (Figure S1).

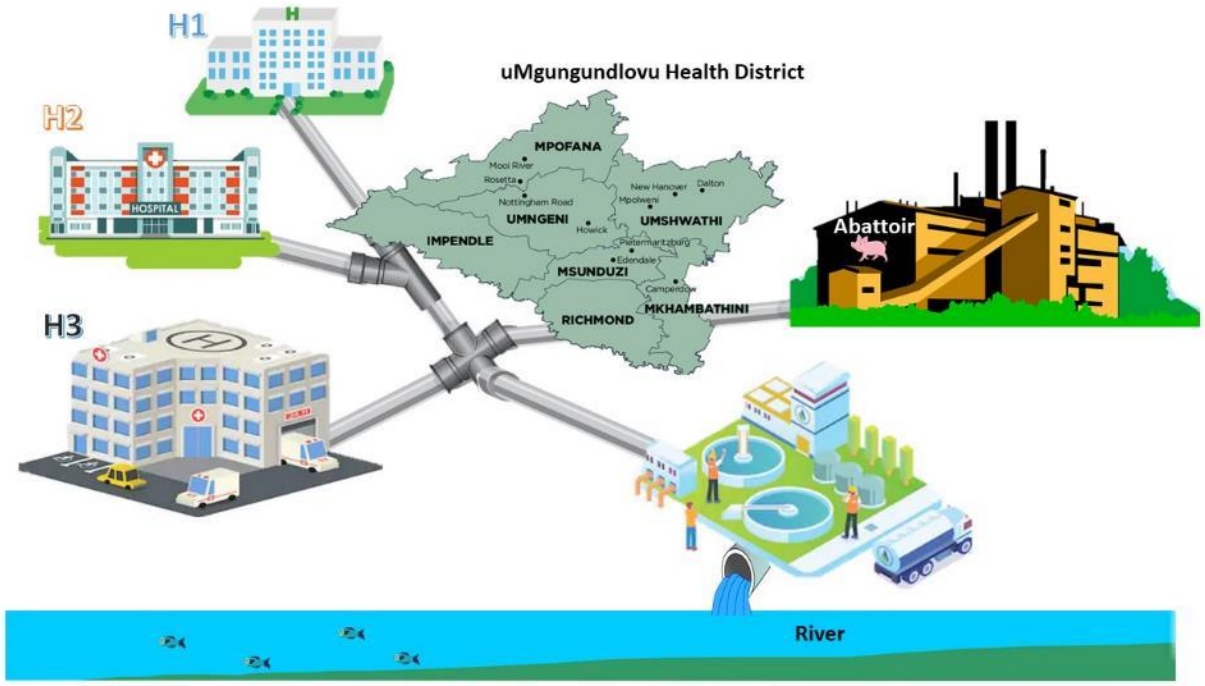

Figure 2. Graphical representation of the sites for the One Health Study: uMgungundlovu District, KwaZulu-Natal South Africa. H1, H2, H3 represent the district, regional, and tertiary healthcare facilities.

Screening rectal swabs were obtained from humans and pigs with environmental samples collected which consisted of influent, effluent, and upstream and downstream surface water from a wastewater treatment plant. The human health component consisted of rectal screens for CPEs from patients admitted to 3 public healthcare facilities. The animal health component consisted of rectal screens from pigs at slaughter, while the environmental component consisted of influent, effluent, and upstream and downstream surface water within the same geographical area of uMgungundlovu, KwaZulu-Natal, South Africa. Selective ChromID CARBA chromogenic agar (bioMérieux) was used to isolate CRE from all samples. Following automated microbial identification and antibiotic susceptibility testing (Vitek 2, bioMérieux), the isolates were subjected to whole genome sequencing (WGS). Bioinformatics was used to identify the carbapenemases conferring carbapenem resistance, characterize MGEs (plasmids, insertion sequences, integrons, transposons), and determine the resistome from the isolated CRE. Clonal lineages and comparative genomics of CRE isolated from humans, animals, and the environment were also determined. An in-depth description of the methodologies used in this study will follow in chapters 2, 3, and 4.

5.0. Thesis outline

This project is presented in the form of submitted manuscripts, consisting of the following five chapters:

Chapter 2. Manuscript 1: **Triangulating the Molecular Epidemiology of Carbapenem-Resistant Enterobacterales in Humans, Food Animals, and the Environment in a One Health Context.**

This manuscript describes a point prevalence study in the One Health context triangulating the molecular epidemiology of CRE in humans, animals, and the environment in the same geographical area of uMgungundlovu, KwaZulu-Natal, South Africa. The study aimed to comprehensively delineate the molecular epidemiology, nature, and extent of AMR specifically in CRE with associated resistomes and mobilomes in the "One Health" context. The study brings to light the potential for disseminating ARGs with associated MGEs in propagating AMR between human and environmental sectors.

Chapter 3. Manuscript 2: **Pathogenomic Analysis of a Novel Extensively Drug-Resistant *Citrobacter freundii* Isolate Carrying a bla$_{NDM-1}$ Carbapenemase in South Africa.** ***Pathogens*. 2020;9(2):89. Published 2020 Jan 31. doi:10.3390/pathogens9020089**

This manuscript reports the pathogenomic analysis performed on a novel carbapenem-resistant *Citrobacter freundii* isolate from a rectal swab of an adult male patient admitted to a healthcare facility in KwaZulu-Natal, South Africa. The manuscript highlights the value of determining and investigating the antibiotic resistome and MGEs, presenting genomic information for future surveillance of CRE spread in South Africa.

Chapter 4. Manuscript 3: Comparative pathogenomics of *Aeromonas veronii* pigs in South Africa: Dominance of the novel ST657 clone. ***Microorganisms*. 2020;8(12):2008. Published 2020 Dec 16. doi:10.3390/microorganisms8122008**

In this study, we describe, for the first time in Africa, the comparative pathogenomics of five novel carbapenem-resistant *Aeromonas veronii (A. veronnii)* strains recovered from pigs in the KwaZulu-Natal Province of South Africa. All the isolates had similar resistance phenotypes, which corroborated with their resistomes (bla$_{CPHA3}$ and bla$_{OXA-12}$), conferring resistance to selected agents from the β-lactam antibiotics emphasizing the need for AMR surveillance in the animal sector. The isolates belonged to the novel sequence type, ST657 (a satellite clone). Estimation of pathogenicity predicted a higher probability ($P_{score} \approx 0.60$), supporting their pathogenic potential to humans with the novel strain possessed a battery of putative virulence factors. Reporting findings of the resistome of *Aeromonas* spp. about phenotype, genotype, genomic, and epidemiological data is important in understanding the origins of antibiotic resistance and the transfer of antibiotic-resistant organisms with the potential to cause significant infections in humans. *Aeromonas* spp. has public health implications as it is a pathogen related to the environment, food industry, and aquaculture.

Chapter 5

Conclusion: this chapter captures the summary and significance of the work, presents the limitations and recommendations for future work.

6.0. References

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CHAPTER 2 - MANUSCRIPT 1

Carbapenem-Resistant Enterobacterales in South Africa: A One Health Point Prevalence Study

Author contributions

- Y. Ramsamy, as the principal investigator, co-conceptualized the study, put forth ethical applications, undertook the literature search, sample collection and the laboratory work, analyzed the genomic data, undertook drafting of manuscript for journal submission and collated all references.

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- Akebe L. K. Abia, assisted environmental and animal sample collection, assisted data analysis, and undertook a critical revision of the manuscript.

- A. Ismail curated the genomic data results and undertook critical revision of the manuscript.

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- Mbanga J. genomic data and bioinformatics analysis and undertook critical revision of the manuscript.

- R. Singh aided in laboratory work and undertook critical revision of the manuscript.

- S.Y. Essack, as co-supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, facilitated data collection and analysis, vetted the results, and undertook critical revision of the manuscript.

Carbapenem-Resistant Enterobacterales in South Africa: A One Health Point Prevalence Study

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Abstract

Introduction

Although antimicrobial resistance (AMR) occurs naturally but is exacerbated by the selection pressure from indiscriminate antimicrobial use in humans and animals, and disposal into the environment, prompting a collaborative One Health approach towards its understanding and containment. This point prevalence study (1) determined colonization with carbapenemase-producing Enterobacterales (CPE) in humans, livestock animals (pigs), and environmental sources within the same geographical area, uMgungundlovu District, KwaZulu-Natal, South Africa and, (2) comprehensively delineated the resistome, mobilome, virulome and phylogeny of the isolates.

Methodology

Following ethical approval, 587 samples, comprising of rectal swabs from humans from three public hospitals (n = 230), rectal swabs from pigs at an abattoir (n = 345) and water samples (n = 12) from a wastewater treatment plant, were collected. Selective chromogenic agar was used to isolate CPE from all samples. The isolates were subjected to microbial identification and antibiotic susceptibility tests followed by whole genome sequencing and bioinformatic analysis to elucidate the resistomes, virulomes, mobilomes, clonality, and phylogenies using various bioinformatics tools in order to triangulate the molecular epidemiology of CPE in a One Health context.

Results

A total of 19/587 (3.2%) samples, i.e., 15/19 humans and 4/19 environmental, yielded carbapenem-resistant Enterobacterales (CRE), noting that 12 environmental samples and 587 human samples were initially screened for CRE from the environment. No CRE were isolated from the pig samples. The CRE identified were *Klebsiella pneumoniae* 9/19 (47%), *Enterobacter hormaechei* 6/19 (32%), *Klebsiella quasi-pneumoniae* 2/19 (11%), a novel ST498 *Citrobacter freundii* 1/19 (5%) and *Serratia marcescens* 1/19 (5%). Eleven isolates were extensively drug-resistant (XDR), and eight were multidrug-resistant (MDR). *Klebsiella pneumoniae* (ST17) and *E. hormaechei* (ST90) were the predominant CREs isolated from humans and the environment. Sixteen of the 19 isolates were CPE harbouring the $bla_{\text{OXA-181}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-484}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{GES-5}}$ carbapenemase-encoding genes. The $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ carbapenemase encoding genes with respective mobile genetic elements (MGEs) were found in multiple species/clones from both sectors. Notably, the IncFIB(K) plasmid replicon was found in most of the *K. pneumoniae* strains (7/9) from humans, and all isolates of *K. quasipneumoniae* from the environment, with the majority of the *K. pneumoniae* isolates were OXA-181 (5/9) producers. The (Col440I) plasmid replicon, identified in 11 (26.82%) isolates, mainly *E. hormaechei* (n = 6) was the most frequently isolated plasmid replicon across both sectors. A significant number of β lactamase-encoding genes were associated with mobile genetic class 1 integrons *IntI*1, insertion sequences (IS)

(IS91, IS5075, IS30, IS3000, IS3, IS19, ISKpn19, IS5075) and transposons (*Tn*3). The IncL/M(pMU407) and IncL/M(pOXA48) plasmid replicons were found exclusively in *K. pneumoniae* across human and environmental sectors, and all but one strain of these strains were OXA-181 producers. A total of 80 virulence genes were found in the eleven *Klebsiella* spp. genomes. Phylogenetic analysis was undertaken with other carbapenemase-producing *K. pneumoniae*, *E. hormaechei*, *S. marcescens*, and *C. freundii* isolates from different sources (animals, environmental sources, and humans) in South Africa. The outcome revealed various relationships such as clustering according to clinical isolates, clustering according to sequence type, and some species belonging to the same clonal node as other clinical isolates previously identified. There were five *Aeromonas* spp. isolates, part of a novel sequence type – ST657, and habouring the bla_{CPHA3} and $bla_{\text{OXA-12}}$ genes obtained from pigs during the screening process of this One Health point prevalence study. These isolates were not CPE but carried the bla_{CPHA3} and $bla_{\text{OXA-12}}$ genes, which conferred resistance to imipenem. No MGEs identified were identified in these isolates.

Conclusion

This One Health Study delineated the resistome, mobilome, virulome, and phylogeny of CPE in human and the environment sectors, highlighting the potential propagation of carbapenemase antibiotic resistance genes via diverse MGEs across the sectors. Such genomic fluidity highlights the need for comprehensive integrated genomic surveillance in a One Health context to address AMR successfully.

1.0. **Introduction**

Antibiotic resistance (ABR) is a complex, global issue that threatens the health and wellbeing of humans, animals, and the environment. Combatting and containing ABR requires collaborative efforts from all three sectors, ideally in the form of a "One Health" approach [1]. Humans, animals, and the environment are interconnected in the causes and consequences of ABR, either directly or indirectly. Undoubtedly, indiscriminate and inappropriate use of antibiotics in humans and animals is a major factor contributing to the increased development and spread of ABR [2]. Other contributory factors include a) increasing global demand for food of animal origin, b) the poor regulation (access and quality) of antibiotics in many parts of the world; c) increasing human and domestic animal populations; d) increasing globalization and international trading [3]. Thus, ABR is widespread in humans, animals, and the environment, with transmission occurring between these sectors via complex pathways [4].

The exponential increase in ABR resulted in the WHO identifying a list of priority pathogens for the research and development of new antibiotics. For example, carbapenem-resistant Enterobacterales (CRE), *Acinetobacter baumannii,* and *Pseudomonas aeruginosa* are critical priority pathogens [5]. Of the CREs, carbapenemase-producing Enterobacterales (CPE) are the most common with other non-specific mechanisms resulting in carbapenem resistance, including the production of other ß-lactamases, efflux pumps, and porin loss. In addition, carbapenemase-encoding genes are located on mobile genetic elements (MGEs) such as transposons, integrons, and plasmids that can be acquired by horizontal gene transfer (HGT), facilitating their dissemination within and between different bacteria and within and between humans, animals, and the environment [5].

Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes(ARGs) can be transferred from animals to humans via the food chain through food contamination during the handling process and the subsequent consumption of raw or undercooked food [6]. ARB harboring ARGs can be discharged via drainage, solid waste, and wastewater from animal farms into receiving environments [7]. This contamination of the environment with ARGs presents a serious threat to human health, the natural environment, and animals. ARBs and ARGs from animals lead to contamination of agricultural (soil, crops) sites and wild animals; ARBs and ARGs may also enter into and pollute nearby surface water through run-off [8]. River water polluted by agriculture and aquaculture activities propagates the spread of ARB spreading to more than one food chain. This fluidity and transmission of ARGs between different environments and bacteria propagate the spread of ABR, threatening all three sectors [9]. Therefore, establishing "One Health" surveillance/monitoring systems incorporating humans, animals, and the environment is critical.

Although the epidemiology, transmission, and spread of CRE and CPE in humans are well described, little is known about their dissemination in livestock and the environment [10,11]. This study delineates the molecular epidemiology of CPEs carried by humans, pigs, and wastewater processing plant

associated with the hospital and abattoir, drawing genomic comparisons between the three sectors within the same geographical area, uMgungundlovu in South Africa. To the best of our knowledge, this is the first study triangulating the molecular epidemiology of CRE in a One Health context within Africa.

2.0. Material and Methods

2.1. Ethical approval

This study was approved by the Biomedical Research Ethics Committee (**BE599/16**, Sub-study of **BCA444/16**) and the Animal Research Ethics Committee (**AREC/079/018D**) of the University of KwaZulu-Natal (UKZN). Permission for animal sampling was obtained from the Department of Agriculture, Forestry and Fisheries (**Ref 12/11/15**). Permission to conduct water sampling was obtained from Umgeni water.

2.2. Study setting

The South African healthcare system is stratified into four levels, depending on the degree of specialisation and location (Figure S1) [12].

This point prevalence study was conducted in three public sector hospitals, a food processing plant with an abattoir, and a wastewater processing plant located within the same geographically area of uMgungundlovu, South Africa (Figure 1). The three healthcare facilities were designated H1 (Level 1; District Hospital), H2 (Level 2; Regional Hospital), H3 (Level 3; Provincial Hospital). The healthcare facilities, levels, number of beds, and patients screened are contained in Table S1 (15).

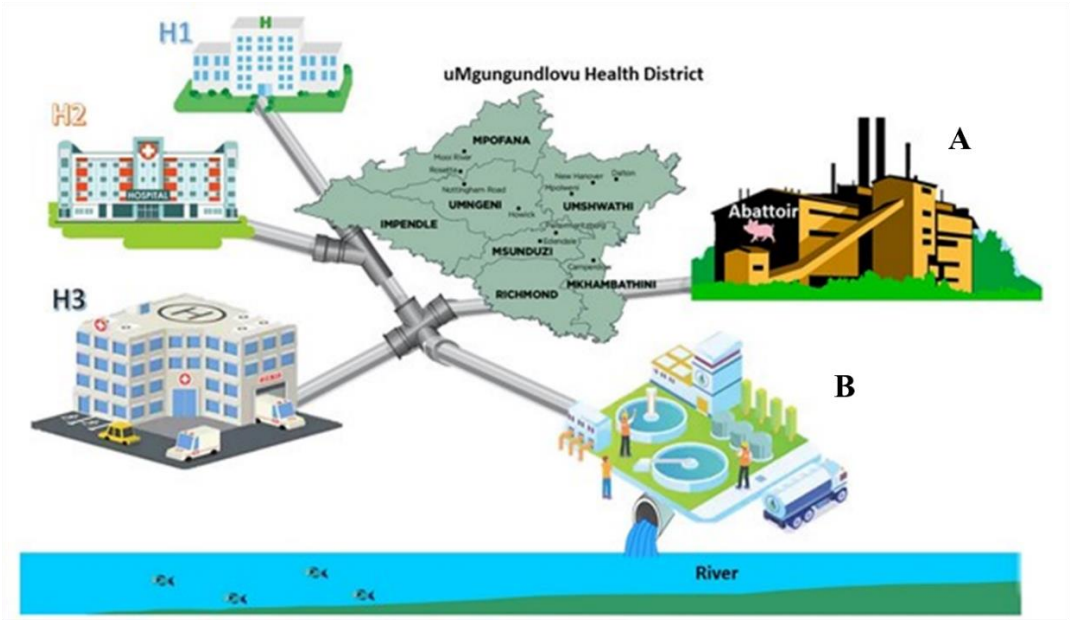

Figure 1. The connection between the three sectors of the One Health Study. A = abattoir, B = wastewater processing plant, and H1, H2, and H3 represent the district, regional and tertiary healthcare

facilities within the same geographical location.

2.3. Sampling

Human samples: A total of 230 rectal swabs were collected from human participants after explicit voluntary written consent was obtained, using nylon flocked swab systems with 5ml of Amies gel transport medium. Three hundred forty-five (345) rectal swabs were collected from pigs 1-5 minutes' post-slaughter. Environmental samples were obtained in triplicates (n = 12) from a wastewater processing plant in the same geographical area as the healthcare facilities and abattoir. Three samples were obtained from each of four areas, the raw influent, final treated effluent, upstream from the WWTP discharge point, and downstream from the WWTP discharge point. Samples were transported on ice to two local laboratories authorized to prcess samples from animals and the environment. All samples were processed within 8 hours of sample collection. Both laboratories partipate in a laboratory Proficiency Testing Scheme (PTS) and are accredited by the South African National Accreditation System (SANAS).

2.4. Isolation and identification of Carbapenemase-producing Enterobacterales (CPE)

Rectal swabs were cultured on ChromID CARBA chromogenic agar medium (BioMérieux, Marcy l'Étoile, France) as previously described [13,14]. This selective chromogenic media allows for rapid and reliable identification of all CPE, particularly NDM-1, OXA-48, and KPC-producers [15]. All plates were incubated for 18 to 24 h at 37 °C under aerobic conditions. Carbapenemase-negative *Klebsiella pneumoniae* ATCC 700603 and carbapenemase-positive *K. pneumoniae* ATCC BAA-1705 were included as controls. For the water samples, 100 µl volumes from each sample were plated in triplicate on the same chromogenic media and incubated under similar conditions.

Presumptive CPE colonies displaying the characteristic phenotypic appearance on chromogenic selective agar (green, blue and pink colonies) were sub-cultured onto MacConkey plates to obtain pure colonies. Following growth on MacConkey plates, isolates were identified using the VITEK 2 (bioMérieux, Marcy l'Étoile, France) automated platform. All Enterobacterales from the rectal swabs with decreased susceptibilities to carbapenems were investigated for carbapenemase production and subjected to further genotypic analysis.

2.4.1. Antimicrobial susceptibility testing (AST)

Antimicrobial susceptibility testing was performed using the microdilution approach on the VITEK 2 automated platform using the VITEK 2 AST-N255 card (BioMérieux Marcy l'Étoile, France). The universal antimicrobial test panel included: ampicillin (AMP), amoxicillin-clavulanate (AMC), ceftriaxone (CRO), cefepime (FEP), cefuroxime (CXM), cefoxitin (FOX), ceftazidime (CAZ),

imipenem (IMI), meropenem (MEM), ertapenem (ERT), piperacillin-tazobactam (TZP), amikacin (AMK), gentamicin (GEN), nitrofurantoin (NIT), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), and tigecycline (TGC). Isolates were categorized as susceptible or resistant using current CLSI-approved breakpoints [16].

2.5. DNA extraction and whole genome sequencing

Genomic DNA (gDNA) was extracted using the GenElute® bacterial genomic DNA kit (Sigma–Aldrich, St. Louis, MO, United States) according to the manufacturer's instructions. The quantity and quality of the extracted gDNA were determined on a Nanodrop spectrophotometer, Qubit (Thermo Scientific, Waltham, MA, USA). Multiplexed paired-end libraries (2 × 300 bp) were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, United States), and sequences were determined on an Illumina MiSeq platform with 100X coverage at the National Institute of Communicable Diseases Sequencing Core Facility, South Africa.

2.6. Genomic analyses and annotation

The resulting raw reads were checked for quality, trimmed, and *de-novo* assembled into contigs using the CLC Genomics Workbench version 10 (CLC, Bio-QIAGEN, Aarhus, Denmark) [17]. The *de-novo* assembled reads were uploaded in GenBank and annotated using the NCBI prokaryotic genome annotation pipeline [18]. The resulting fasta files were deposited at GenBank under the Bioproject **PRJNA564235**. Resistance and virulence genes, plasmids, integrons, transposons, insertion sequences, and prophages were determined using online databases including ResFinder[19], BacWGSTdb [20], PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) [21], INTEGRALL [22], RAST SEEDVIEWER (https://rast.nmpdr.org/seedviewer.cgi), ISFinder (https://isfinder.biotoul.fr/) [23] and PHASTER (https://phaster.ca/) [24], respectively. MLST sequence types for the assembled genomes were determined using the Centre for Genomic Epidemiology (CGE) MLST 1.8 database (http://cge.cbs.dtu.dk/services/MLST/). Virulence genes were assayed using VirulenceFinder 2.0 on the CGE website (https://cge.cbs.dtu.dk/services/VirulenceFinder/).

2.7. Phylogenomics and Phylogeography

Whole genome sequences of *K. pneumoniae*, *Enterobacter* spp., *Citrobacter freundii* and *Serratia marcescens* from South Africa curated at the Pathosystems Resource Integration Center (PATRIC) website (https://www.patricbrc.org/), and National Centre for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/pathogens/) were downloaded and compared with study isolates for epidemiological and evolutionary analysis. The generated phylogenetic trees were visualized, annotated, and edited using Randomized Axelerated Maximum Likelihood (RAxML) and Figtree (http://tree.bio.ed.ac.uk/software/figtree/) [25]. The geographical location of resistant clones/clades was

mapped manually.

3.0. Results

3.1. Demographics and characteristics of CPE in a One Health context

A total of 3.2% (19/587) samples yielded isolates that were resistant to carbapenems. Of the 230 human samples, 15/230 (6,5%) isolates were positive during the screening, and most were from patients admitted to the H2, regional level hospital 9/15 (60%). The ages of the patients ranged from 33 to 91 years. The proportion of males to females in the study were similar, with seven isolates from males and eight isolates from females. No CPE was isolated from animal samples. Also, four CPE (4/12; 33%) were isolated from the water samples (Table 1). Of the four positive water samples, three samples were from the influent, and one was obtained upstream from the WWTP. The most isolated CRE identified in the study was *K. pneumoniae* 9/19 (47%), followed by *Enterobacter hormaechei* 6/19 (32%) (Table 1).

3.2. Antibiotic Phenotypes

All the CREs detected were resistant to at least one of the carbapenems tested. All 19 (100%) isolates were resistant to AMC, CXM, CTX, CAZ, CRO, and ERT, while the lowest resistance was observed against AMK (4/19; 21%) (Figure 2).

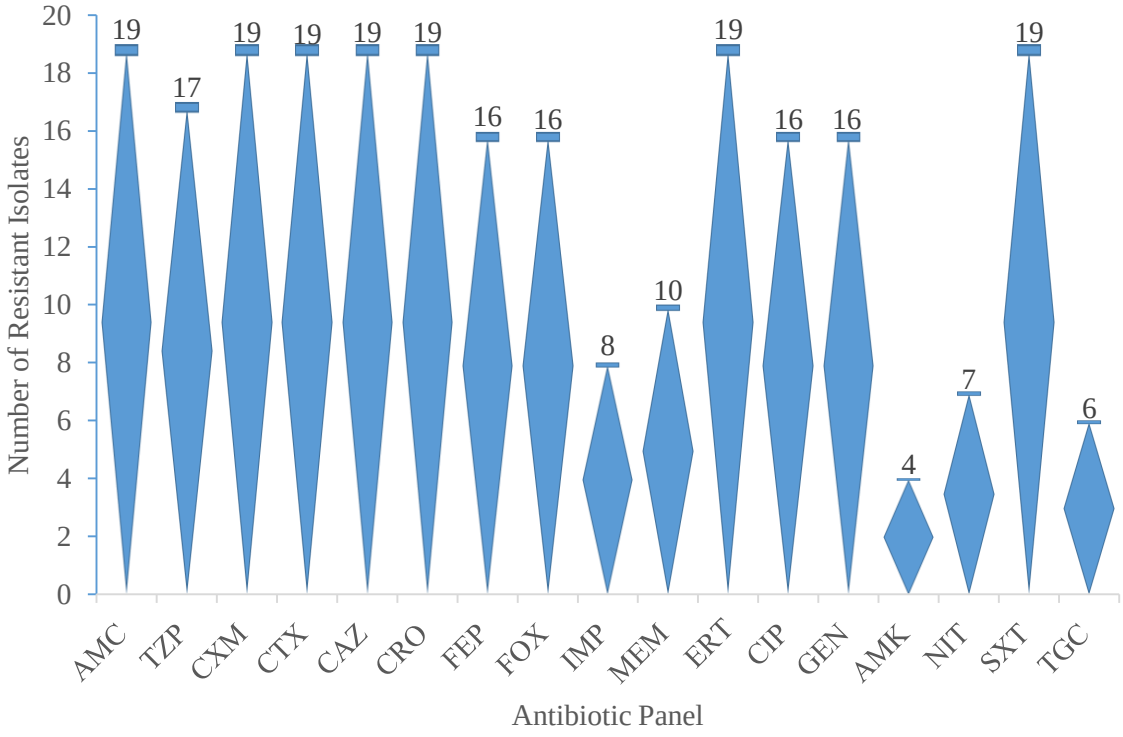

Figure 2. Resistance pattern of isolates to selected antibiotics.

Six isolates showed intermediate susceptibility to imipenem, and three isolates showed intermediate susceptibility to meropenem. The isolates were multidrug-resistant (MDR), with a substantial number

being extensively drug-resistant (XDR) (Table 1). MDR and XDR catergorized according to standard criteria [26]. Of note, all the environmental samples were XDR.

3.3. Genome features

The genomic features of the sequences, in terms of size, coverage, L50, N50, CRISPR arrays, GC content, number of RNAs, and coding sequences are shown in (Table S2).

3.4. Molecular typing (sequence types and capsular polysaccharide serotypes)

MLST analyses revealed 12 sequence types (STs). The CR-*K. pneumoniae* isolates from human and water samples were multiclonal belonging to 5 different STs namely, ST17 (n = 4), ST15 (n = 2), ST152 (n = 1), ST607 (n = 1), and ST39 (n = 1). The two *K. quasipneumoniae* strains belonged to two different STs (ST3318 and ST1889). Similar diversity was observed in the six CR-*E. hormaechei* strains which belonged to four STs; ST90 (n = 3), ST78 (n = 1), ST88 (n = 1) and ST231 (n = 231). The *C. freundii* isolate belonged to a novel sequence type (ST489). The *Serratia marcescens* had no MLST typing scheme (Table 2). The CR-*K. pneumoniae* isolates of the same sequence type possessed the same capsular serotypes (K and O loci) types (Figure S2) and were clone-specific (Table 2). The STs of the different species differed in humans and the environment. There were no similar ST between both sectors.

3.5. Antibiogram-resistome associations of the CREs

Resistome analysis showed that 16/19 isolates resistant to at least one carbapenem contained carbapenemase-encoding genes. Five different carbapenemase encoding genes belonging to class A, B, and D were identified (Table 1). The most prevalent carbapenemase-encoding gene was $bla_{\text{OXA-181}}$ (9/16), followed by $bla_{\text{NDM-1}}$ (4/16) with singletons of $bla_{\text{OXA-48}}$, $bla_{\text{OXA-484,}}$ and $bla_{\text{GES-5}}$. The $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-181}}$ were common to both human and environmental isolates. Isolates containing the $bla_{\text{OXA-181}}$ carbapenemase gene (9/16) displayed varying susceptibility to ertapenem, imipenem, and meropenem (Table 1). All the isolates carrying $bla_{\text{NDM-1}}$ were resistant to all the carbapenems and demonstrated high-level resistance to meropenem. Only one isolate demonstrated high-level resistance to imipenem. The $bla_{\text{NDM-1}}$ gene was found in all the species except *E. hormaechei*. Of the six *E. hormaechei* strains, three harboured the $bla_{\text{OXA-181}}$ carbapenemase gene while carbapenemase genes were not detected in the remaining three *E. hormaechei* isolates. Additionally, isolates harboring $bla_{\text{OXA-48}}$ and $bla_{\text{GES-5}}$ genes displayed resistance to all carbapenems with the $bla_{\text{GES-5}}$-harbouring isolate showing high-level resistance for meropenem and imipenem, respectively. The $bla_{\text{OXA-484}}$-containing isolate exhibited high-level resistance to imipenem and intermediate susceptibility to meropenem. None of the isolates contained more than one carbapenemase (Table 1).

The resistomes of the sequenced CPE isolates were diverse and correlated with their respective

antibiograms (Table 1). Genes conferring resistance to aminoglycoside, quinolones, chloramphenicol, tetracycline, and trimethoprim were also identified (Table 2). Chromosomal mutations conferring high-level ciprofloxacin (*GyrA* and/or *ParC*), and tigecycline (*acrR*, *ramR*, and *marR*) resistance were only observed in some species (Table S3).

TABLE 1. MICs and carbapenemases

Isolate ID (MLST)	**MIC (µg/ml)**			**Category**	**CP+**	**Carbapenemase**	**Class**	**Other β-lactamases**	**Species**
	IMI	**MER**	**ERT**						
Human									
H2730R (ST498)	≥16 (R)	≥16 (R)	≥8 (R)	XDR	+	*bla*$_{NDM-1}$	B	*bla*$_{CMY-48}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-10}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$	*C. freundii*
H8767R (ST90)	1 (S)	1 (S)	≥8 (R)	MDR		-	-	*bla*$_{ACT-15}$	*E. hormaechei*
H8871 (ST78)	1 (S)	1 (S)	≥8 (R)	MDR	+	*bla*$_{OXA-181}$	D	*bla*$_{CTX-M-71}$, *bla*$_{ACT-5}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$	*E. hormaechei*
H8825R (ST90)	1 (S)	1 (S)	4 (R)	MDR		-	-	*bla*$_{CTX-M-15}$, *bla*$_{ACT-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$	*E. hormaechei*
H2741 (ST90)	1 (S)	0.5 (S)	4 (R)	MDR		-	-	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$	*E. hormaechei*
H2716R4 (ST231)	2 (I)	4 (R)	≥8 (R)	XDR	+	*bla*$_{OXA-181}$	D	*bla*$_{ACT-7}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{OXA-9}$, *bla*$_{TEM-1B}$	*E. hormaechei*
H8860 (ST15)	≥16 (R)	≥16 (R)	≥8 (R)	XDR	+	*bla*$_{OXA-181}$	D	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *bla*$_{SHV-28}$	*K. pneumoniae*
H8844R (ST17)	≥16 (R)	2 (I)	4 (R)	XDR	+	*bla*$_{OXA-484}$	D	*bla*$_{SHV-94}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-10}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$	*K. pneumoniae*
H8773R (ST17)	2 (I)	2 (I)	4 (R)	XDR	+	*bla*$_{OXA-181}$	D	*bla*$_{SHV-94}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$	*K. pneumoniae*
H8770 (ST17)	2 (I)	8 (R)	≥8 (R)	XDR	+	*bla*$_{OXA-181}$	D	*bla*$_{SHV-94}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$	*K. pneumoniae*

H4767 (ST39)	2 (I)	1 (S)	4 (R)	MDR	+	*bla*$_{OXA-181}$	D	*bla*$_{CTX-M-3}$, *bla*$_{SHV-40}$, *bla*$_{TEM-1B}$	*K. pneumoniae*
H2850 (ST15)	2 (I)	≤0.25 (S)	4 (R)	MDR	+	*bla*$_{OXA-181}$	D	*bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-28}$, *bla*$_{OXA-1}$	*K. pneumoniae*
H2745 (ST607)	4 (R)	≥16 (R)	≥8 (R)	MDR	+	*bla*$_{NDM-1}$	B	*bla*$_{OXA-10}$, *bla*$_{DHA-1}$, *bla*$_{TEM-1B}$, *bla*$_{SHV-65}$, *bla*$_{CTX-M-15}$	*K. pneumoniae*
H2737 (ST17)	2 (I)	2 (I)	4 (R)	XDR	+	*bla*$_{OXA-181}$	D	*bla*$_{SHV-94}$, *bla*$_{OXA-10}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$	*K. pneumoniae*
H8980R (NA)	≥32 (R)	≥16 (R)	≥8 (R)	XDR	+	*bla*$_{NDM-1}$	B	*bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *bla*$_{SRT-1}$, *bla*$_{OXA-10}$	*S. marcescens*
Environment									
INF3-a (ST88)	≥16 (R)	4 (R)	≥8 (R)	XDR	+	*bla*$_{OXA-181}$	D	*bla*$_{ACT-7}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$	*E. hormaechei*
US2 (ST152)	≥16 (R)	4 (R)	≥8 (R)	XDR	+	*bla*$_{OXA-48}$	D	*bla*$_{CTX-M-15}$	*K. pneumoniae*
INF3b (ST1889)	≥16 (R)	≥16 (R)	≥8 (R)	XDR	+	*bla*$_{NDM-1}$	B	*bla*$_{OXA-10}$, *bla*$_{CTX-M-10}$, *blaOKP-B-9*, *bla*$_{SHV-12}$	*K. quasipneumoniae*
INF1-a (ST3318)	≥16 (R)	≥16 (R)	≥8 (R)	XDR	+	*bla*$_{GES-5}$	A	*bla*$_{CTX-M-3}$, *bla*$_{OKP-B-3}$, *bla*$_{TEM-1B}$	*K. quasipneumoniae*

Table 2. Resistome and mobilome characteristics of the isolates**.**

Plasmid type and sample code MLST clone) a	**Species**	**Resistome**	**Plasmid replicons**	**pMLST**	**Integron (s)**
IncF[K-:A13:B-]					
IncF[K-:A13:B-] H2745 (ST607) [KL25-O1]	*K.pneumoniae*	*bla*$_{NDM-1}$, *bla*$_{OXA-10}$, *bla*$_{DHA-1}$, *bla*$_{TEM-1B}$, *bla*$_{SHV-65}$, *bla*$_{CTX-M-15}$*; aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, aadA1; qnrB4, oqxA, oqxB; fosA; dfrA14; ARR-2; sul2, sul1*	Col440I, ColRNAI, FIA(pBK30683), IncFIB(K), IncR, IncT	IncF[K-:A13:B-]	IntI1[b]
IncF[K-:A13:B-] H8770 (ST17) [KL25-O5]	*K. pneumoniae*	*bla*$_{OXA-181}$, *bla*$_{SHV-94}$, *bla*$_{CTX-M-15}$, *word1; aac(3)-Iia, aadA16, aac(6')-Ib3, aph(6)-Id, aph(3'')-Ib; qnrB6, oqxA, oqxB, aac(6')-Ib-cr; fosA; dfrA27,dfrA17; ARR-3; catB3; sul2,sul1; tet(D)*	Col440I, FIA(pBK30683), IncFIB(Mar), IncL/M(pMU407), IncR	IncF[K-:A13:B-]	
IncF[K-:A13:B-] H8773R (ST17) [KL25-O5]	*K. pneumoniae*	*bla*$_{OXA-181}$, *bla*$_{SHV-94}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$*; aadA16, aac(3)-Iid, aac(3)-Iia, aph(6)-Id, aph(3'')-Ib, aac(6')-Ib-cr; qnrB6, oqxA, oqxB, aac(6')-Ib-cr; fosA; dfrA27, dfrA17; ARR-3; sul2, sul1; tet(D)*	Col440I, ColKP3, FIA(pBK30683), IncL/M(pMU407), IncR	IncF[K-:A13:B-]	
IncF[K-:A-:B-]					
IncF[K-:A-:B-] H8871 (ST78)	*E. hormaechei*	*bla*$_{OXA-181}$, *bla*$_{CTX-M-71}$, *bla*$_{ACT-5}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$*; aadA1, aph(3'')-Ib, aac(3)-Iia, aac(3)-Iid, aph(6)-Id, aadA5; qnrS1, qnrB1; fosA; dfrA14, dfrA15, dfrA17; ARR-3; catB3, cmlA1; sul2, sul1; tet(A)*	Col440I, Col440II, ColKP3, IncFIB(pHCM2), IncHI2, IncHI2A, IncN, IncU, IncX3, RepA	IncF[K-:A-:B-]	
IncF[K-:A-:B-] H4767 (ST39) [KL149-O1]	*K. pneumoniae*	*bla*$_{OXA-181}$, *bla*$_{CTX-M-3}$, *bla*$_{SHV-40}$, *bla*$_{TEM-1B}$*; aac(6')-Ib3, aadA16; aac(6')-Ib-cr, oqxB, oqxA; fosA; dfrA14, dfrA27, dfrA17; ARR-3; floR; sul1; tet(D), tet(A)*	Col440I, ColKP3, IncFIB(K), IncL/M(pMU407), IncR	IncF[K-:A-:B-]	
IncF[K13:A13:B-]					

IncF[K13:A13:B-] H2737 (ST17) [KL25-O5]	*K. pneumoniae*	*bla*$_{OXA-181}$, *bla*$_{SHV-94}$, *bla*$_{OXA-10}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$; *aac(6')-Ib3, aac(3)-Iia, aph(6)-Id, aadA1, aac(3)-Iid, aadA16, aph(3'')-Ib; aac(6')-Ib-cr, oqxA, oqxB, qnrB1; fosA; dfrA23, dfrA27, dfrA14; catB3, cmlA1; sul2, sul1; tet(A)*	ColKP3, FIA(pBK30683), IncA/C2, IncFIB(K), IncFII(K), IncR, IncX3	IncF[K13:A13:B-]	
IncF[K13:A13:B-] H8844R (ST17) [KL25-O5]	*K. pneumoniae*	*bla*$_{OXA-484}$, *bla*$_{SHV-94}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-10}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$; *aac(6')-Ib3, aac(3)-Iia, aph(6)-Id, aadA1, aac(3)-Iid, aadA16, aph(3'')-Ib; aac(6')-Ib-cr, oqxA, oqxB, qnrB1; fosA; dfrA23, dfrA27, dfrA14; catB3, cmlA1; sul2, sul1; tet(A)*	ColKP3, FIA(pBK30683), IncA/C2, IncFIB(K), IncFII(K), IncR, IncX3	IncF[K13:A13:B-]	
IncF[K9:A-:B-]					
IncF[K9:A-:B-] H2850 (ST15) [KL112-O1]	*K. pneumoniae*	*bla*$_{OXA-181}$, *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-28}$, *bla*$_{OXA-1}$; *aac(6')-Ib-cr, aac(3)-Iia, aph(6)-Id, aph(3'')-Ib; aac(6')-Ib-cr, oqxA, oqxB, qnrB1; fosA; dfrA14; catB3; sul2; tet(A)*	Col(MGD2), ColKP3, ColpVC, ColRNAI, IncFIB(K), IncFII, IncL/M(pMU407)	IncF[K9:A-:B-]	
IncF[K9:A-:B-] H8860 (ST15) [KL112-O1]	*K. pneumoniae*	*bla*$_{OXA-181}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *bla*$_{SHV-28}$; *aac(6')-Ib-cr, aac(3)-Iia, aph(6)-Id, aph(3'')-Ib; aac(6')-Ib-cr, oqxA, oqxB, qnrB1; fosA; dfrA14, dfrA17; catB3; sul2, sul1; tet(A)*	Col(MGD2), ColKP3, ColpVC, ColRNAI, IncFIB(K), IncFII, IncL/M(pMU407)	IncF[K9:A-:B-]	
IncF[Y3:A13:B-] H2716R4 (ST231)	*E. hormaechei*	*bla*$_{OXA-181}$,*bla*$_{ACT-7}$,*bla*$_{CTX-M-15}$,*bla*$_{OXA-1}$,*bla*$_{OXA-9}$,*bla*$_{TEM-1B}$; *aph(3'')-Ib, aph(6)-Id, aadA24, aac(3)-Iid, aadA5; qnrS1; fosA; dfrA14, dfrA17; ARR-2; catB3, cmlA1, catA2; sul2, sul1*	Col440I, Col440II, ColKP3, ColpVC, ColRNAI, FIA(pBK30683), IncFII(Yp), IncR, IncX3	IncF[Y3:A13:B-]	
IncHI2-ST-1 H2741 (ST90)	*E. hormaechei*	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$; *aac(6')-Ib3, aadA1, aac(3)-Iia, aadA16, aadA2, aph(6)-Id, aph(3'')-Ib, aac(6')-Ib-cr, qnrB1; fosA; dfrA12, dfrA27; ARR-3; catA1, catB3; sul2, sul1; tet(A)*	Col440I, Col440II, IncHI2, IncHI2A, RepA	IncHI2-ST-1	
IncHI2	*E. hormaechei*	*bla*$_{CTX-M-15}$, *bla*$_{ACT-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$; *aac(6')-Ib-cr, aadA1,*	Col440I, Col440II, IncHI2,	IncHI2	

H8825R (ST90)		*aac(3)Iia, aadA16, aadA2, aph(6)-Id, aph(3'')-Ib; aac(6')-Ib-cr, qnrB1; fosA; dfrA12, dfrA27; ARR-3; catB3, catA1; sul2, sul1; tet(A)*	IncHI2A, RepA		
IncF[Y4:A-:B36] H2730R (ST498)	*C. freundii*	*bla*$_{NDM-1}$, *bla*$_{CMY-48}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-10}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$*; aph(3')-Ia, aac(6')-Ib-cr, aac(3)-Iia, aph(6)-Id, aadA1, aac(3)-Iid, rmtC, aph(3'')-Ib; aac(6')-Ib-cr, qnrB1; fosA; dfrA23, dfrA7, dfrA14; ARR-2; catB3, cmlA1; sul2, sul1; tet(A)*	IncA/C2, IncFIB(pB171), IncFII(Yp), IncQ1	IncF[Y4:A-:B36]	
N/A H8767R (ST90)	*E. hormaechei*	*bla*$_{ACT-15}$*; aadA2; fosA; dfrA12*	Col440I, Col440II	N/A	
N/A H8980R (No ST)	[c]*S. marcescens*	*bla*$_{NDM-1}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *bla*$_{SRT-1}$, *bla*$_{OXA-10}$*; rmtC, aadA1, aac(6')-Ib-cr, aac(6')-Ic, aac(3)-Iid, aph(6)-Id, aph(3'')-Ib; aac(6')-Ib-cr; dfrA23; ARR-2; catB3, cmlA1; sul2, sul1*	N/A	N/A	IntI1[b]
Environment					
IncF[K29:A-:B36] INF3b (ST1889) [KL14-O12]	*Klebsiella quasipneumoniae*	*bla*$_{NDM-1}$, *bla*$_{OXA-10}$, *bla*$_{CTX-M-10}$, *bla*$_{OKP-B-9}$, *bla*$_{SHV-12}$*; aac(3)-I, aph(6)-Id, aph(3'')-Ib, aph(3')-VI, aac(6')-Iic, aadA24, aadA2; oqxA, oqxB, qnrB2; fosA; dfrA23, dfrA19; ARR-2; catA2, cmlA1, floR; sul2, sul1; tet(A), tet(D).*	Col440I, Col440II, ColRNAI, IncA/C2, IncFIB(K), IncFIB(pB171), IncFII(K), IncFII(Yp),IncHI2,IncHI2A,IncR, RepA	IncF[K29:A-:B36]	
IncF[K12:A-:B36] US2 (ST152) [KL149-O4]	*K. pneumoniae*	*bla*$_{OXA-48}$, *bla*$_{CTX-M-15}$*; aac(3)-IIa, aph(3'')-Ia, aadA16, aph(3)-Ib, aac(6')Ib-3, aph(6)-Id; oqxA, oqxB, qnrB6, aac(6')-Ib-cr; fosA; dfrA27; ARR-3; catA1; sul2, sul1*	IncL/M(pOXA48), IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI	IncF[K12:A-:B36]	
IncF[Y3:A-:B70] INF3-a (ST88)	*E. hormaechei*	*bla*$_{OXA-181}$, *bla*$_{TEM-1B}$, *bla*$_{ACT-7}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$*; aph(6)-Id, aadA1, aadA16, aac(6')-Ib-cr, aac(3)-Iid, aadA2, aph(3'')-Ib; aac(6')-Ib-cr, qnrB1,qnrS1; dfrA12,dfrA27; ARR-3; catA1, catB3;*	Col440I, Col440I, RepA, ColKP3, IncFIB(pB171), IncFII(Yp), IncHI2, IncHI2A,	IncF[Y3:A-:B70]	

		sul2,sul1; tet(A), tet(D)	IncX3		
IncF[K4:A-:B-] INF1-a (ST3318) [KL146-O12]	*K. quasipneumoniae*	*bla*$_{GES-5}$, *bla*$_{CTX-M-3}$, *bla*$_{OKP-B-3}$, *bla*$_{TEM-1B}$*; oqxA, oqxB, qnrS1; dfrA14; ARR-3*	Col(MGD2), Col440I, Col440II, ColRNAI, FII(pBK30683), IncFIB(K), IncFIB(pQil), IncFII,IncFII(K), IncN, IncR, IncU,pSL483	IncF[K4:A-:B-]	

[a]MLST-Multilocus sequence typing, *K* and *O* typing-*Klebsiella* surface polysaccharide capsule characterization (K-loci and O-loci) typing scheme; [b]class 1 integron-integrase gene (intI1). [c]The *S. marcescens* had no MLST typing scheme.

3.6. Mobilome (Plasmids, Insertion sequences, Intact prophages, and Integrons) and Associated ARGs

A total of 42 plasmid replicons were identified amongst 19 isolates, with most of the isolates (89.47%) having more than five plasmid replicons (Table 2). The two *K. quasipneumoniae* isolates (from WWTP influent) showed the highest multi replicon combination. The most frequently isolated plasmid replicon, Col440I, was identified in 11 (26.82%) isolates (Table 2). No plasmid replicons were found in the *bla*$_{\text{NDM-1}}$-producing *S. marcescens* isolate - H8980R (Table 4). Also, the Col440I, IncFIB(K), IncHI2, IncHI2A, and IncN plasmid replicon were found in human and environmental isolates. The IncQ1 plasmid replicon was found in the *bla*$_{\text{NDM-1}}$-producing *C. freundii* isolate only. The IncL/M(pMU407) and IncL/M(pOXA48) plasmid replicons were found exclusively in *Klebsiella pneumoniae,* and all but one strain of these strains were OXA-181 producers (Table 2).

Antibiotic resistance genes were carried on transposable elements and/or insertion sequences; a few were found on class 1 integrons (Figure S3) (Table 3). In all OXA-181-producing isolates (H8860, H8773R, H8871, H8770, H4767, H2850, H2737, INF3-a) except one (H2716R4), the genetic environment of the *bla*$_{\text{OXA-181}}$ gene had the *ereA* gene which codes for an erythromycin esterase that confers high-level resistance to erythromycin (Table 3). The *bla*$_{\text{OXA-181}}$ gene was consistently associated with insertion sequences (ISKpn19, IS3000), transposons (Tn3), and a recombinase. All the contigs harbouring the *bla*$_{\text{OXA-181}}$ gene had high similarity (99-100%) to plasmids in GenBank.

The genetic environment of the isolates highlighting the AMR genes and associated MGEs were mapped (Figure S3) and tabulated (Table 3). The genetic environment of the *bla*$_{\text{NDM-1}}$ gene consistently had insertion sequences, with IS91 occurring in all contigs bearing the gene and were probably carried on plasmids based on the high similarity (99-100%) of the source contigs to plasmids in GenBank. There were no transposons, integrons, or insertion sequences associated with *bla*$_{\text{OXA-484}}$, *bla*$_{\text{OXA-48}}$, and *bla*$_{\text{GES-5}}$. The genetic environment of the *bla*$_{\text{TEM-1b}}$ consisted of other ARGs, including those encoding resistance to β-lactams, aminoglycoside, and sulphonamides. Different combinations of the resistance genes were co-carried on the Tn3 transposon, especially those harbouring the *bla*$_{\text{TEM-1b}}$ and CTX-M-3 or CTX-M - 15 genes. The Tn3 carried ARG cassettes were also consistently associated with a recombinase, and the insertion sequences IS91 and/or ISEc9 (Figure S3, Table 3). The class 1 integron in isolate H8980R was flanked by the insertion sequence IS91 and had ARGs encoding resistance to trimethoprim, sulphonamide, rifamycin, chloramphenicol, and β-lactams. The disinfectant resistance gene (*qacEΔ1*) which encodes for resistance to quartanry ammonium compound disinfectants, *AadA1*, *bla*$_{\text{OXA-10}}$, and *Arr-2* were common to both isolates with the the class 1 integron. The trimethoprim resistance gene, *DfrA14*, was unique to the class 1 integron in isolate H2745. The genetic environment of ARGs was similar for both the environmental and human isolates.

Table 3. Mobile genetic elements associated with antibiotic resistance

Sample	MLST	Beta lactam - ARG	Contigs	Synteny of resistance genes and MGE	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
US2	ST152	*bla*$_{OXA-48}$	253	OXA-48	*K. pneumoniae* strain KPC160132 chromosome (CP040023.1) (99%)
		bla$_{CTX-M-15}$	196	IS110:ISEc9:*bla*$_{CTX-M-15}$:wbuC(metalloprotein):IS4321:ISPa38 (Tn3)	*K. pneumoniae* strain E17KP0027 chromosome (CP052239.1) (99%)
INF3b	ST1889	*bla*$_{NDM-1}$	159	*bla*$_{NDM-1}$:Ble-MBL(bleomycin binding protein)::::::IS91:IS30:IS3	*K. quasipneumoniae*strain QD1501 plasmid pQD1501-Ct1 (MN310375.1) (99%)
		bla$_{OXA-10}$	205	Arr2(rifampin resistance):CmlA5(chloramphenicol efflux MFS transporter):*bla*$_{OXA-10}$	*K. quasipneumoniae*SNI47 plasmid pTMSNI47-1 (AP019688.1) (100%)
		bla$_{SHV-12}$	187	*bla*$_{SHV-12}$	*K. quasipneumoniae*subsp. quasipneumoniae strain M17277 plasmid p17277A_477 (CP043927.1) (100%)
		bla$_{CTX-M-10}$	55	wbuC(metalloprotein):*bla*$_{CTX-M-37}$	*K. quasipneumoniae*strain NCTC9170, chromosome (LR588411.1) (99.04%)
		bla$_{OKP-B-9}$	25	*bla*$_{OKP-B-9}$	*K. quasipneumoniae*strain Pinhead Larry chromosome (CP065467.1) (99%)
INF3-a	ST88	*bla*$_{TEM-1B}$	158	*bla*$_{TEM-1B}$	*E. hormaechei* strain AMS-38 plasmid pAMS-38c (CP051135.1) (100%)
		bla$_{OXA-1}$	145	catB:*bla*$_{OXA-1}$	*E. hormaechei* subsp. hoffmannii strain Eh1 plasmid p1 (CP034755.1) (100%)
		bla$_{CTX-M-15}$	115	*bla*$_{CTX-M-15}$:wbuC(metalloprotein):Tn3	*E. hormaechei* strain AUH-ENM30 chromosome (CP045611.1) (100%)
		bla$_{OXA-181}$	90	*bla*$_{OXA-1}$81:EreA(Ery resistance)::ISKpn19:recombinase:pRiA4b ORF-3 (plasmid protein)::recombinase:QnrS1:transposase	*E. hormaechei* subsp. xiangfangensis M206 plasmid pM206-OXA181 (AP018831.1) (100%)
		bla$_{ACT-7}$	2	sugE(DRG)::*bla*$_{ACT-17}$:AmpR(transcription regulator)	*E. hormaechei* strain EB_P6_L3_02.19 chromosome (CP043853.1
INF1-a	ST3318	*bla*$_{GES-5}$	491	*bla*$_{GES-5}$	*K. quasipneumoniae* strain KPN 47 plasmid p3 (CP066863.1) (99%)
		bla$_{OKP-B-3}$	324	*bla*$_{OKP-B-3}$	*K. quasipneumoniae* strain NCTC11357, Chromosome (LR134196.1) (99%)
		bla$_{TEM-1B}$, *bla*$_{CTX-M-3}$	144	conjugative relaxase::Tn3:ISEc9:*bla*$_{CTX-M-3}$:wbuC(metalloprotein):*bla*$_{TEM-1B}$:recombinase	*K. quasipneumoniae* strain L22 plasmid pL22-2 (CP031259.1) (99%)
Human					
H8980R	N/A	*bla*$_{OXA-1}$	60	catB:*bla*$_{OXA-1}$:AAC(6')-Ib-cr5	*E. coli* strain AH01 plasmid pAH01-4 (CP055255.1) (99%)
		bla$_{NDM-1}$	53	*bla*$_{NDM-1}$:Ble-MBL::::::IS91	*K. pneumoniae* subsp. pneumoniae strain ST2017:950142398

					plasmid p18-43_01 (CP023554.1) (100%)
		bla$_{OXA-10}$	43	tyrosine-type recombinase:recombinase:Tn3:IS5075:::DfrA23:::IS91:Sul1:qacEΔ1:AadA1:*bla*$_{OXA-10}$:CmlA5:Arr-2:IntI1	*Vibrio cholerae* strain YA00120881 plasmid Pya00120881 (MT151380.1) (99%)
		bla$_{TEM-1B}$	23	ISEc9::recombinase:*bla*$_{TEM-1B}$:IS91:APH(6)-Id:APH(3")-Ib:Sul2::ParA:repB	*Edwardsiella ictaluri* strain MS-17-156 plasmid pEI-MS-17-156-1 (CP028814.1) (100%)
		bla$_{SRT-1}$	1	*bla*$_{SRT-1}$	
H8871	ST78	*bla*$_{CTX-M-71}$	125	*bla*$_{CTX-M-71}$** class A *bla* gene not specified	*E. hormaechei* strain E70 plasmid pE70-TEM1 (CP046275.1) (100%)
		bla$_{TEM-1B}$	107	*bla*$_{TEM-1B}$	*E. hormaechei* strain AMS-38 plasmid pAMS-38c (CP051135.1) (100%)
		bla$_{OXA-1}$	98	catB:*bla*$_{OXA-1}$:AAC(6')-Ib	*E. hormaechei* subsp. hoffmannii strain Eh1 plasmid p1 (CP034755.1) (100%)
		bla$_{OXA-181}$	86	EreA:*bla*$_{OXA-1}$81	*E. hormaechei* subsp. steigerwaltii strain ST190 plasmid pLAU_ENM17_OXA181 (MN792919.1) (100%)
		bla$_{ACT-5}$	10	*bla*ACT-24::sugE	*E. hormaechei* subsp. hoffmannii OIPH-N069 DNA, chromosome (AP019817.1) (99%)
H8860	ST15	*bla*$_{OXA-1}$	110	CatB:*bla*$_{OXA-1}$:AAC(6')-Ib-cr5	*K. pneumoniae* strain B17KP0069 plasmid pB17KP0069-1 (CP052491.1) (100%)
		bla$_{CTX-M-15}$	100	Tn3:wbuC:*bla*$_{CTX-M-15}$	*K. pneumoniae* strain MS14393 plasmid pMS14393B (CP054305.1) (100%)
		bla$_{TEM-1B}$	88	ISEc9::recombinase:*bla*$_{TEM-1B}$:IS91:APH(6)-Id:APH(3")-Ib:sul2:	*K. pneumoniae* strain E16KP0288 plasmid pE16K0288-1 (CP052263.1) (100%)
		bla$_{OXA-181}$	47	*bla*$_{OXA-1}$81:EreA::ISKpn19:recombinase:pRiA4b ORF-3:::TrbA(conjugal transfer protein)	*K. pneumoniae* strain 721005 plasmid p721005-3 (CP030295.1) (99%)
		bla$_{SHV-28}$	35	*bla*$_{SHV-28}$	*K. pneumoniae* strain B17KP0069 chromosome (CP052490.1) (100%)
H8844R	ST17	*bla*$_{OXA-484}$	125	*bla*$_{OXA-484}$** class D *bla* gene not specified	*K. pneumoniae* strain 50595 plasmid p50595_OXA_181 (CP050375.1) (100%)
		bla$_{OXA-1}$	91	catB:*bla*$_{OXA-1}$:AAC(6')-Ib-cr5	*K. pneumoniae* strain MS14393 plasmid pMS14393B (CP054305.1) (99.48%)

		bla$_{OXA-10}$	77	CmlA5:*bla*$_{OXA-10}$:AadA1:qacEΔ1:sul1	*K. pneumoniae* strain AR_0139 plasmid tig00000006 (CP021961.1) (100%)
		bla$_{TEM-1B}$, *bla*$_{CTX-M-15}$	69	*bla*$_{TEM-1B}$:recombinase::ISEc9:*bla*$_{CTX-M-15}$:WbuC:Tn3	*K. pneumoniae* strain D16KP0122 plasmid pD16KP0122-2 (CP052364.1) (100%)
		bla$_{SHV-94}$	12	*bla*$_{SHV-11}$	*K. pneumoniae* isolate KSB1_9J-sc-2280309, chromosome (LR890434.1) (99%)
H8825R	ST90	*bla*$_{OXA-1}$	60	CatB:*bla*$_{OXA-1}$	*E. hormaechei* subsp. hoffmannii strain Eh1 plasmid p1 (CP034755.1) (100%)
		bla$_{CTX-M-15}$, *bla*$_{TEM-1B}$	38	Tn3:WbuC:*bla*$_{CTX-M-15}$:ISEc9::recombinase:*bla*$_{TEM-1B}$:IS91:APH(6)-Id:APH(3")-Ib:Sul2	*E. hormaechei* strain Eho-10 plasmid pEcl10-1 (CP048704.1) (99%)
		bla$_{ACT-15}$	5	AmpR (transcriptional regulator):*bla*ACT-56::sugE	*E. hormaechei* strain EB_P9_L5_03.19 chromosome (CP043766.1) (99.97%)
H8773R	ST17	*bla*$_{OXA-181}$	48	Tn3:IS3000:*bla*$_{OXA-1}$81:EreA	*K. pneumoniae* strain 709 plasmid pKP709-OXA181 (MN227183.1) (100%)
		bla$_{TEM-1B}$	38	ISKpn12::IS3::ISKpn11::AAC(3)-IIe:*bla*$_{TEM-1B}$:recombinase:	*K. pneumoniae* plasmid pKp848CTX (LM994717.1) (99%)
		bla$_{CTX-M-15}$	22	WbuC:*bla*$_{CTX-M-15}$	*K. pneumoniae* strain F93-2 chromosome, (CP026157.1) (99%)
		bla$_{SHV-94}$	3	*bla*$_{SHV-11}$	*K. pneumoniae* isolate KSB1_9J-sc-2280309, chromosome (LR890434.1) (99%)
H8770	ST17	*bla*$_{CTX-M-15}$	157	*bla*$_{CTX-M-15}$:WbuC	*K. pneumoniae* strain MS14393 plasmid pMS14393B (CP054305.1) (100%)
		bla$_{OXA-1}$	152	CatB:*bla*$_{OXA-1}$	*K. pneumoniae* strain MS14393 plasmid pMS14393B (CP054305.1) (100%)
		bla$_{OXA-181}$	126	EreA:*bla*$_{OXA-1}$81	*K. pneumoniae* strain 50595 plasmid p50595_OXA_181 (CP050375.1) (100%)
		bla$_{SHV-94}$	33	*bla*$_{SHV-11}$	*K. pneumoniae* strain YML0508 chromosome, (CP045193.1) (99%)
H8767R	ST90	*bla*$_{ACT-15}$	2	*bla*$_{ACT-56}$::sugE	*E. hormaechei* strain EB_P9_L5_03.19 chromosome (CP043766.1) (99%)
H4767	ST39	*bla*$_{OXA-181}$	179	*bla*$_{OXA-1}$81:EreA::ISKpn19:recombinase:pRiA4b ORF-3	*K. pneumoniae* strain 50595 plasmid p50595_OXA_181 (CP050375.1) (100%)
		bla$_{CTX-M-3}$	137	transposase:tetR(A):tet(A):IS91:LysR:FloR(chloramphenicol/florfenicol efflux)::Tn3:recombinase:*bla*$_{TEM-1B}$:WbuC:*bla*$_{CTX-M-3}$:	*K. pneumoniae* strain HKU49 plasmid pHKU49_CIP (MN543570.1) (100%)

		bla$_{SHV-40}$	19	*bla*SHV-11	*K. pneumoniae* strain LH94 chromosome (CP035202.1) (99%)
H2850	ST15	*bla*$_{OXA-1}$	94	CatB:*bla*$_{OXA-1}$:AAC(6')-Ib-cr5:	*K. pneumoniae* strain B17KP0069 plasmid pB17KP0069-1 (CP052491.1) (100%)
		bla$_{TEM-1B}$	75	Recombinase:*bla*$_{TEM-1B}$:IS91:APH(6)-Id:APH(3")-Ib:Sul2	*K. pneumoniae* strain E17KP0053 plasmid pE17KP0053-2 (CP052219.1) (100%)
		bla$_{CTX-M-15}$	44	ISEc9:*bla*$_{CTX-M-15}$:WbuC	*K. pneumoniae* strain C16KP0065 chromosome (CP052451.1) (99%)
		bla$_{OXA-181}$	42	*bla*$_{OXA-1}$81:EreA::ISKpn19:recombinase:pRiA4b ORF-3	*K. pneumoniae* strain KP4368 plasmid pKP4368 (KX783441.1) (99%)
		bla$_{SHV-28}$	3	*bla*$_{SHV-28}$	*K. pneumoniae* strain B17KP0069 chromosome (CP052490.1) (100%)
H2745	ST607	*bla*$_{NDM-1}$	73	IS91:IS30:*bla*$_{NDM-1}$:Ble-MBL:	*K. pneumoniae* strain NK29 plasmid Pk29 (EF382672.1) (100%)
		bla$_{OXA-10}$	72	qacEΔ1:AadA1:*bla*$_{OXA-10}$:Arr-2:DfrA14:IntI1	*K. pneumoniae* plasmid pHM881QN DNA (LC055503.1) (99%)
		bla$_{DHA-1}$	65	HypA(nickel metallochaperone):AmpR:*bla*$_{DHA-1}$	*K. pneumoniae* strain E16KP0102 plasmid pE16KP0102-1 (CP052310.1) (99%)
		bla$_{TEM-1B}$, *bla*$_{CTX-M-15}$	58	*bla*$_{TEM-1B}$:recombinase::ISEc9:*bla*$_{CTX-M-15}$:WbuC:Tn3	*K. pneumoniae* strain NMBU-W07E18 plasmid pNMBU-W07E18_01 (CP042883.1) (99%)
		bla$_{SHV-65}$	1	*bla*$_{SHV-1}$	
H2741	ST90	*bla*$_{OXA-1}$	107	CatB:*bla*$_{OXA-1}$	*E. hormaechei* subsp. hoffmannii strain Eh1 plasmid p1 (CP034755.1) (100%)
		bla$_{CTX-M-15}$,*bla*$_{TEM-1B}$	73	Tn3:WbuC:*bla*$_{CTX-M-15}$:ISEc9::recombinase:*bla*$_{TEM-1B}$:IS91:APH(6)-Id:APH(3")-Ib:Sul2	*E. hormaechei* subsp. xiangfangensis strain GENC200 plasmid pGENC200 (CP061495.1) (100%)
H2737	ST17	*bla*$_{OXA-181}$	61	pRiA4b ORF-3:recombinase:ISKpn19::EreA:*bla*$_{OXA-1}$81	*K. pneumoniae* strain 50595 plasmid p50595_OXA_181 (CP050375.1) (100%)
		bla$_{SHV-94}$	9	*bla*$_{SHV-11}$	*K. pneumoniae* strain LH94 chromosome (CP035202.1) (99%)
		bla$_{OXA-10}$	68	CmlA5:*bla*$_{OXA-10}$:AadA1	*K. pneumoniae* strain AR_0139 plasmid tig00000006 (CP021961.1) (100%)
		bla$_{CTX-M-15}$, *bla*$_{TEM-1B}$	33	IS91:*bla*$_{TEM-1B}$:recombinase::ISEc9:*bla*$_{CTX-M-15}$:WbuC:Tn3	*K. pneumoniae* isolate 83554f92-b38d-11e9-8998-68b599768938, plasmid: p14ARS_MMH0055-5 (LR697126.1) (99%)
		bla$_{OXA-1}$	74	CatB:*bla*$_{OXA-1}$:AAC(6')-Ib-cr5	*K. pneumoniae* strain MS14393 plasmid pMS14393B (CP054305.1) (99.48%)

H2730R	UK	*bla*$_{NDM-1}$	22	IS1182:IS3:IS30:IS91::::::Ble-MBL:*bla*$_{NDM-1}$::IS91:DDE-type integrase:Mu transposase:TniB:TniQ:qacEΔ1	*C. freundii* strain ZY198 plasmid pZY-NDM1 (CP055250.1) (99%)
		bla$_{CMY-48}$	5	SugE::*bla*$_{CMY-48}$:AmpR	
		bla$_{CTX-M-15}$, *bla*$_{TEM-1B}$	26	*bla*$_{TEM-1B}$:recombinase::ISEc9:*bla*$_{CTX-M-15}$:WbuC:Tn3	*C. freundii* strain SL151 plasmid unnamed1 (CP017058.1) (99%)
		bla$_{OXA-10}$	35	Arr-2:CmlA5:*bla*$_{OXA-10}$:ANT(3")-Ia	*C. freundii* isolate *C. freundii* str. U2785 plasmid 2 (LS992184.1) (100%)
		bla$_{OXA-1}$	40	CatB:$_{OXA-1}$:AAC(6')-Ib-cr5	*C. freundii* MH17-012N plasmid pMH17-012N_3 (AP018569.2) (99%)
H2716R4	ST231	*bla*$_{OXA-181}$	134	*bla*$_{OXA-181}$	*E. hormaechei* subsp. steigerwaltii strain ST190 plasmid pLAU_ENM17_OXA181 (MN792919.1) (100%)
		bla$_{TEM-1B}$	146	*bla*$_{TEM-1B}$	*E. hormaechei* strain AMS-38 plasmid pAMS-38c (CP051135.1) (100%)
		bla$_{OXA-9}$	143	ANT(3"):*bla*$_{OXA-9}$	*E. hormaechei* strain 189 plasmid pECL189-1 (CP047966.1) (100%)
		bla$_{OXA-1}$	122	CatB:*bla*$_{OXA-1}$:AAC(6')-Ib	*E. hormaechei* subsp. hoffmannii strain Eh1 plasmid p1 (CP034755.1) (100%)
		bla$_{CTX-M-15}$	104	ISEc9:*bla*$_{CTX-M-15}$:WbuC	*E. hormaechei* subsp. xiangfangensis MY458 plasmid (LC511996.1) (100%)
		bla$_{ACT-7}$	5	SugE::*bla*$_{ACT-25}$:AmpR	*E. hormaechei* strain C126 chromosome (CP041054.1) (99%)

3.7 Virulome

The virulence determinants were predicted only for the *Klebsiella* spp (n = 11). A total of 80 virulence genes were found in the eleven genomes (Figure S4). Comparatively, the human isolates had more virulence genes than environmental isolates. There was no association between the carbapenemases encoded and the set of virulence factors; however, the isolates harboring the class D carbapenemases contained more virulence factors (n_{range} = 66-71) than class A/D (n_{range} = 49-59). Of note, the virulence factors were clonally related as all the isolates belonging to the ST17 harboured virulence determinants (Figure S4).

3.7. Methylome

Three different restriction-modification systems (RMS) composed of both methyltransferases (MTases) and restriction enzymes (REs) were found in the isolates. Type II (RMS) was the most abundant (n = 40), followed by Type I (n = 4) and Type III (n=3). The RMS encoded were not clonally related. Similarly, there was no association between the carbapenemases encoded and RMS in the isolates. However, different species contained specific RMS. For instance, the *C. freundii* and *E. hormaechei* contained only Type II RMS, whiles the *Klebsiella* spp. had different combinations of Type I, II and III RMS. The *S. marcescens* did not possess any RMS.

Generally, methyltransferases (MTases, n = 7) were more predominant than restriction enzymes (REs, n = 1) in the isolates' RMS. Of note, most of the MTases were species-specific; *M.Ecl34399Dcm* (*E. hormaechei*), *M.Kpn34618Dcm* and *M.EcoJA03PI* (*Klebsiella* spp.). However, the MTases (*M.EcoRII*) and REs (*Eco128I*) were found in *C. freundii*, *E. hormaechei* and *K. pneumoniae*. All the Type II MTases/REs (*M.EcoRII, M.Ecl34399Dcm, M.Kpn34618Dcm,* and *Eco128I*) and Type I REs (*M.EcoJA03PI*) had identical recognition sequences; CCWGG (Accession numbers: CP010392, NEBM64, AY618907) and GATGNNNNNCTG (Accession number: JMIZ01000008), respectively. Similarly, the Type III MTases (*M.Kpn1420I, M.Kpn1420I,* and *M.Sen16III*) had the recognition sequences; CCGAG (Accession numbers: CP012091).

3.8. Phylogenomics and resistome

The *C. freundii*, *Klebsiella* spp., and *S. marcescens* isolates were phylogenetically closely aligned to other human isolates (Figure S5, Figure 3, Figure S6), while the *E. hormaechei* isolates showed diverse phylogenetic relationships. Isolates obtained from humans (H2741, H8825R, H8767R) belonging to ST 90 clustered together and were in the same clade as other clinical isolates (K006, K130, K063), albeit on separate branches. Isolates mostly clustered according to sequence type with H2716R4 (ST 231) clustering with clinical isolates (Ec009, Ec010) of the same sequence type (Figure S7). The environmental isolates were closely related to other environmental isolates (Figure S7).

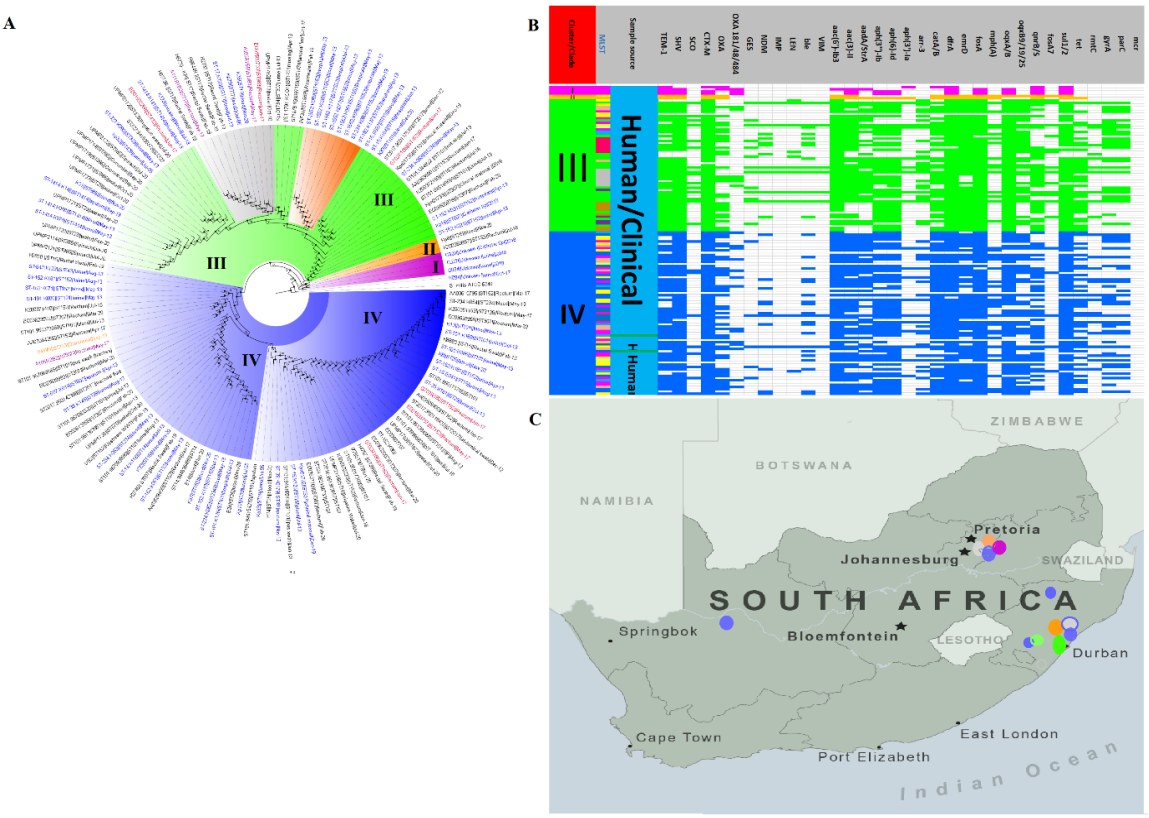

Figure 3. Phylogenomics of *Klebsiella pneumoniae*

Discussion

Antimicrobial resistance is a complex global issue that threatens human, animal, and environmental health, the global economy, and global security. It has been described as the quintessential One Health problem requiring a One Health approach for its containment. The One Health approach, as defined by the American Veterinary Medical Association, is "the collaborative effort of multiple disciplines—working locally, nationally and globally—to attain optimal health of people, animals, and our environment." Evidence suggests an interplay of antibiotic resistance determinants between humans, animals, and the environment [1,27–29].

This point prevalence study sought to triangulate carbapenem resistance, specifically carbapenemase-mediated resistance among Enterobacterales from humans, animals, and the environment taking a One Health approach. The study was conducted within a single district in the same geographical area in South Africa and highlighted multiple resistance and virulence determinants and MGEs (plasmids, transposons, integrons, and ISs) mobilising ARGs. Phylogenetic analyses also highlighted the relatedness of isolates from this study to other South African isolates.

The *bla*$_{\text{OXA-181}}$ gene was the commonest carbapenemase isolated from humans. This contrasts with a

2015 publication from the National Institute for Communicable Diseases (NICD) in South Africa, reporting a high prevalence of *bla*$_{NDM-1}$-producing *K. pneumoniae* in Kwazulu-Natal [30]. In 2016, Perovic et al. also observed a high prevalence of *bla*$_{NDM-1}$ in the Gauteng Province among human isolates [31]. In 2019, however, Lowe et al. described the spread of a carbapenemase-producing clone, *K. pneumoniae* ST307 *bla*$_{OXA-181}$, across 23 cities and towns within six South African provinces [32]. Perovic and colleagues, in a later study, also noted a significant increase in the number of *bla*$_{OXA-48}$ and variants (52%) compared to *bla*$_{NDM-1}$ (34%) carbapenemase encoding genes, with *K. pneumoniae (*78%) being the predominant species among the CRE [33]. Similarly, the predominant isolate in this study was *K. pneumoniae* bearing the *bla*$_{OXA-181}$ gene. The *bla*$_{OXA-181}$ gene was found in both human and environmental *E. hormaechei* isolates associated with similar plasmids and plasmid replicons, intimating the potential movement of MGEs carring this ARG between these two sectors in the same species (Table 2). Additionally, the association of the *bla*$_{OXA-181}$ gene with insertion sequences, transposons, and recombinases on the same contigs further attests to the potential mobilization of these carbapenemase encoding genes across species and sectors (Figure S3). The *bla*$_{NDM-1}$ gene from isolates in both sectors was similarly closely associated with common insertion sequences, and transposons (Figure S3). Other carbapenemase-encoding genes isolated in this study included *bla*$_{NDM-1}$ (4/16), *bla*$_{OXA-484}$ (1/16), *bla*$_{OXA-48}$ (1/16), *bla*GES-5 (1/16). The bla$_{OXA-48}$ *and bla*$_{GES-5}$ carbapenemase-encoding genes were only found in isolates from environmental water samples. The other two isolates from the water samples contained *bla*$_{OXA-181}$ and *bla*$_{NDM-1}$ carbapenemase genes. Isolates recovered from both sectors (humans and environmental) containing the similar *bla*$_{NDM-1}$ and *bla*$_{GES-5}$ carbapenemase encoding genes generally had higher MICs to carbapenems than those with *bla*$_{OXA-181}$ (Table 1), most likely due to the lower hydrolytic activity of the OXA group of carbapenemases [34].

The presence carbapenemase encoding genes in the environment resonates with the recent views of Ebomah and Okoh, who highlighted the need for more research investigating CRE isolates in different environmental niches in South Africa [35].

The carbapenemase encoding genes were located on MGEs as evidenced by their association with plasmid replicons, transposons insertion sequences, and less frequently, integrons which in some instances were similar in human and environmental isolates (Table 3). Studies have highlighted the fluidity of resistomes between humans, animals, and the environment. In the case of the NDM-1 carbapenemase, Walsh and colleagues described its worldwide dissemination outside of its country of origin, India [36]. Apart from being isolated from surface and tap water samples in India and other countries, NDM-1 has been found in the arctic soil of a Norwegian archipelago, raising the possibility of spread by feces from migratory birds [36-38]. In this study, *bla*$_{NDM-1}$ was found in both human and environmental sectors in different bacterial species associated with similar MGEs highlighting the potential mobilization carbapenemase encoding genes between sectors.

Similarly, the OXA-48 and OXA-48-like enzymes have appeared in many countries worldwide following their original discovery. Genomic surveillance programs have shown that the OXA-48-like enzymes are the 2nd most common carbapenemases among Enterobacterales globally. OXA-48, OXA-181, OXA-232, OXA-204, OXA-162, and OXA-244 are the most common enzymes in the OXA-48-like carbapenemase group and are endemic to regions such as the Middle East and North Africa, with hospital outbreaks reported in Europe, Australia, Mexico, China, and South Africa. OXA-181 carbapenemases are endemic to countries of the sub-continent such as India, Bangladesh, Pakistan, and Sri Lanka. Their dissemination is associated with the *bla* genes located on plasmids making them highly transmissible [38,39]. A Belgian study identified $bla_{\text{OXA-48}}$ genes in the Zenne River due to continuous discharge from WWTPs [40]. Different ST types among *E. coli* and *K. pneumoniae* harboring $bla_{\text{OXA-48}}$ were isolated from rivers in Algeria [41]. A systematic review of 68 publications on the PubMed database between 1980-2017 of CRE isolated from samples obtained from wildlife, companion animals, and food-producing animals was published in 2018 by Kock et al. It was found that VIM, KPC, NDM, OXA, and IMP were the predominant carbapenemases isolated, mainly from genera of *Escherichia* and *Klebsiella* spp. Two studies reported that 33-67% of exposed humans from poultry farms carried CP-CRE resembling isolates from the farm environment. Following selective screening for CRE, 27 studies demonstrated a CRE prevalence of <1% among livestock and companion animals in Europe, 2-26% in Africa, and 1-15% in Asia. Gulls from Europe and Australia, reflective of wildlife, carried CRE in 16-19% [42]. Lowe et al. described the spread of a carbapenemase-producing (OXA-181) clone, *K. pneumoniae* ST307, that contained the IncX3 plasmid bearing the $bla_{\text{OXA-181}}$ gene across cities and towns within provinces in South Africa over time [32]. The plasmid p72_X3_OXA181 was identical to other IncX3 plasmids carrying the $bla_{\text{OXA-181}}$ gene reported from China and Angola [32]. Similar to the findings of Lowe et al., five isolates bearing the $bla_{\text{OXA-181}}$ gene carried by the IncX3 plasmid were identified in this study. Significantly, this occurred in isolates of *E. hormaechei* from human and animal sectors. Finding the $bla_{\text{OXA-181}}$ gene in different bacterial species across the human and environmental sectors suggests potential mobilization of carbapenemase-encoding genes across the sectors and between species. Since the $bla_{\text{OXA-181}}$ gene in association with IncX3 has been found in other parts of South Africa, it is plausible that our finding is part of the continuous dissemination of the $bla_{\text{OXA-181}}$ gene in South Africa. If so, the movement of these carbapenem resistance genes is occurring between bacterial species and across the human and environmental sectors. IncL/M, IncF, and ColKP3 plasmids play a significant role in the dissemination of $bla_{\text{OXA-48}}$ genes and variants among *K. pneumoniae* globally [39]. The greater proportion of $bla_{\text{OXA-181}}$ genes in this study was found in association with IncL/M, IncF, and ColKP3 plasmids suggesting continuous mobilization and fluidity of these carbapenemase encoding genes. Therefore, it is unsurprising that the $bla_{\text{OXA-181}}$ carbapenemase gene in Enterobracterales has increased in recent years and has become widely disseminated across all South African provinces [33]. This is of great concern warranting continuous surveillance in the human,

environmental, and animal sector. Despite not isolating CPE among animals which were only recently culled pigs during this study, the close association of farmed animals with humans and environment/environmental sources makes a compelling case for AMR surveillance using a One Health approach.

The *bla*$_{\text{NDM-1}}$ genes found on *K. pneumoniae, K. quasipneumoniae,* and *C. freundii* were mostly associated with IncF (FII and FIB) and Inc A/C plasmid replicons, similar to other South African reports [25]. The insertion sequences, transposons, and prophages found in the human and environmental carbapenemase-producing provide an environment conducive to horizontal genetic exchange, enabling the acquisition and spread of resistance genes. Importantly, our findings suggest that the mobilization of resistance genes, owing to various mobile genetic elements, could potentially occur between bacterial species and across the human and environmental sectors. As previously highlighted, the animal sector is a component that closely interacts with humans and the environment, so ARB and ARGs' potential spread is likely. The large pool of MGEs in the genetic environment of the human and environmental isolates increases the propensity for bacterial HGT of ARGs within and between different isolates, species, and environments.

Phylogenetic analysis revealed that the *C. freundii* isolate was closely aligned to other human isolates recovered in South Africa with closely related ARGs. Additionally, the single *Serratia marcescens* isolate was on the same clonal node as other *bla*$_{\text{NDM-1}}$ positive clinical isolates from South Africa. These findings suggest a clonal spread of these isolates, which could have significant implications in transmitting infections caused by carbapenemase-producing strains of *C. freundii* and *S. marcescens* within healthcare settings resulting in widespread outbreaks. This finding highlights the need for continuous surveillance within healthcare settings, monitoring the spread of infections caused by MDR organismsas patients who were colonized with CPE were from different healthcare institutions and not necessarily from patients admitted to an intensive care unit (ICU) which is the focus of CRE studies. The *E. hormaechei* isolates showed diverse phylogenetic relationships with clustering of similar sequence types having many ARGs in common.

The phylogenetic analysis of the *K. pneumoniae* isolates highlighted a range of relationships, according to MLST schemes. Some isolates with different STs clustered together (Figure 3). The environmental isolate (US2) which was upstream from the WWTP, clustered with other environmental isolates, with US2 being closely related to a water isolate (UPMP1728). This finding could indicate that these isolates are endemic to South African waters and could potentially have implications to humans and animals using these water sources, especially in resource-limited and rural settings.

This study demonstrates the potential for the dissemination of ARB and ARGs between humans and the

environment facilitated by MGEs. Not finding CPE amongst the pigs sampled may suggest that pigs might not pose a significant risk at this point in time, however, consideration must be given to the animal sector as there are close interactions between humans and animals with the environment which could facilitate the dissemination of MDR organisms and associated mobile genetic elements to the animal sector. So herein lies the possible situation of circulation, transfer and sharing of ARB and antibiotic resistance determinants between the three sectors. Surveillance of AMR should therefore be performed across all three sectors in a One Health context to enhance our understanding of the dissemination of AMR.

Conclusion

This One Health study identified common CPE with carbapenemase-encoding genes and resistomes in humans and the environment. The concurrent One Health sampling study design presented here provides an overarching screening tool on which to base further efforts to elucidate transmission routes, characterize ARG bacterial reservoirs, and inform policy for promoting good stewardship and combating the spread of resistance among and between animals, humans, and the environment. While the results presented in this study are crucial to understanding the spread of AMR within the One Health context, they should not be generalised due to the limited number of isolates obtained. Nevertheless, the established association between carbapenemase-encoding genes and MGEs lends itself to the possibility of resistome and mobilome propagation across the sectors. Further research with a larger sample size investigating the dissemination of CPE in a One Health context and in different geographical locations is therefore needed.

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Figure S1. Levels of healthcare in South Africa.

Table S1. Healthcare institutions with the designated level of care, number of beds, and number of patients screened

Hospital	Level	Number of beds	Number of patients screened
H1	District Hospital (N)	384	57
H2	Regional Hospital (E)	872	120
H3	Provincial Tertiary Hospital (G)	530	53

Table S2. Demographic and genomic characteristics of the *Carbapenem-resistant Enterobacteriaceae* (CRE) isolates

Sample code	Accession no.	Age	Sex*	Hospital	Species	Size (Mb)	GC%	Contigs	No. of RNAs	No. of coding sequences	N_{50}	L_{50}	Coverage (X)	No. of CRISPR arrays
Human														
H2730R	VWTQ00000000	N/A	M	H3	*C. freundii*	5.3	51.8	58	77	5258	518,368	4	97	1(0)
H8767R	VWTK00000000	50	M	H3	*E. hormaechei*	4.8	55.4	90	81	4722	116,725	12	98	1(0)
H8871	VWTE00000000	91	F	H2	*E. hormaechei*	5.3	54.4	156	77	5263	142,312	12	87	0(0)
H8825R	VWTH00000000	37	M	H2	*E. hormaechei*	5.1	54.9	71	81	5028	260,202	6	92	2(0)
H2741	VWTO00000000	68	M	H2	*E. hormaechei*	5.1	54.9	124	74	5089	101,015	15	92	3(0)
H2716R4	VWTR00000000	34	M	H2	*E. hormaechei*	5.0	54.9	172	75	5033	97,655	18	98	2(0)
H8860	VWTF00000000	38	M	H2	*K. pneumoniae*	5.7	57.0	129	76	5645	98,968	19	92	3(1)
H8844R	VWTG00000000	58	F	H2	*K. pneumoniae*	5.7	57.0	133	75	5683	128,778	13	87	3(0)
H8773R	VWTI00000000	43	F	H3	*K. pneumoniae*	5.6	57.2	96	77	5540	338,720	6	90	2(0)
H8770	VWTJ00000000	63	F	H3	*K. pneumoniae*	5.8	56.7	195	69	5884	90,833	20	85	2(1)
H4767	VWTL00000000	34	F	H1	*K. pneumoniae*	5.6	57.2	293	62	5781	41,176	42	95	4(1)
H2850	VWTM00000000	33	M	H1	*K. pneumoniae*	5.6	57.0	116	76	5585	125,258	13	93	2(1)
H2745	VWTN00000000	62	F	H2	*K. pneumoniae*	5.7	57.0	140	81	5797	148,872	14	89	3(1)
H2737	VWTP00000000	N/A	F	H2	*K. pneumoniae*	5.7	57.0	105	76	5680	183,937	10	87	2(0)
H8980R	VWTD00000000	45	F	H2	*S. marcescens*	5.4	59.5	74	75	5342	172,767	9	94	0(0)
Environment														
INF3-a	VWTB00000000	N/A	N/A	Influent	*E. hormaechei*	5.2	54.8	184	65	5173	66,382	24	98	1(0)
US2	VWSZ00000000	N/A	N/A	Upstream	*K. pneumoniae*	5.6	57.2	333	49	5756	34,590	51	100	4(0)

INF3b	VWTA00000000	N/A	N/A	Influent	*K. quasipneumoniae*	5.9	57.0	298	74	6050	44,452	35	84	1(0)
INF1-a	VWTC00000000	N/A	N/A	Influent	*K. quasipneumoniae*	5.8	56.8	702	31	6354	19,632	51	90	3(0)

*M = Male, F = Female, N/A = Not applicable

Table S3. Point mutations on the fluroquinolone and tigecycline chromosomal resistance genes of the isolates

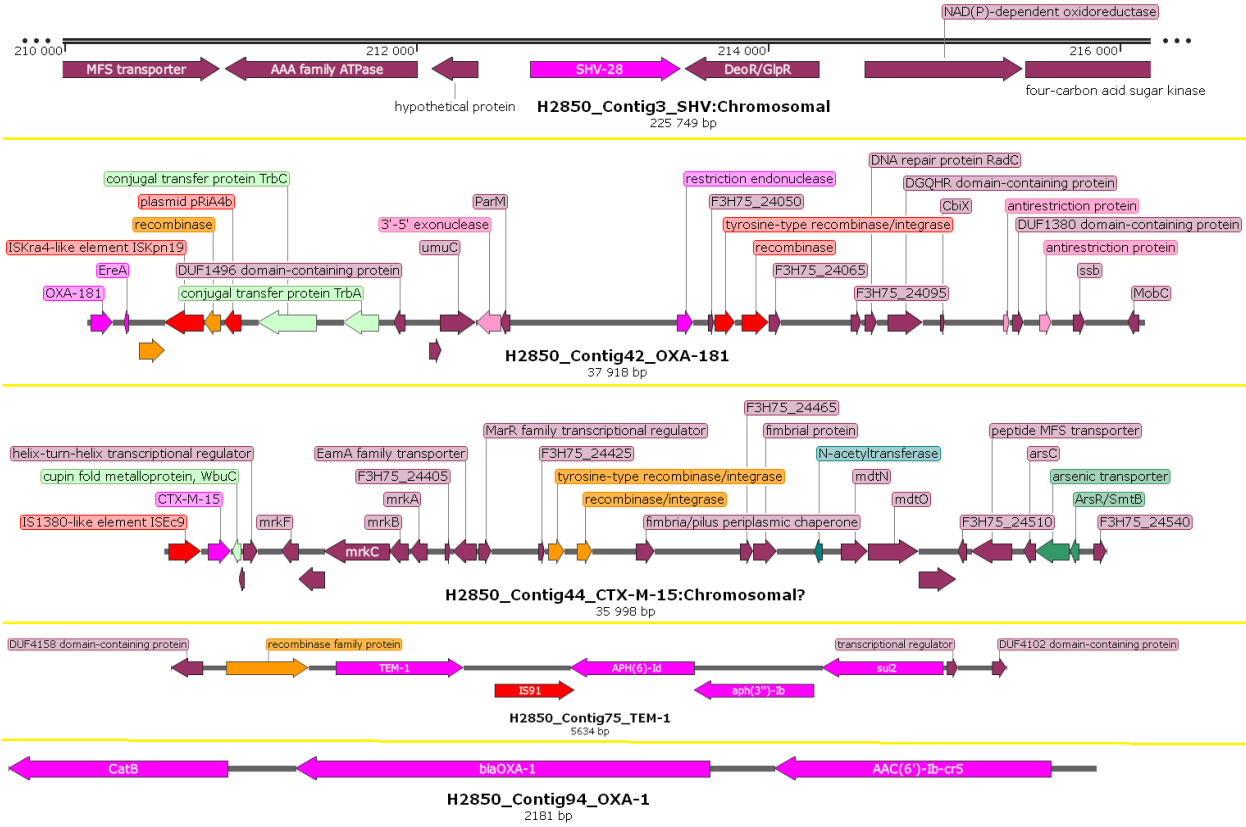
Isolate ID (MLST)	**Species**	**MIC (µg/ml)**	**Gene Mutation(s) in:**				**MIC (µg/ml)**	**Gene Mutation(s) in:**		
		Ciprofloxacin	***GyrA***	***GyrB***	***ParC***	***ParE***	**Tigecycline**	***acrR***	***ramR***	***marR***
Human										
H2730R (ST498)	*C. freundii*	≥4 (R)	S83I	-	-		1 (S)	-	-	-
H8767R (ST90)	*E. hormaechei*	2 (R)	-	-	-	-	≥8 (R)	-	-	-
H8871 (ST78)	*E. hormaechei*	≥4 (R)	S83I		-		2 (S)	-	-	-
H8825R (ST90)	*E. hormaechei*	≥4 (R)	-		-		≥8 (R)	-	-	-
H2741 (ST90)	*E. hormaechei*	≥4 (R)	-		-		4 (R)	-	-	-
H2716R4 (ST231)	*E. hormaechei*	≥4 (R)	S83F		-		2 (S)	-	-	-
H8860 (ST15)	*K. pneumoniae*	≥4 (R)	S83F		S80I		≥8 (R)	-	A19V	-
H8844R (ST17)	*K. pneumoniae*	2 (R)	-		-		2 (S)	-	-	-
H8773R (ST17)	*K. pneumoniae*	2 (R)	-		-		≤0.5 (S)	-	-	-
H8770 (ST17)	*K. pneumoniae*	2 (R)	-		-		1 (S)	-	-	-
H4767 (ST39)	*K. pneumoniae*	≤0.5 (S)	-	-	-	-	≤0.5 (S)	-	-	-
H2850 (ST15)	*K. pneumoniae*	≤ 0.25 (S)	-	-	-	-	2 (S)	-	-	-
H2745 (ST607)	*K. pneumoniae*	≤0.5 (S)	-	-	-	-	1 (S)	-	-	-
H2737 (ST17)	*K. pneumoniae*	2 (R)	-		-	-	2 (S)	-	-	-
H8980R	*S. marcescens*	≥4 (R)	-		-	-	2 (S)	-	-	-

(NA)										
Environment										
INF3-a (ST88)	*E. hormaechei*	≥4 (R)	-	-	-	-	4 (R)	-	-	-
US2 (ST152)	*K. pneumoniae*	≥4 (R)	S83F	-	S80I		≤0.5 (S)	-	-	-
INF3b (ST1889)	*K. quasipneumoniae*	≥4 (R)	-	-	-	-	≥8 (R)	Y114F, V165I	-	-
INF1-a (ST3318)	*K. quasipneumoniae*	≥4 (R)	-	-	-	-	≥8 (R)	Y114F, V165I	-	S3N

† Reported Genes for fluoroquinolone (*GyrB* and *ParE*), and tigecycline (*acrA, acrB, TolC, SoxS, SoxR, ramA, marA, marB and rarA)* resistance which there were no known mutations were omitted from the Table.

Figure S2. Types and distribution of K and O capsule types among the *K. pneumoniae* strains.

H2850


NAD(P)-dependent oxidoreductase
210 000
212 000
214 000
216 000
MFS transporter
AAA family ATPase
SHV-28
DeoR/GlpR
four-carbon acid sugar kinase
hypothetical protein
H2850_Contig3_SHV:Chromosomal
225 749 bp
conjugal transfer protein TrbC
plasmid pRiA4b
recombinase
ISKra4-like element ISKpn19
EreA
OXA-181
DUF1496 domain-containing protein
conjugal transfer protein TrbA
ParM
3'-5' exonuclease
umuC
restriction endonuclease
F3H75_24050
tyrosine-type recombinase/integrase
recombinase
F3H75_24065
DNA repair protein RadC
DGQHR domain-containing protein
CbiX
F3H75_24095
antirestriction protein
DUF1380 domain-containing protein
antirestriction protein
ssb
MobC
H2850_Contig42_OXA-181
37 918 bp
F3H75_24465
helix-turn-helix transcriptional regulator
cupin fold metalloprotein, WbuC
CTX-M-15
IS1380-like element ISEc9
mrkF
mrkB
mrkC
EamA family transporter
F3H75_24405
mrkA
MarR family transcriptional regulator
F3H75_24425
tyrosine-type recombinase/integrase
recombinase/integrase
fimbria/pilus periplasmic chaperone
fimbrial protein
N-acetyltransferase
mdtN
mdtO
peptide MFS transporter
arsC
arsenic transporter
ArsR/SmtB
F3H75_24510
F3H75_24540
H2850_Contig44_CTX-M-15:Chromosomal?
35 998 bp
DUF4158 domain-containing protein
recombinase family protein
TEM-1
IS91
APH(6)-Id
aph(3")-Ib
transcriptional regulator
sul2
DUF4102 domain-containing protein
H2850_Contig75_TEM-1
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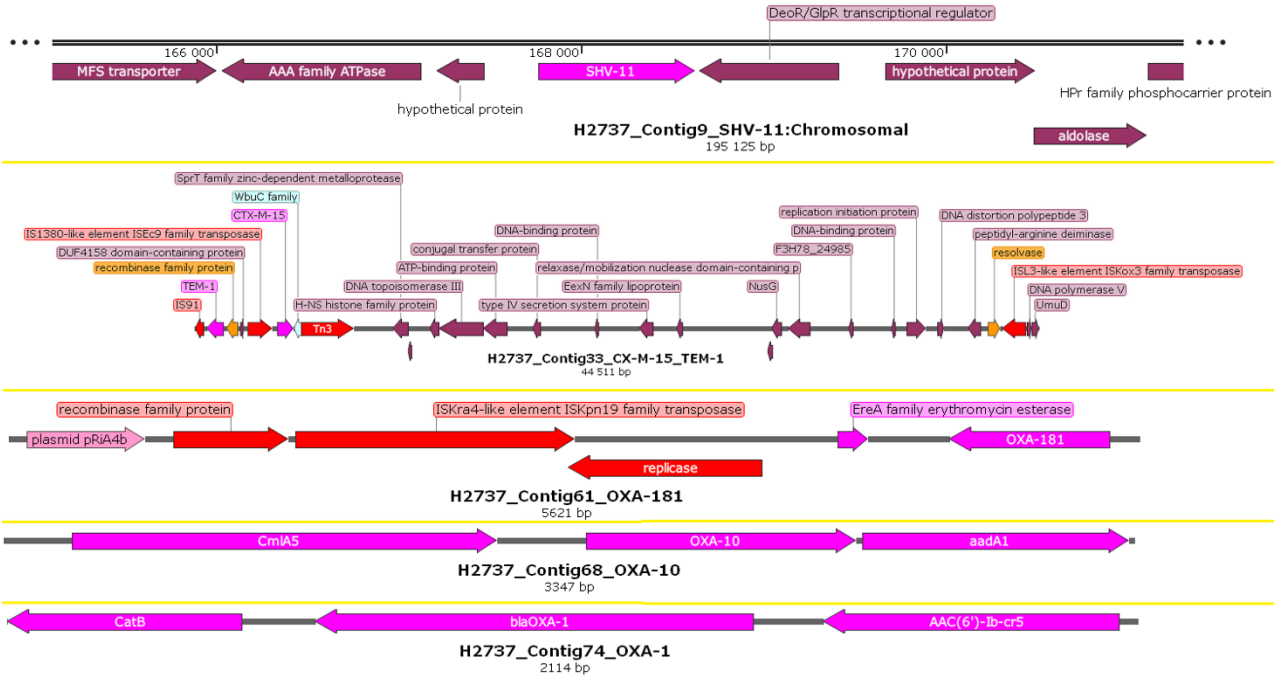
CatB
blaOXA-1
AAC(6')-Ib-cr5
H2850_Contig94_OXA-1
2181 bp

H2737


DeoR/GlpR transcriptional regulator
166 000
168 000
170 000
MFS transporter
AAA family ATPase
SHV-11
hypothetical protein
HPr family phosphocarrier protein
hypothetical protein
H2737_Contig9_SHV-11:Chromosomal
195 125 bp
aldolase
SprT family zinc-dependent metalloprotease
WbuC family
CTX-M-15
IS1380-like element ISEc9 family transposase
DUF4158 domain-containing protein
recombinase family protein
TEM-1
IS91
Tn3
H-NS histone family protein
DNA topoisomerase III
DNA-binding protein
conjugal transfer protein
ATP-binding protein
relaxase/mobilization nuclease domain-containing p
EexN family lipoprotein
type IV secretion system protein
NusG
replication initiation protein
DNA-binding protein
F3H78_24985
DNA distortion polypeptide 3
peptidyl-arginine deiminase
resolvase
ISL3-like element ISKox3 family transposase
DNA polymerase V
UmuD
H2737_Contig33_CX-M-15_TEM-1
44 511 bp
recombinase family protein
plasmid pRiA4b
ISKra4-like element ISKpn19 family transposase
replicase
EreA family erythromycin esterase
OXA-181
H2737_Contig61_OXA-181
5621 bp
CmlA5
OXA-10
aadA1
H2737_Contig68_OXA-10
3347 bp
CatB
blaOXA-1
AAC(6')-Ib-cr5
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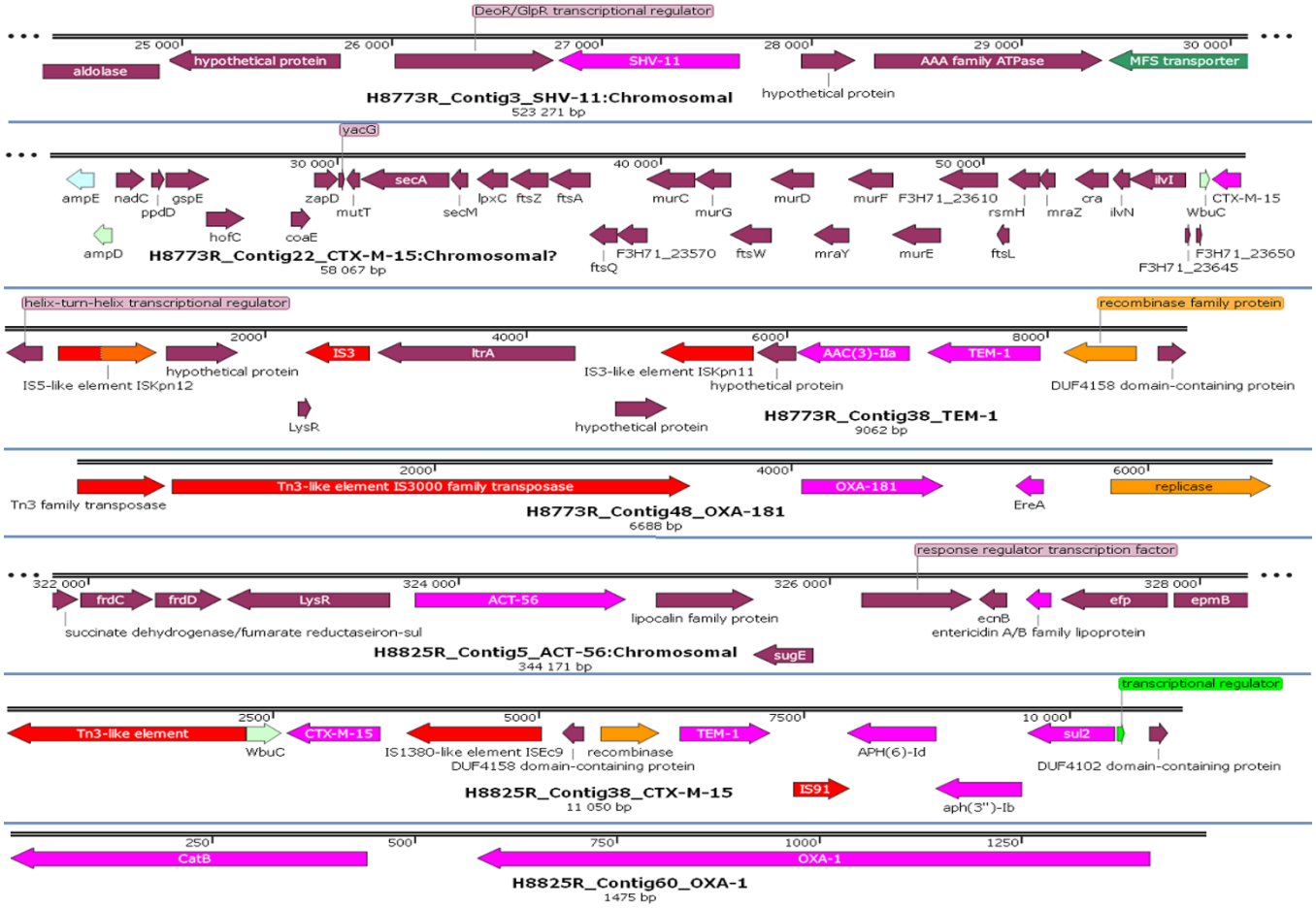
2114 bp

H8773R and H8825R



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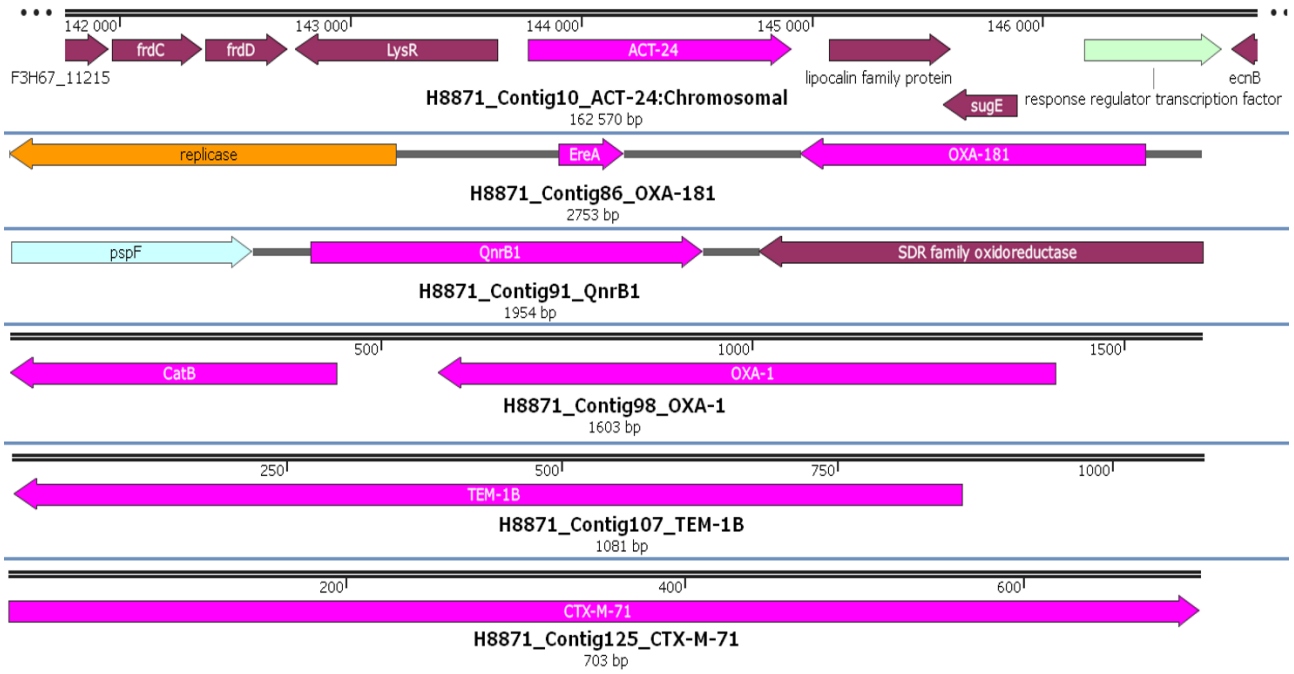

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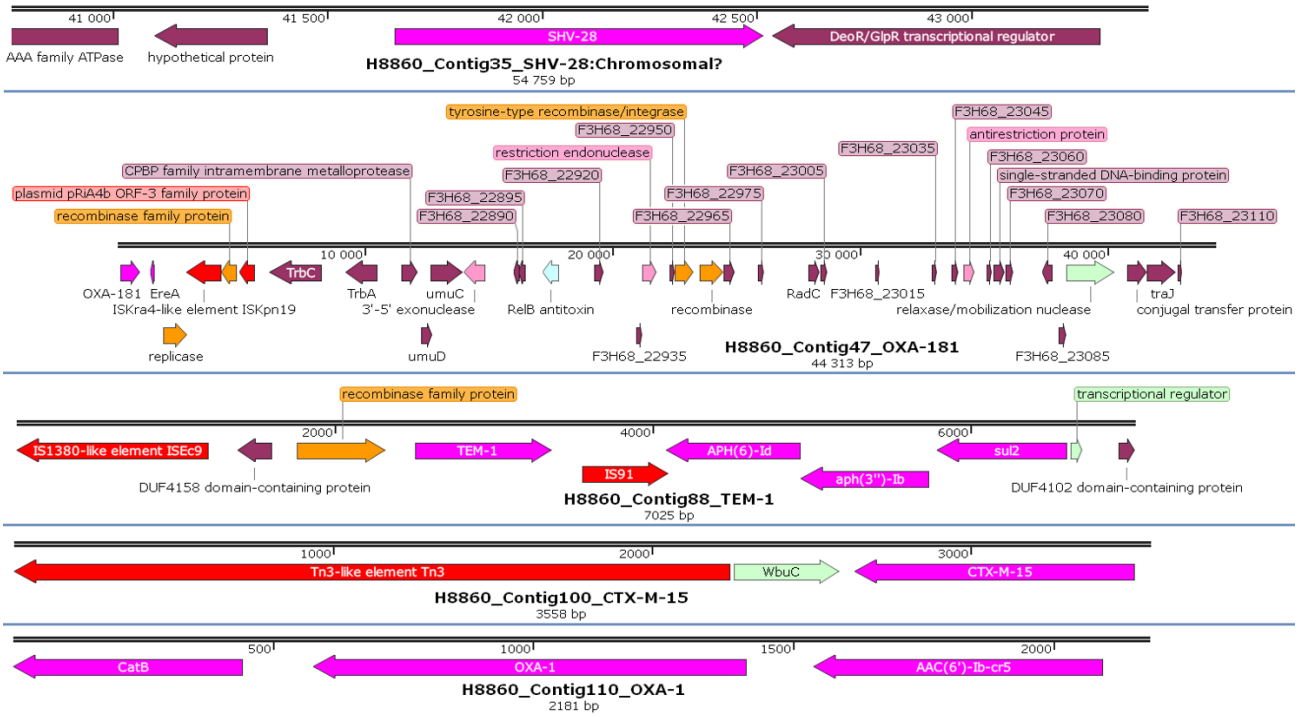
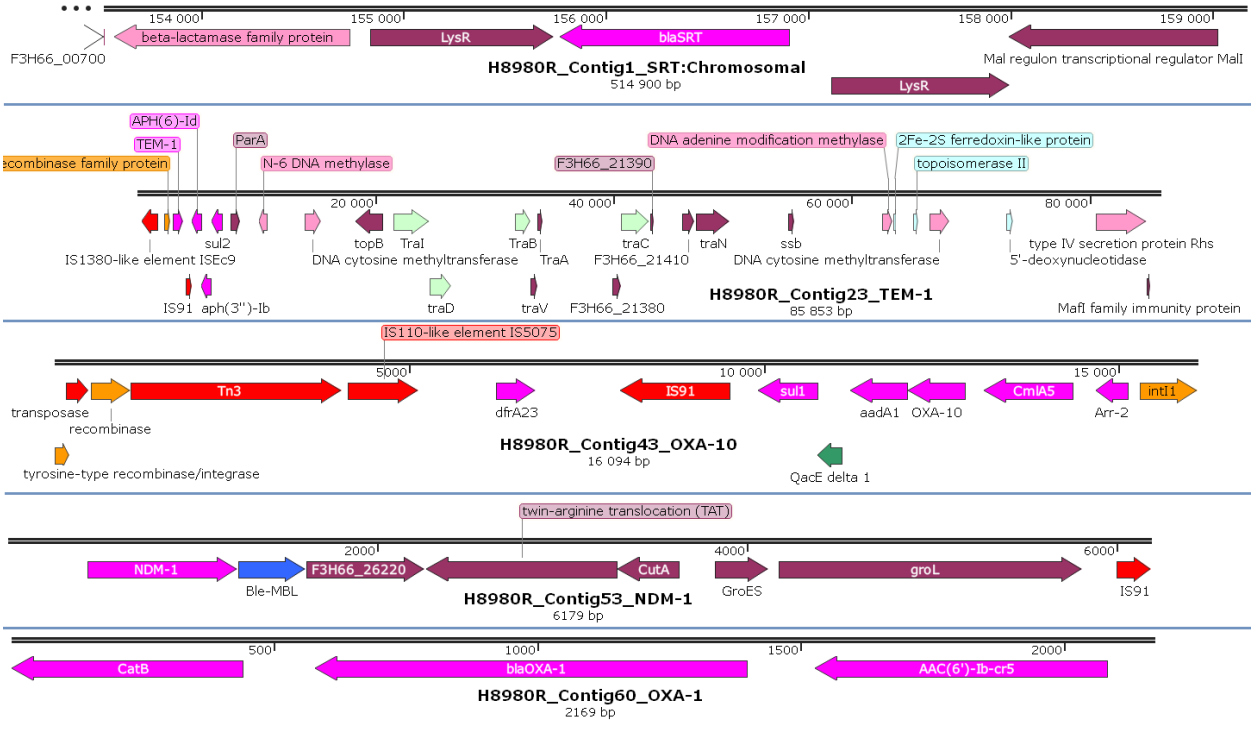


H8980R



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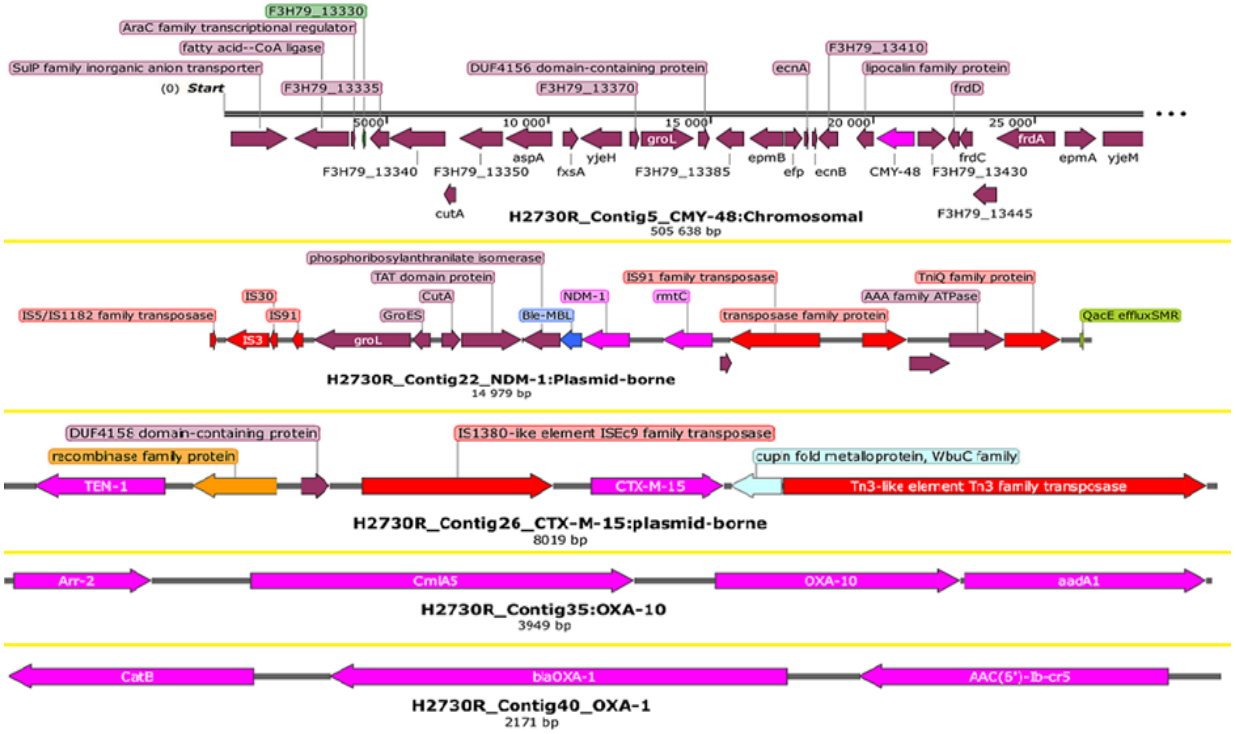
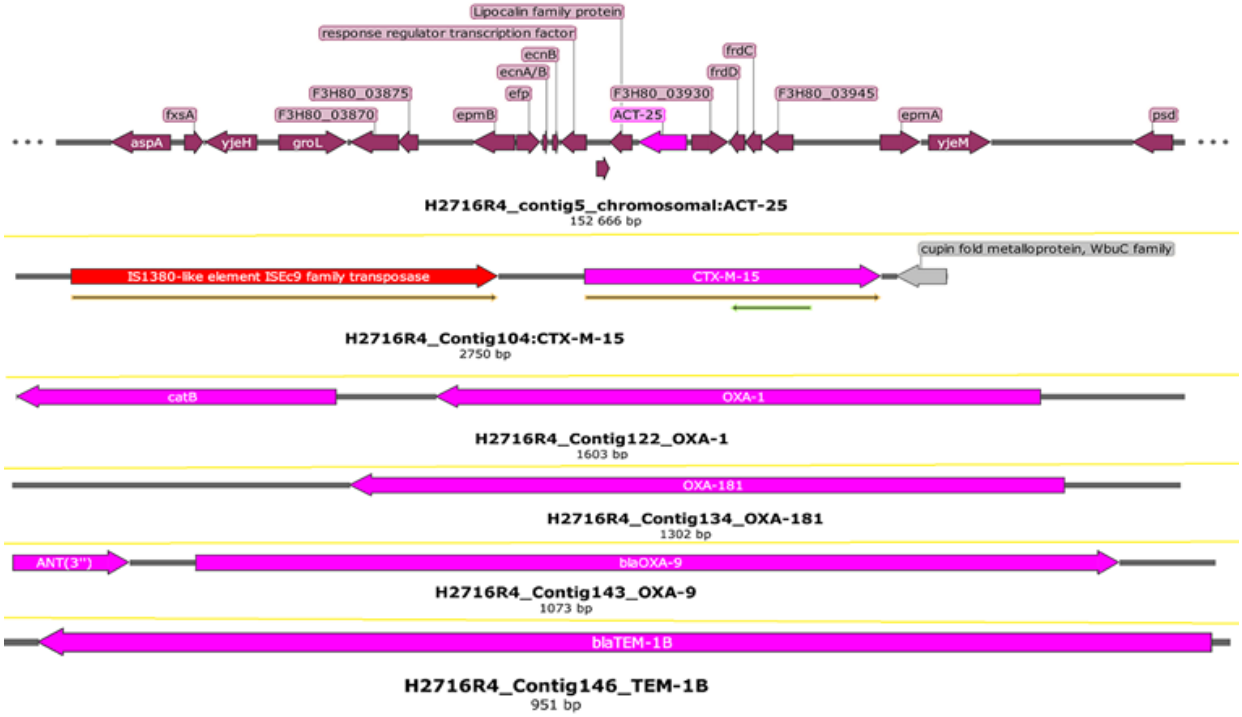
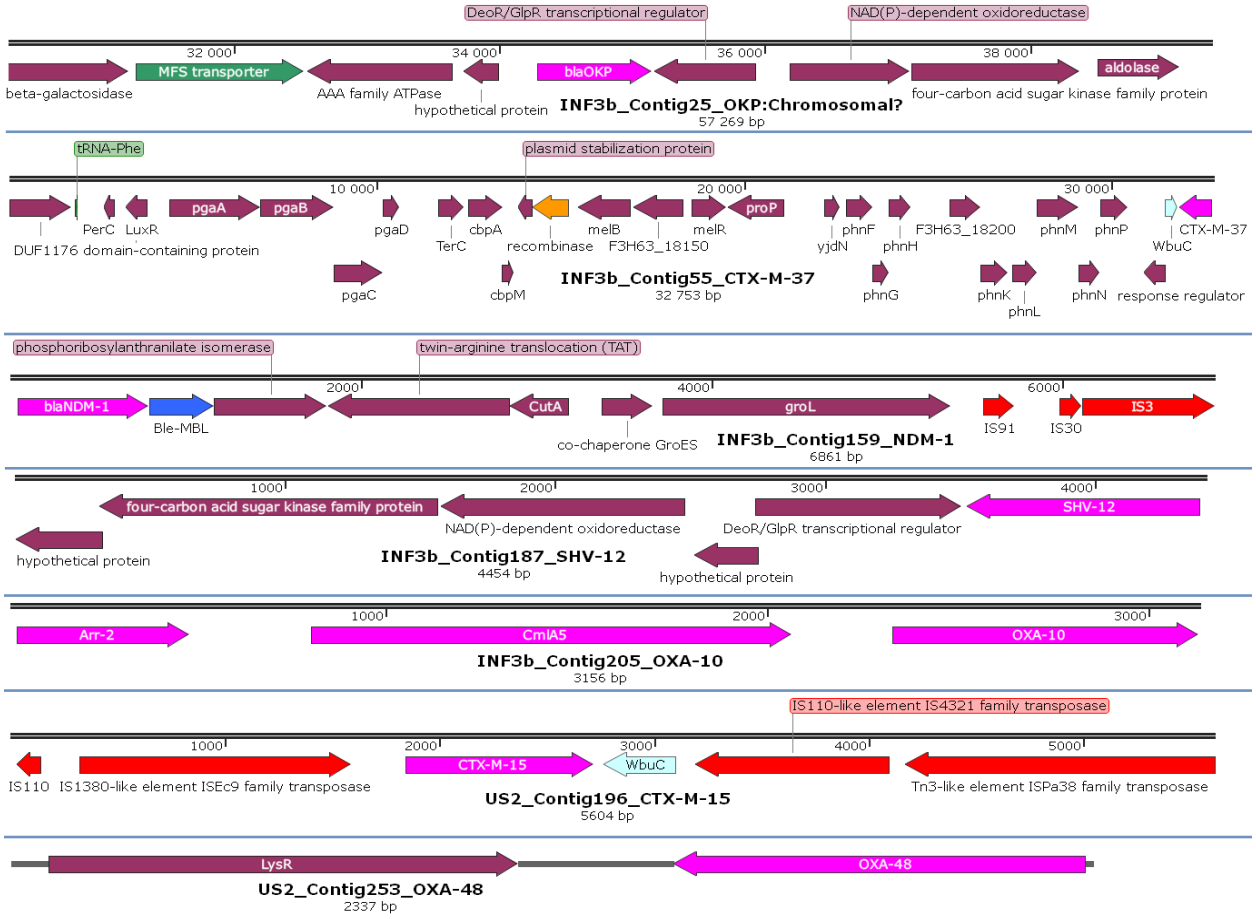


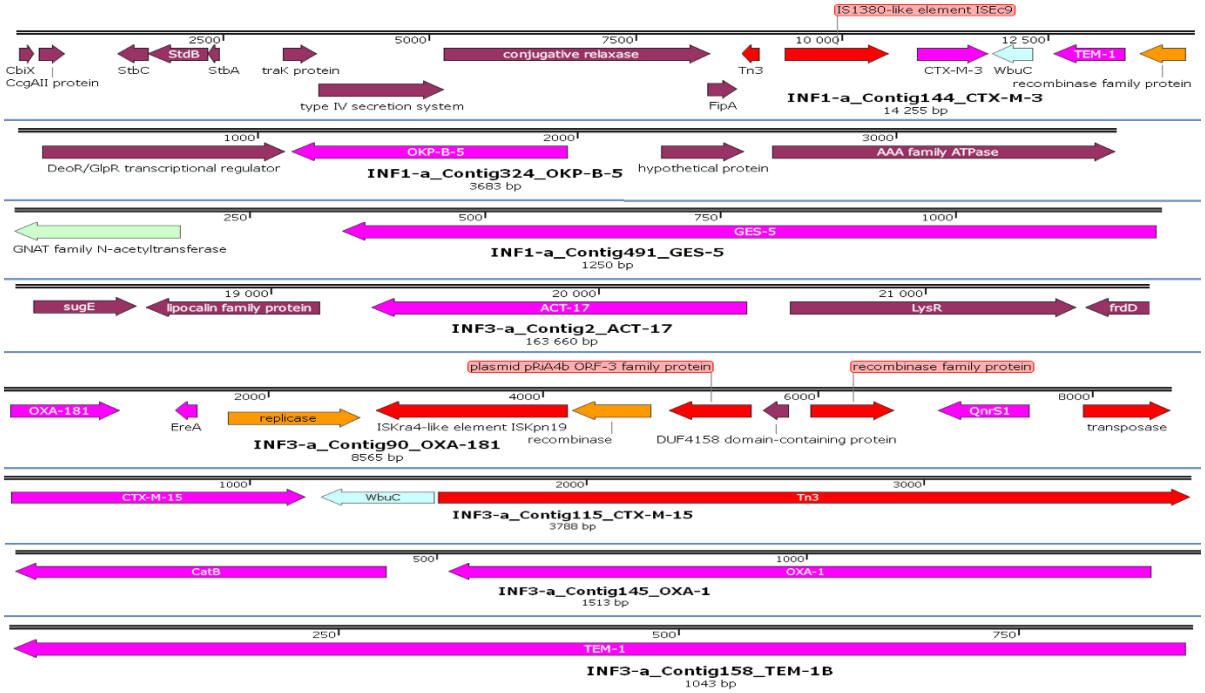
H2716R4



INF3b, US2

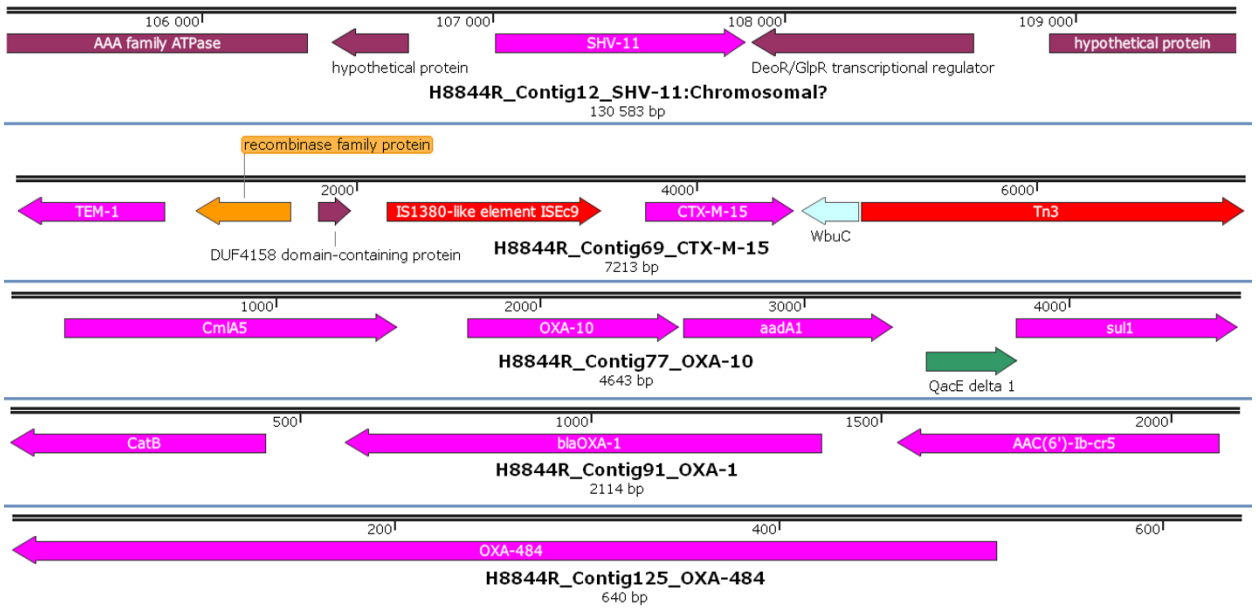


INF1-a and INF3-a



H4767, H8767, H8779

H8844R



H2741 and H2745

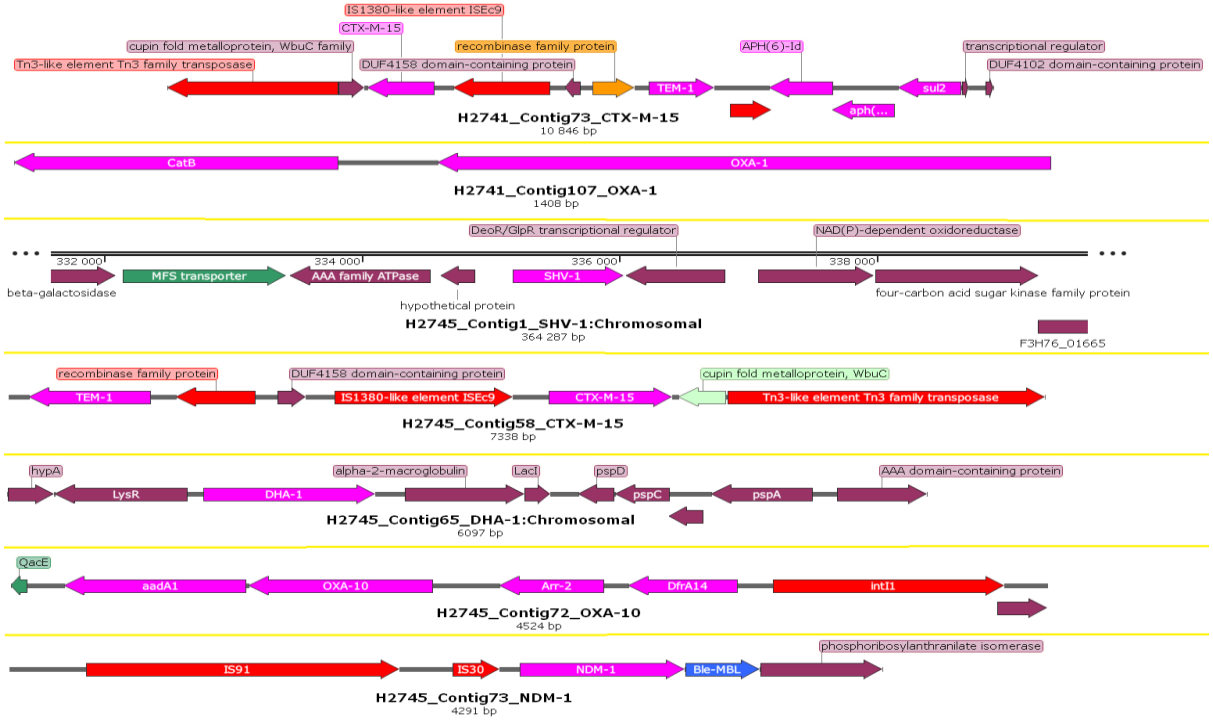


Figure S3. Mapping of AMR genes and MGEs in isolates from this study

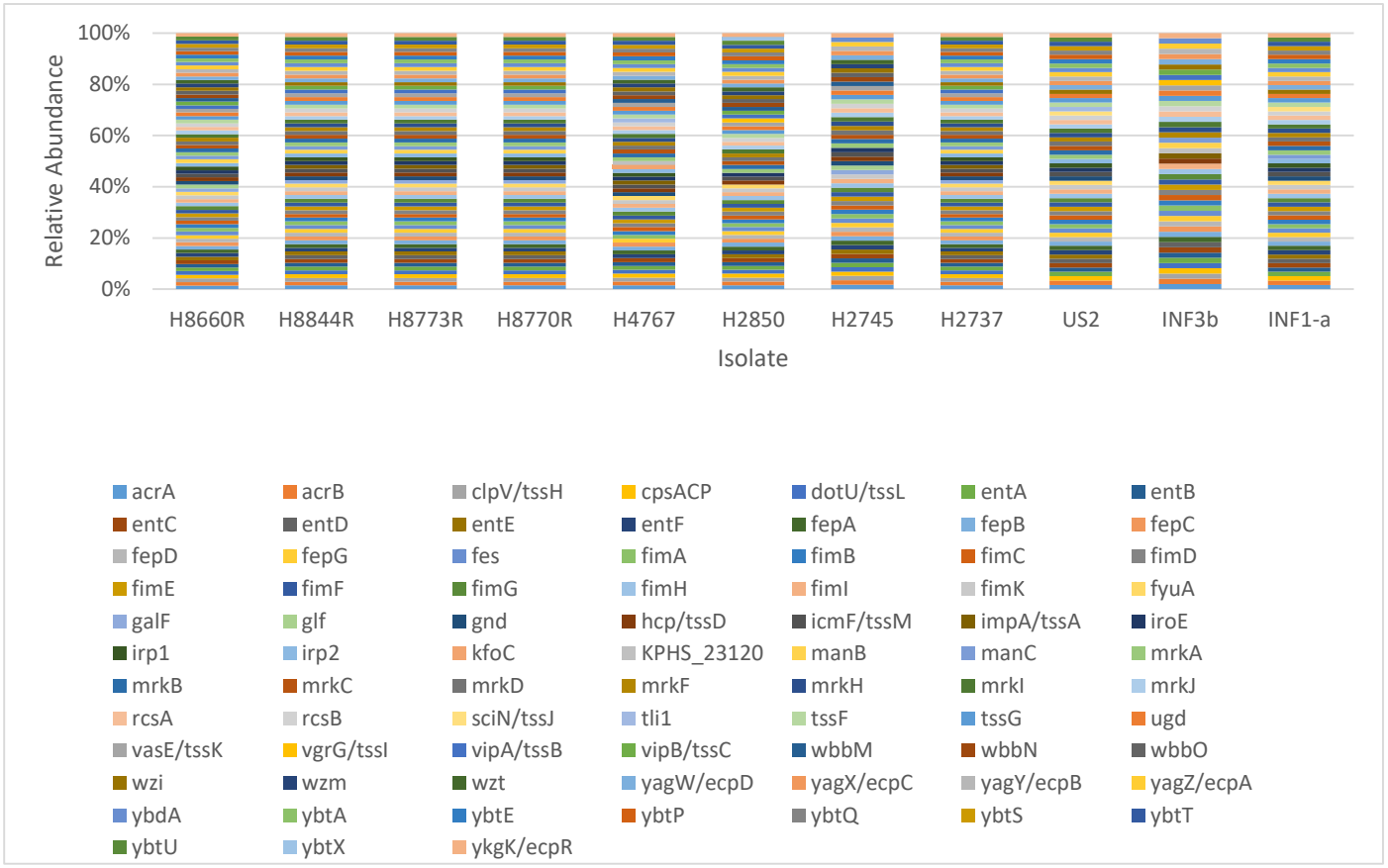


Figure S4. Virulence factors found in the genome of the Carbapenem-resistant *Klebsiella* spp.

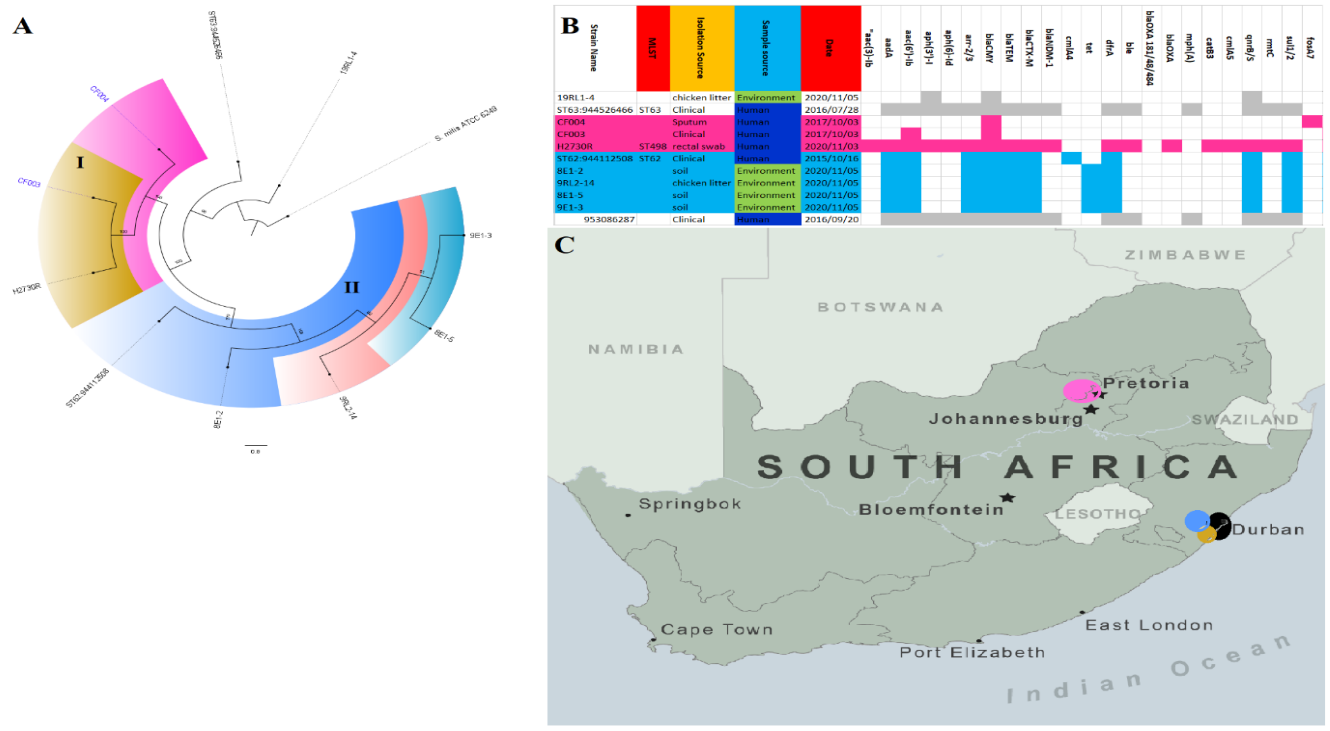


Figure S5. Phylogenomics of *Citrobacter freundii*

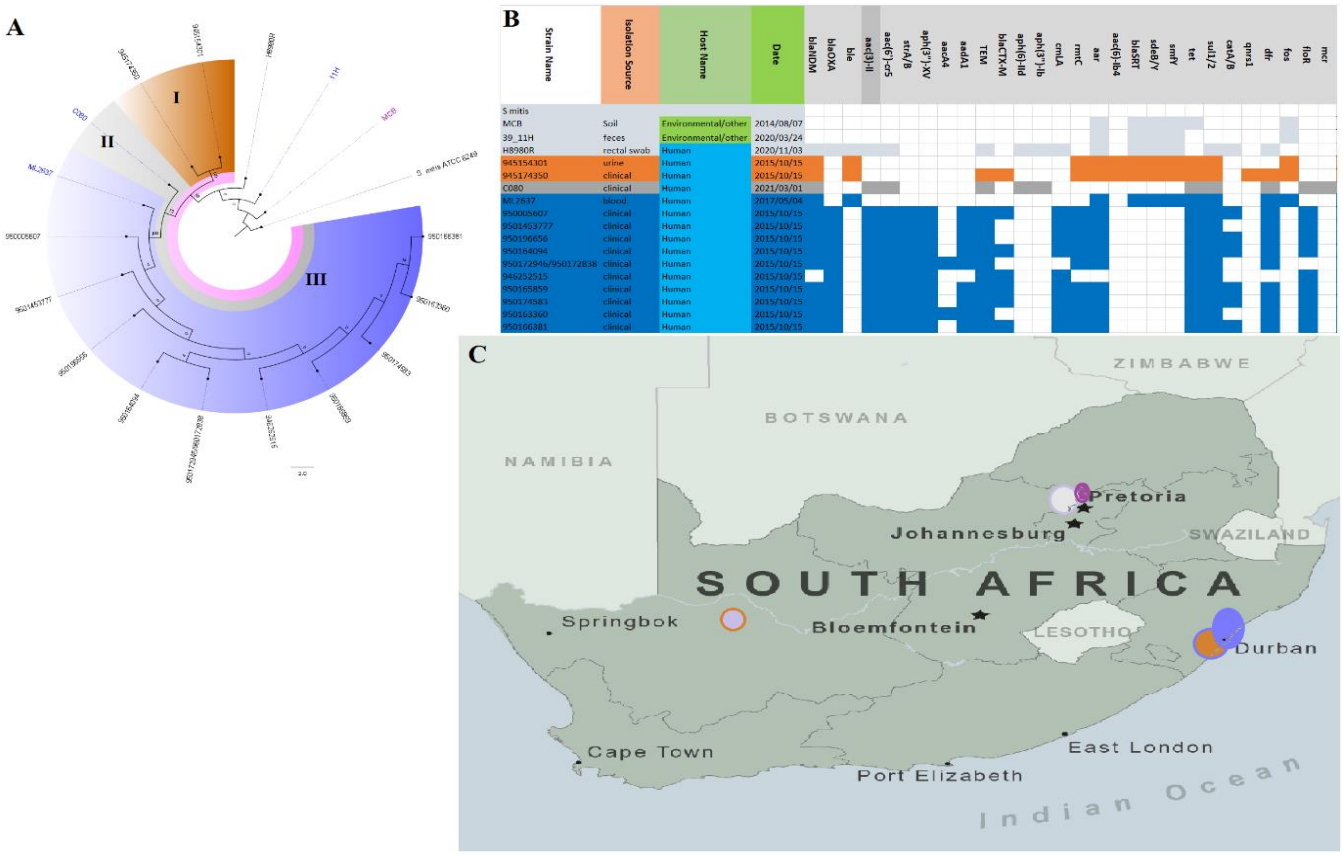


Figure S6. Phylogenomics of *Serratia marcescens*

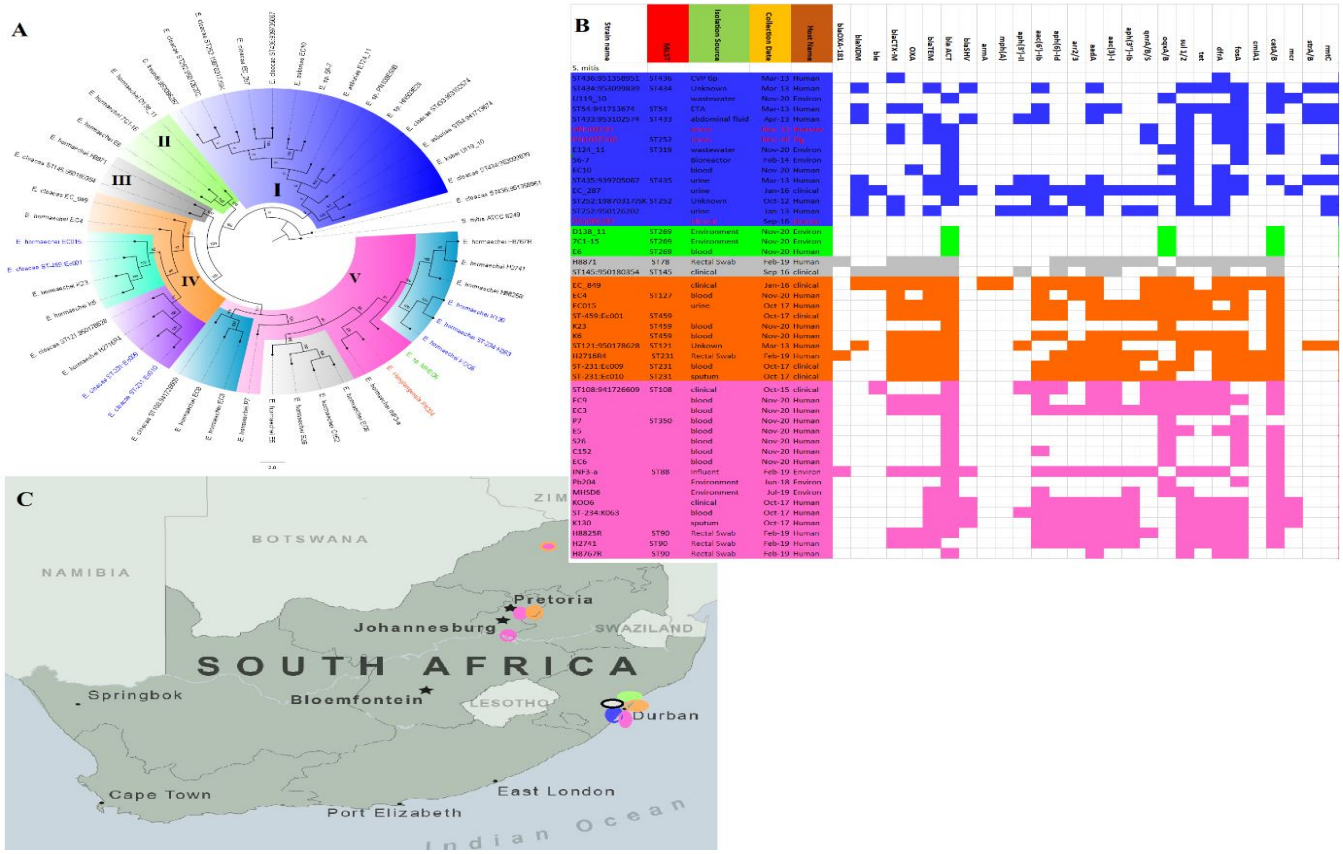


Figure S7. Phylogenomics of *Enterobacter hormaechei*

CHAPTER 3 - MANUSCRIPT 2

Pathogenomic Analysis of a Novel Extensively Drug-Resistant Carbapenem-resistant *Citrobacter freundii* Isolate carrying a bla$_{NDM-1}$ Carbapenemase in South Africa

Author contributions

- Y. Ramsamy, as the principal investigator, co-conceptualized the study, put forth ethical applications, undertook the literature search, facilitated sample collection, performed laboratory work, analysed the genomic data, undertook drafting of manuscript for journal submission and collated all references.

- S.Y. Essack, as co-supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, facilitated data collection and undertook critical revision of the manuscript.

- K.P. Mlisana, as principal supervisor, co-conceptualized the study, guided the literature review. Facilitated the laboratory work and undertook critical revision of the manuscript.

- Daniel. G. Amoako assisted in genomic data curation, analysis and undertook critical revision of the manuscript.

- M. Allam curated the genomic data results and undertook critical revision of the manuscript.

- A. Ismail curated the genomic data results and undertook critical revision of the manuscript.

- Akebe L. K. Abia assisted in genomic data analysis and undertook critical revision of the manuscript.

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Keywords: Pathogenomics; Novel; Mobilome; Carbapenem-resistant; *Citrobacter freundii; bla*NDM-1; Carbapenemase; South Africa.

Summary

In this report, we describe a whole-genome sequence of a novel sequence type of carbapenem resistant *Citrobacter freundii*, isolated from a patient during a One Health study to triangulate the molecular epidemiology of CRE in humans, food animals and the environment. This study reports the comprehensive pathogenomics of an XDR *C. freundii* strain isolated from South Africa. The multi-replicon plasmid p18-43_01 encoding the $_{bla\text{NDM-1}}$ reported for carbapenem resistance coupled with the diversity of the resistome, virulome, and mobilome of this novel pathogen necessitates continuous resistance surveillance programs, including rectal screening, stringent infection control measures, and antibiotic stewardship policies to curb further emergence and spread of these antibiotic-resistant bacteria.

pathogens

MDPI

Article

Pathogenomic Analysis of a Novel Extensively Drug-Resistant *Citrobacter freundii* Isolate Carrying a blaNDM-1 Carbapenemase in South Africa

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Abstract: Pathogenomic analysis was performed on a novel carbapenem-resistant *Citrobacter freundii* isolate (H2730R) from a rectal swab of an adult male patient admitted to a tertiary hospital, Durban, South Africa. H2730R was identified using selective media and API 20e kit. Confirmatory identification and antibiotic susceptibility testing were performed using the VITEK II. H2730R was whole-genome sequenced on the Illumina MiSeq platform. H2730R was resistant to all tested antibiotics except tigecycline and was defined as ST498 by the *C. freundii* multilocus sequence typing (MLST) database. The estimated pathogenic potential predicted a higher probability ($P_{score} \approx 0.875$), supporting H2730R as a human pathogen. H2730R harbored 25 putative acquired resistance genes, 4 plasmid replicons, 4 intact prophages, a class 1 integron (IntI1), 2 predominant insertion sequences (IS3 and IS5), numerous efflux genes, and virulome. BLASTn analysis of the blaNDM-1 encoding contig (00022) and its flanking sequences revealed the blaNDM-1 was located on a plasmid similar to the multireplicon p18-43_01 plasmid reported for the spread of carbapenem resistance in South Africa. Phylogenomic analysis showed clustering of H2730R with CF003/CF004 strains in the same clade, suggesting a possible association between *C. freundii* strains/clones. Acquiring the p18-43_01 plasmid containing blaNDM-1, the diversity, and complex resistome, virulome, and mobilome of this pathogen makes its incidence very worrying regarding mobilized resistance. This study presents the background genomic information for future surveillance and tracking of the spread of carbapenem-resistant *Enterobacteriaceae* in South Africa.

Keywords: Pathogenomics; Novel; *Citrobacter freundii*; blaNDM-1; Mobilome; South Africa; Carbapenemase

1. Introduction

Citrobacter freundii (C. freundii) is a rod-shaped, Gram-negative, aerobic member of the Enterobacterales family that is an intestinal commensal in humans and animals [1,2]. It is a known cause of nosocomial infections associated with the biliary tract, respiratory tract, urinary system, and

central nervous system, including meningitis, neonatal sepsis, and infectious diarrhea [3,4]. *C. freundii* can persist in their hosts for a long time and acquire resistance mechanisms, making the treatment of their infections challenging [4]. The emergence and dissemination of drug-resistant *C. freundii* in humans, animals, and the environment have previously been described, making this pathogen a potential reservoir for the spread of antimicrobial-resistant genes [1,2,4–7].

Carbapenem resistance is a growing concern in *C. freundii*, as carbapenems are regarded as antimicrobial agents of last resort used to treat life-threatening infections caused by these pathogens [8]. The easy mobilization of carbapenemase encoding genes, due to their locations on mobile genetic elements (MGEs), has favored the acquisition of carbapenem resistance by diverse *Enterobacterales* [9]. These MGEs, specifically plasmids, prophages, insertion sequences (IS), and integrons, are known to influence microbial virulence and pathogenicity via horizontal gene transfer (HGT) [10,11]. HGT allows new genomic traits to be acquired from other unrelated bacteria and remains the most effective means of bacterial evolution, causing the dissemination of antibiotic resistance and associated disease pathogenesis [12,13]. In this study, we present the emergence of a novel carbapenem-resistant sequence type (ST498) of *C. freundii* isolated from a rectal swab of an adult male patient admitted to a tertiary healthcare facility in Durban, South Africa, harboring a diverse resistome, virulome, and mobilome. This investigation forms part of a broader study specifically designed to isolate carbapenemase-producing *Enterobacteriaceae* (CPE) for patients at admission in a public hospital in the province. We also show the phylogenomic relationship between this novel isolate (H2730R) and all the deposited *C. freundii* genomes from South Africa at the Pathosystems Resource Integration Center (PATRIC) online platform.

2. Results

2.1. Isolation, Phenotypic Confirmation of Carbapenem Resistance, and Antibiotic Susceptibility Testing (AST)

The carbapenem-resistant *C. freundii* strain (H2730R) was isolated using the selective media and confirmed using the VITEK II AST-N255 automated platform. Antibiotic susceptibility testing (AST) revealed that H2730R was extensively drug-resistant (XDR) (Table 1).

Table 1. Susceptibility Pattern of Isolate H2730R.

Antibiotic	AST Profile	Minimum Inhibitory Concentrations (mg/L)
Amoxicillin-clavulanic acid	R	(32/16)
Piperacillin-tazobactam	R	(128/4)
Cefuroxime	R	(32)
Cefotaxime	R	(4)
Ceftriaxone	R	(4)
Ceftazidime	R	(16)
Cefepime	R	(16)
Cefoxitin	R	(32)
Imipenem	R	(4)
Meropenem	R	(4)
Ciprofloxacin	R	(1)
Gentamicin	R	(16)
Amikacin	R	(64)
Nitrofurantoin	R	(128)
Trimethoprim-sulfamethoxazole	R	(4/76)
Tigecycline	S	(2*)

S, susceptible; I, intermediate; R, resistant. * CLSI 2017 used for interpretation of resistance breakpoints for all antibiotics except that of Tigecycline where EUCAST resistance breakpoints (v 7.1) were used for interpretation.

2.2. General Genomic Features of H2730R

The CG Viewer Server was used for the genomic visualization of the isolate (Figure A1; Appendix A). The *C. freundii* (H2730R) genomic features are shown in Table A1. The size, GC content, number of contigs, N50, and L50 of the H2730R genome were 5.29 Mbp, 51.80%, 58, 518368, and 4, respectively. Annotation with RAST and PGAP resulted in 5006 protein-coding genes, 70 RNAs, and 12 tRNAs (Table A1). The protein-coding genes (CDSs) and non-protein-coding genes were assigned to 27 clusters of orthologous groups (COG) and functional categories (Table A1).

2.3. WGS-Based Confirmation and Multilocus Sequence Typing (MLST)

The identification of H2730R isolate was confirmed with generated genomic data via the Global Platform for Genomic Surveillance (Pathogenwatch). In silico determination of the classical sequence type from WGS data using the *C. freundii* MLST scheme resulted in a previously undescribed MLST comprising a new allelic combination for *arca_5*, *aspc_16*, *clpx_14*, *dnag_54*, *fadd_103*, *lysp_5*, *mdh_15*. Allele sequences were submitted for curation and assigned to the new ST 498 by the *C. freundii* PubMLST database. BURST (Based Upon Related Sequence Types) analysis identified the novel ST498 as a satellite clone (more distantly related strain) with no single-locus variant (SLV) or double-locus variant (DLV) of globally curated *C. freundii* STs. Of note, the new ST had different allelic profiles from the five deposited *C. freundii* genomes from South Africa.

2.4. WGS Analysis of Resistance Genes and Genetic Support

WGS analysis via the different databases revealed 25 putative acquired antibiotic resistance genes responsible for resistance to the various antibiotics found in the isolate, which corroborated the phenotypic profile (Table 2). Chromosomal mutation in GyrA [S83I] and the plasmid-mediated quinolone resistance genes (*aac (6')-Ib-cr and qnrB1*) were found. Most of the resistance genes were plasmid-borne (Table 2). Resistance to carbapenems was linked to the New Delhi metallo-β-lactamase-1 (*bla*$_{NDM-1}$) belonging to the sub-class B1. The BLASTn analysis of the bla$_{NDM-1}$ carbapenemase containing contig (00022) and its flanking sequences matched the 212.3 Kbp multireplicon plasmid p18-43_01 (GenBank accession number **CP023554.1**). The PlasmidFinder v1.3 identified four plasmid replicons (Inc A/C2, Inc FIB(pB171), Inc FII(Yp), and Inc Q1).

The PHAge Search Tool (PHAST) detected four intact phages (Escher_HK639, Entero_c_1, Salmon_RE_2010, and Salmon_SJ46). The insertion sequences in the genomes belonging to the IS3 family (IS2) and IS5 family (IS903) were predicted by BLAST searches against contigs on the ISFinder database. In silico analysis identified the class 1 integron-integrase gene (IntI1) in H2730R. The relationship between the IntI1 integrase in H2730R and other closely related IntI1 integrases in diverse species obtained from the RAST Server was shown as a phylogenetic tree using the MAFFT online tool (Figure 1). The Intl1 of the H2730R strain had the closest similarity with the *Salmonella enterica subsp. enterica serovar Choleraesuis* plasmid pOu7519, as depicted in Figure 1.

Furthermore, 10 efflux pump systems known to be involved in drug resistance were identified (Table 2). They belonged to three main efflux systems, namely: ABC, ATP-binding cassette; MFS, major facilitator superfamily; and RND, resistance–nodulation–division (Table 2).

Table 2. Resistance gene profile and efflux pump systems of Isolate H2730R.

Antibiotic class	Genes	Genomic location
β-lactams	*blaNDM-1, blaCMY-48, blaCTX-M-15, blaOXA-10, OXA-1, blaTEM-1B*	Plasmid
Aminoglycosides	*aph(3')-Ia, aac(6')-Ib-cr, aac(3)-IIa, aph(6)-Id, aadA1, aac(3)-IId, rmtC, aph(3'')-Ib*	Plasmid
Fluroquinolones	*aac(6')-Ib-cr, qnrB1, GyrA(S83I)*	Plasmid and Chromosome (*GyrA*)
Fosfomycin	*fosA*	Chromosome
Trimethoprim	*dfrA23, dfrA7, dfrA14*	Plasmid
Rifampicin	*ARR-2*	Plasmid
Phenicols	*catB3, cmlA1*	Plasmid
Sulphonamides	*sul2, sul1*	Plasmid
Tetracycline	*tet(A)*	Plasmid
Efflux pump systems		
ATP-binding cassette (ABC)	*msbA*	Chromosome
Major facilitator superfamily (MFS)	*mdfA, mdtG*	Chromosome
Resistance–nodulation–division (RND)	*marA, H-NS, mdtC, baeR, acrA, acrB, CRP*	Chromosome

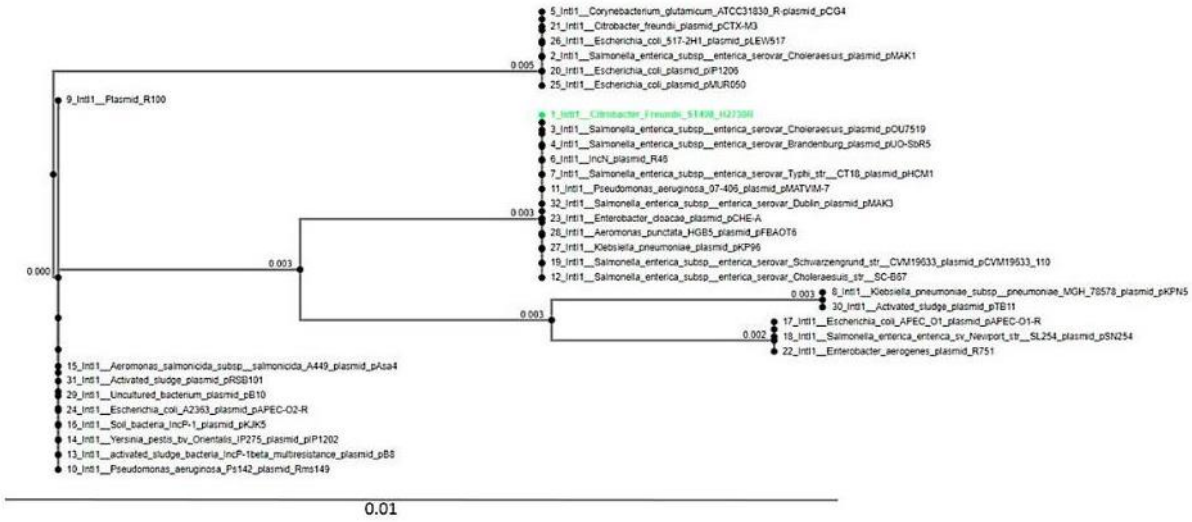

Figure 1. A phylogenetic tree showing the association between Integron_Integrase_IntI1 of *C. freundii* H2730R and other predicted closely related *IntI1* integrases in diverse species.

2.5. Pathogenicity, Defence Systems Mechanisms, and Virulome Predictions

H2730R had a 0.875 probability of being pathogenic to humans and was found to match with 110 pathogenic families. All the 110 pathogenic families were linked to members of the Enterobacterales, of which *Citrobacter koseri* ATCC BAA-895 was the organism with the highest possible pathogen linkage. The CRISPRCasFinder predicted two clustered, regularly interspaced, short palindromic repeat (CRISPR) arrays with no Cas systems. Of note, the in silico analysis revealed a Type II restriction-modification (R-M) defense system consisting of *Eco128I* (restriction enzyme) and *M. EcoRII* (methyltransferase)]. The whole-genome virulome analysis predicted virulence-encoded pathogenesis-associated proteins predominantly made up of adherence determinants, regulation, toxin, motility, antiphagoctyosis, invasion, and biofilm formation (Table 3).

Table 3. Genomic characterization of putative virulence factors in the isolate H2730R*.

No.	Putative Virulence Factors	Genes	Organisms [Highest Homology]
1	Fimbrial adherence determinants	*csgA, csgB, csgC, csgD, csgE, csgF, csgG fimA, fimC, fimD, fimF, fimH, fimI, fimW, fimZ, lpfC, pegB, staB, staC, stcA, stcC, stgA, stgB, stkA, stkB, stkC, stkD, stkE and StkF*	*Salmonella enterica*
2	Nonfimbrial adherence determinants	*misL, ratB, shdA and sinH*	*Salmonella enterica*
3	Regulation	*phoP and phoQ*	*Salmonella typhimurium*
4	Toxin	*usp*	*Escherichia coli*
5	Motility	*flaA*	*Bordetella bronchiseptica*
6	Antiphagocytosis	*uge*	*Klebsiella pneumoniae*
7	Invasion	*ibeB*	*Escherichia coli*
8	Biofilm formation	*pgaC*	*Acinetobacter baumannii*

*The fimbrial adherence determinants consisted of the thin aggregative fimbriae (*csgA, csgB, csgC, csgD, csgE, csgE, csgG*), Type 1 fimbriae (*fimA, fimC, fimD, fimF, fimH, fimI, fimW, fimZ*), long polar fimbriae (*lpfC*), plasmid-encoded fimbriae (*pegB*), and other unique fimbriae (*staB, staC, stcA, stcC, stgA, stgB, stkA, stkB, stkC, stkD, stkE, and StkF*). The nonfimbrial adherence determinants included unique fimbriae (*misL, ratB, shdA,* and *sinH*). The regulation factor, toxin factor, and motility factor consisted of a two-component system (*phoP and phoQ*), colicin-like *usp*, and flagellin (*flaA*), respectively. The antiphagoctyosis, invasion, and biofilm formation determinants were composed of the capsule (*uge*), invasion of brain endothelial cell (*ibeB*), and biofilm-associated protein (*pgaC*), respectively.

2.6. Phylogenomic Analysis and Metadata Insights of Reported Citrobacter Freundii Isolates from South Africa

The phylogenetic relationship and epidemiological distribution of all deposited *C. freundii* genomes on GenBank from South Africa are depicted in Figure 2 in which isolates of the same cluster are highlighted in the same color. The tree analysis, coupled with metadata, revealed two clusters (A and B) of *C. freundii* with insights into the diversity of clones in South Africa (Table A2). Of note, the H2730R was related to the CF003 and CF004 strains (Cluster B). All the isolates were from human hosts.

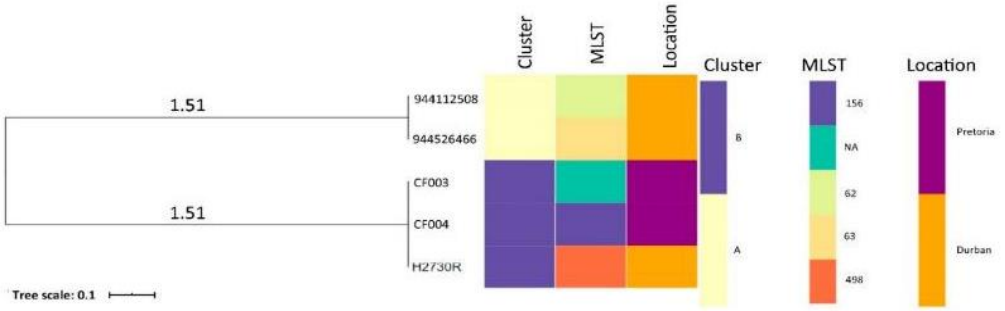

Figure 2. The whole-genome phylogenomic branch and metadata [MLST and Demographics (Location and Host)] coupled using Phandango showing the relationship between H2730R and all deposited *Citrobacter freundii* strains (n = 4) from South Africa. All the isolates were from a human host.

3. Discussion

Carbapenem resistance in Enterobacteriaceae has become a major public health threat that requires urgent attention. In this study, we use whole-genome sequencing (WGS) and bioinformatics analysis to describe the emergence of a novel carbapenem-resistant sequence type (ST498, satellite clone) of *C. freundii* isolated from the rectal swab of an adult male patient admitted to a tertiary healthcare facility in Durban, South Africa, harboring a diverse resistome, virulome, and mobilome.

H2730R was categorized as XDR due to its phenotypic resistance to all tested antibiotics except tigecycline [14] (Table 1). The carbapenem phenotypes of the strain, as identified on selective chromogenic screening medium, were confirmed by the presence of carbapenemase (bla_{NDM-1}) (Tables 1 and 2). The 100% concordance between phenotypes and predicted resistome annotation data reiterated the reliability of WGS for the accurate prediction of antibiotic resistance [15,16]. The *C. freundii* H2730R genome harbored a total of 25 putative resistance genes, which were predominantly plasmid-borne (Table 2), indicating an acquisition and the potential for spread via horizontal resistome transfer between H2730R and other species. Resistance to ciprofloxacin was mediated by GyrA [S83I] chromosomal mutation, plasmid-mediated quinolone resistance genes (PMQR) (*aac(6')-Ib-cr and qnrB1*), and efflux genes (Table 2) [17]. Sekyere and Amoako et al. (2017) [15] reported that high-level fluroquinolone resistance was mediated by efflux, PMQR genes, and gyrA mutations in Enterobacterales, including *Citrobacter freundii*, which supported our results. More so, efflux genes belonging to three main efflux pump systems (Table 2) reported to play vital roles in antibiotic resistance in Enterobacterales were also identified [18,19]. However, the mere presence of these efflux genes does not directly implicate resistance, and hence further studies on their expression levels will be needed to ascertain the possibility of efflux pump hyperexpression [18]. The diverse resistance mechanisms (plasmid-borne, chromosomal, and efflux) involved in H2730R resistance indicate the various processes the organism expresses to survive the effects of antibiotics and other biocides (Table 2).

The National Center for Biotechnology Information (NCBI) microbial nucleotide BLAST search of the bla_{NDM-1} carbapenemase containing contig (00022) and its flanking sequences in the isolate revealed the bla_{NDM-1} was located on a plasmid similar to the 212.3 Kbp multireplicon p18-43_01 plasmid (accession no. **CP023554.1**) reported for the spread of carbapenemases in Enterobacterales (including *K. pneumoniae* [n = 4], *Citrobacter freundii* [n = 1], *Serratia marcescens* [n = 11], and *Enterobacter* family complex [n = 7]) in the private [20] and public healthcare sector [21] in Durban, South Africa. This implicates a possible bla_{NDM-1} acquisition as well as nosocomial spread and development of an XDR genetic lineage via this local plasmid across sectors in the KZN province, which is very worrisome. The four plasmid replicon genes (Inc A/C2, Inc FIB(pB171), Inc FII(Yp), and Inc Q1) identified via the PlasmidFinder supported the multireplicon plasmid nature of the p18-43_01. This global spread of carbapenem resistance through the acquisition of plasmid encoding carbapenemase has been highly documented in the literature [22–24].

Moreover, H2730R contained two insertion sequences (IS3 family and IS5 family) whose genetic transposition could help in the plasticity and concomitant adaptability of H2730R phenotypic traits, including catabolism, pathogenicity, resistance to antibacterial agents, and virulence [25]. The IS2 (IS3 family) has been reported to cause erythromycin and fluoroquinolone resistance through the formation of hybrid promoters [25]. Additionally, the IS930 (IS5 family) has been implicated in carbapenem and metal ion (silver) resistance in *Enterobacter aerogenes* [26] and *E. coli*, respectively. Further analysis of H2730R revealed prophage with possible origination from diverse sources (*E. coli* and *Salmonella*) [27]. For instance, the two prophages Salmon_RE_2010 and Salmon_SJ46 were initially identified in *Salmonella enterica subsp. enterica serovar Enteritidis* and Salmonella *enterica subsp. enterica serovar Indiana*, respectively [28]. This supports a possible exchange of genetic traits via horizontal transfer between Enterobacterales. Class 1 integrons, reported to be associated with multiple classes of antibiotic resistome, including β-lactams, quinolones, and aminoglycosides worldwide, were found [29]. However, the full mobile gene cassettes and integron genetic structure could not be determined as they were truncated into different contigs during the assembly process.

The pathogenic potential (P_{score}) is a machine learning algorithm used for the in silico prediction of the possibility of a strain being a human pathogen with the probability ranging from 0 to 1. This estimation of the pathogenic potential using trained algorithms to differentiate between pathogenic or commensal strains predicted a higher probability (P_{score} ≈0.875) suggesting H2730R as a potential human pathogen. Further, in silico analysis of H2730R predicted the presence of two CRISPR arrays and a Type II restriction–modification (R-M) defense system which offers bacterial protection against viral attacks and thus increases its survival and adaptability in the microbial landscape [30,31].

Moreover, the genomic virulome analysis revealed a battery of different virulence encoded proteins from diverse sources. These virulence determinants were predominantly made up of adherence determinants which aid in the complement-independent attachment to host mammalian cells [32]. H2730R possessed toxins to induce cell membrane damage, trigger cytokine formation, and reduce or kill neutrophils [33]. Interestingly, the strain also harbored the *ibeB* and *pgaC* genes, which contribute to invasion, biofilm formation, and adhesion to eukaryotic host cells. Additionally, H2730R harbored the two-component regulation system (*PhoPQ*) activated by low divalent cations magnesium (Mg^{2+}) and calcium (Ca^{2+}) levels and required for intracellular survival, cationic antimicrobial peptides (CAMPs) resistance, and stimulation of cytokine secretion [34]. The higher virulence factor homology existing between H2730R and other Gram-negative species (Table 3) suggests that they were possibly obtained horizontally, necessitating further studies on transconjugation and/or transcriptomics to ascertain this assertion. The detection of the virulome can inform vaccine development by delineating possible bacterial protein targets.

Phylogenomic analysis, coupled with metadata, showed clustering of H2730R (Durban) with CF003/CF004 strains in the same cluster (B) suggesting a possible association of *C. freundii* strains/clones. However, not much inference could be drawn from the tree analysis due to the small number of deposited *C. freundii* genomes from South Africa. It is thus recommended that more studies be carried out in all the sectors (human, animal, and environment) to monitor the emergence and spread of XDR *C. freundii* using high throughput technologies such as whole-genome sequencing (WGS). Moreover, there was limited data on the clinical information, history and/or clinical examination of the patient, and all deposited *C. freundii* genomes obtained from the human host, which could be very useful in comparative phylogenomic analysis. Ultimately, a combination of demographics, clinical information, and WGS data coupled with graphical analysis should be applied to offer insights into the spread of pathogens and increase confidence during molecular epidemiological investigations [35].

4. Materials and Methods

4.1. Ethical Approval

This study was approved by the Biomedical Research Ethics Committee (approval no: **BE599/16**, a substudy of **BCA444/16**), College of Health Sciences, University of KwaZulu-Natal (UKZN).

4.2. Identification of the Isolate

Selective chromogenic screening medium, CHROMID® CARBA (BioMérieux, Marcy l'Étoile, France), was used for the isolation of carbapenemase-producing Enterobacteriaceae from a screening rectal swab obtained from an adult patient. The CHROMID® CARBA (BioMérieux, Marcy l'Étoile, France) agar plate was inoculated with the following control strains: carbapenemase-negative *K. pneumoniae* ATCC 700603 and carbapenemase-positive *K. pneumoniae* ATCC BAA-1705.

4.3. Antibiotic Susceptibility Testing (AST)

Confirmatory phenotypic microbial identification and antibiotic susceptibility testing (AST) was performed on the VITEK II AST-N255 automated platform (BioMérieux, Marcy l'Étoile, France). The universal antimicrobial test panel included: penicillin, ampicillin, amoxicillin-clavulanate, ceftriaxone, cefepime, cefuroxime, cefoxitin, ceftazidime, imipenem, meropenem, ertapenem, piperacillin-tazobactam, amikacin, gentamicin, nitrofurantoin, trimethoprim/sulfamethoxazole,

ciprofloxacin, and tigecycline. The isolate was characterized as susceptible or resistant using CLSI-approved breakpoints [36].

4.4. DNA Purification, Genome Sequencing, and Preprocessing of Sequence Data

The isolate was streaked onto a nutrient agar (NA) (Sigma-Aldrich, St. Louis, USA) plate and incubated at 37 °C for 24 h. Following incubation, genomic DNA was extracted from a visibly pure culture using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymogen Research, USA, cat. no. D6005), quantified by Nanodrop 8000 (Thermo Scientific, Waltham, MA) and Qubit, and verified by agarose gel electrophoresis. A paired-end library (2 × 300 bp) was prepared using Illumina Nextera XT DNA Sample Preparation Kit and sequenced on a MiSeq machine (Illumina, USA). The generated sequenced reads were quality assessed and trimmed using the Skesa assembler (version 2.3). Default parameters were used for all software unless otherwise specified. The CheckM tool [37] was used to verify that the sequence reads were not from mixed species using lineage-specific marker sets from other genetically well-characterized, closely related *C. freundii* strains.

4.5. Bioinformatic Analysis

4.5.1. Genome Visualization and Annotation

The raw reads were de novo assembled using the Skesa (version 2.3) assembler [38]. The genomes of the strains were visualized using the CG Viewer Server (Figure A1; Appendix A). The National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP; version 4.3) [39] and Rapid Annotation using Subsystem Technology (RAST) Server (version 2.0) [40] were used for annotation of the size, GC content, number of contigs, N50, L50, average coverage, number of RNAs, and protein-coding sequences of the isolate.

4.5.2. WGS-Based Confirmation and Molecular Typing

The generated contigs from the WGS data were used to confirm the identity of the isolate on the pathogen watch platform [41]. The assembled genome was submitted to the *C. freundii* MLST database, which predicted the allelic profiles of the seven housekeeping genes to assign the new sequence type [42]. An eBURST [43] algorithmic analysis was performed in the MLST database (https://pubmlst.org/cfreundii/) to ascertain whether the novel sequence type (ST) was a single-locus variant (SLV) or double-locus variant (DLV) or satellite (SAT, more distantly related strain) of known STs. The allelic profiles and STs of all deposited *C. freundii* genomes from South Africa in the PATRIC database were also predicted using the MLST database.

4.5.3. Genomic Identification of the Antibiotic Resistome and Mobile Genetic Elements (MGEs)

The bacterial analysis pipeline of GoSeqIt via ResFinder [44], Antibiotic Resistance Gene-Annotation database (ARG-ANNOT) [45], and the comprehensive antibiotic resistance database (CARD) [46] tools were also used to annotate and identify antibiotic resistance genes. To determine if the resistance genes and their associated MGEs were plasmid-borne or chromosomal, the contigs bearing these resistance genes were BLASTed on BLASTn to determine if the closest nucleotide homologies were chromosomal or plasmids. Plasmid replicons were predicted through PlasmidFinder [47]. The PHAge Search Tool [48] server was used for the identification, annotation, and visualization of prophage sequences. Phage regions were extracted and analyzed in the NCBI nucleotide BLASTn service to confirm their identity. Insertion sequences and transposase in the genomes were predicted by blasting contigs on the ISFinder database [49] to find the common insertion sequences. Integrons in the genomes predicted by PGAP and RAST subsystems were blasted on the INTEGRALL database to find the actual integrons. Additionally, using the nucleotide sequences, the MAFFT multiple alignment program [50] was used to show the phylogenetic relationship between predicted and other closely related integrases in diverse species obtained from the RAST Server.

4.5.4. Pathogenicity, Defence Systems, and Virulome Predictions

PathogenFinder [51] was used to predict pathogenicity towards human hosts. The CRISPRCasFinder was used to identify the putative CRISPR loci and Cas cluster in the draft genomes [52]. Restriction Modification Finder predicted the R-M system in the isolate [53]. Virulence determinants (sequences and functions) corresponding to different major bacterial virulence factors associated with *C. freundii* were searched for virulence factors with the pathogenic bacteria database, VFanalyzer [54], and validated by blasting assembled genomes to a pseudomolecule generated by concatenating a set of target genes using the NCBI in-house BLASTN tool.

4.6. Phylogenomic Analyses of *C. freundii* Isolates from South Africa

The genome sequences of all five available genomes were downloaded from GenBank and PATRIC for genomic comparison and phylogenomic analysis via CSI Phylogeny-1.4 [55], an online service which identifies SNPs from WGS data, filters and validates the SNP positions, and then infers phylogeny based on concatenated SNP profiles [55]. A bootstrapped indicator with 100 replicates was applied to identify recombined regions and provide the phylogenetic accuracy in groups with little homoplasy. Figtree was used to edit and visualize the phylogenetic tree. The phylogeny was visualized alongside annotations for isolate demographics and WGS in silico molecular typing metadata using Phandango [56].

4.7. Data Availability

This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under the accession no. VWTQ00000000. The version described in this paper is version VWTQ01000000.

5. Conclusions

This study reports the comprehensive pathogenomics of an XDR *C. freundii* strain isolated from South Africa. The multireplicon plasmid p18-43_01 encoding the bla_{NDM-1} reported for carbapenem resistance coupled with the diversity of the resistome, virulome, and mobilome of this novel pathogen makes this incidence very worrying. This necessitates continuous resistance surveillance programs, including rectal screening, stringent infection control measures, and antibiotic stewardship policies to curb further emergence and spread of these antibiotic-resistant bacteria.

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Appendix A

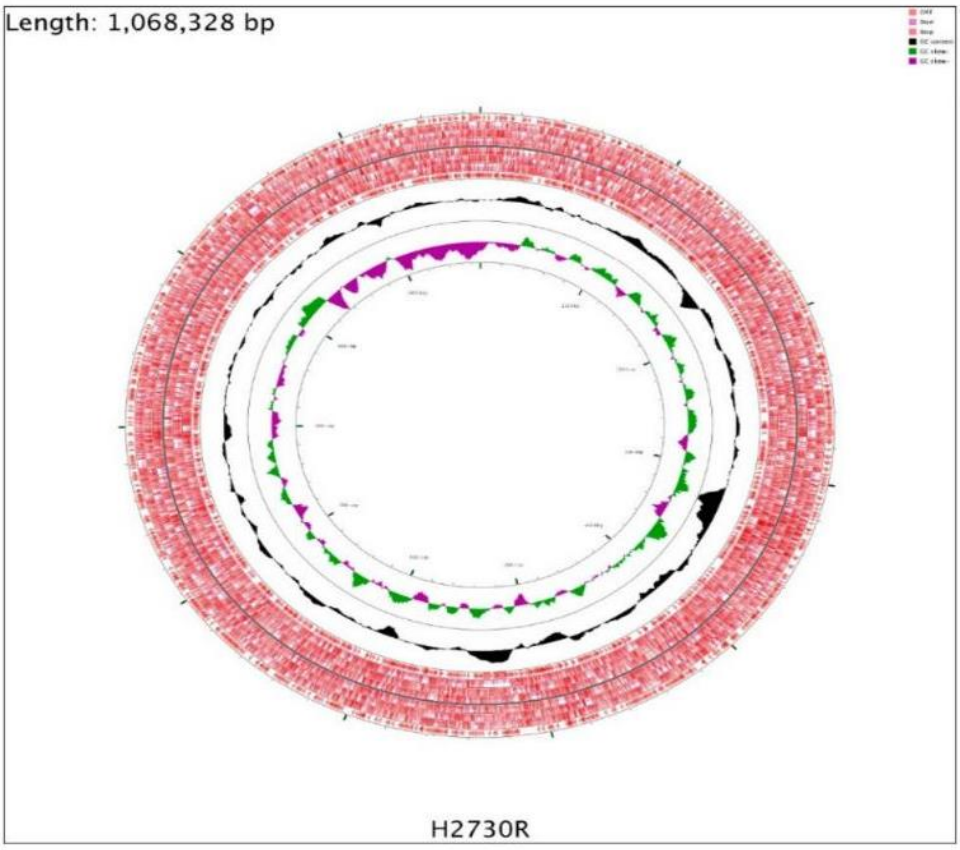

Figure A1. Graphic depiction of the H2730R genome belonging to ST498. The two outer circles show the open reading frame (ORF–in mauve). The inner circle shows the GC skew, with green and purple indicating positive and negative values, respectively. The GC content is indicated in black. This genome map was visualized using the CGView Server.

Appendix B

Table A1. Genomic features of Isolate H2730R.

Attribute	Value
Sequencing platform	Illumina MiSeq machine
Assembler	Skesa (version 2.3)
Assembly accession	VWTQ00000000
No. of Contigs	58
Genome size (bp)	5,299,408
DNA G + C (%)	51.8
Genome coverage (X)	99.0
Number of RNAs genes	70
Number of tRNAs genes	12
23S rRNAs	7
5S rRNAs	5
N50	518,368
L50	4
Number of subsystems	396
Number of CDSs	5135
Genes assigned to COGs	5006
Pseudo Genes	129

Table A2. A table showing the diversity of MLST (ST types) and allelic profiles of the seven housekeeping genes of all deposited *Citrobacter freundii* strains (n = 5) from South Africa.

Isolate	MLST	*arcA*	*aspC*	*clpX*	*dnaG*	*fadD*	*lysP*	*mdh*
944112508	ST156	7	15	63	54	13	5	12
944526466	NA	33	49	50	45	57	16	47
CF003	ST62	32	48	10	6	14	45	13
CF004	ST63	33	49	50	45	57	16	47
H2730R	ST498	5	16	14	54	103	5	15

NA, Not Available.

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CHAPTER 4 - MANUSCRIPT 3

Comparative pathogenomics of *Aeromonas veronii* pigs in South Africa: Dominance of the novel ST657 clone

Author contributions

- Y. Ramsamy, as the principal investigator, co-conceptualized the study, put forth ethical applications, undertook the literature search, facilitated sample collection, performed laboratory work, analysed the genomic data, undertook drafting of manuscript for journal submission and collated all references.

- S.Y. Essack, as co-supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, facilitated data collection and undertook critical revision of the manuscript.

- K.P. Mlisana, as principlal supervisor, co-conceptualized the study, guided the literature review. Facilitated the laboratory work and undertook critical revision of the manuscript.

- Daniel. G. Amoako assisted in genomic data curation, analysis and undertook critical revision of the manuscript.

- M. Allam curated the genomic data results and undertook critical revision of the manuscript.

- A. Ismail curated the genomic data results and undertook critical revision of the manuscript.

- Akebe L. K. Abia assisted in genomic data analysis and undertook critical revision of the manuscript.

- R, Singh aided in laboratory work and undertook critical revision of the manuscript.

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Running title: Pathogenomics of *A. veronii* in Pigs from South Africa

Keywords: Genomics, *Aeromonas veronii*, Pig, Abattoir, Antibiotics, Mobile genetic elements, Global phylogenomic tree.

Summary

The large proportion of antibiotic use in animal husbandry is thought to contribute to the problem of AMR globally. It is therefore vital that monitoring of antibiotic use and AMR within the animal sector is performed. In this report, we describe the pathogenomics of *Aeromonas veronii* strains isolated from food producing animals (pigs) during a One Health study triangulating the molecular epidemiology of CRE in humans, food animals and the environment in KwaZulu-Natal, South Africa. All *Aeromonas veronii* isolated during the study harbored the CphA gene which conferred resistance to β-lactams, in particular imipenem. This study highlights the role of CRE surveillance in animals such that CRE surveillance should not only be confined to the human population as CRE and AMR is widespread between humans, animals and the environment as evidenced by this One Health study. Continous surveillance of AMR across all three sectors is essential in enforcing infection prevention and control measures and formulation of antibiotic stewardship policies required to curb dissemination of resistant bacteria. Monitoring of AMR in food producing animals will ensure consumption of safe meat products. Surveillance using molecular technology such as those used in this study will demonstrate dissemination and evolution of pathogens across all three sectors.

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Comparative Pathogenomics of *Aeromonas veronii* from Pigs in South Africa: Dominance of the Novel ST657 Clone

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Abstract: The pathogenomics of carbapenem-resistant *Aeromonas veronii (A. veronii)* isolates recovered from pigs in KwaZulu-Natal, South Africa, was explored by whole genome sequencing on the Illumina MiSeq platform. Genomic functional annotation revealed a vast array of similar central networks (metabolic, cellular, and biochemical). The pan-genome analysis showed that the isolates formed a total of 4349 orthologous gene clusters, 4296 of which were shared; no unique clusters were observed. All the isolates had similar resistance phenotypes, which corroborated their chromosomally mediated resistome ($bla_{CPHA\,3}$ and $bla_{OXA\text{-}12}$) and belonged to a novel sequence type, ST657 (a satellite clone). Isolates in the same sub-clades clustered according to their clonal lineages and host. Mobilome analysis revealed the presence of chromosome-borne insertion sequence families. The estimated pathogenicity score ($P_{score} \approx 0.60$) indicated their potential pathogenicity in humans. Furthermore, these isolates carried several virulence factors (adherence factors, toxins, and immune evasion), in different permutations and combinations, indicating a differential ability to establish infection. Phylogenomic and metadata analyses revealed a predilection for water environments and aquatic animals, with more recent reports in humans and food animals across geographies, making *A. veronii* a potential One Health indicator bacterium.

Keywords: genomics; *Aeromonas veronii*; intensive pig farming; abattoir; antibiotics; mobile genetic elements; global phylogeny

1. Introduction

Aeromonas spp. are Gram-negative, rod-shaped, non-sporulating, non-acid-fast, and facultative anaerobic bacteria that have been recognized as emerging nosocomial pathogens [1]. They belong to the family Aeromonadaceae, class Gammaproteobacteria that encompasses three genera, viz., *Tolumonas*, *Oceanimonas* and *Aeromonas* [2,3]. *Aeromonas* share many similar biochemical characteristics with *Enterobacterales* but are easily differentiated, with *Aeromonas* being oxidase-positive [4]. The *Aeromonas* genus has a complex, dynamic taxonomy due to the expanding number of new species and its high intra- and interspecies genetic variability [5,6]. This genus currently comprises 36 species, with *A. dhakensis*,

A. hydrophila, *A. caviae*, *A. salmonicidai*, and *A. veronii* being the most clinically relevant and pathogenic species [3,7,8].

Aeromonas spp. have been implicated in a range of diseases in animals and humans, including gastroenteritis, septic arthritis, peritonitis, osteomyelitis, myositis, ocular infections, meningitis, cholangitis, pneumonia, hemolytic uremic syndrome, and urinary tract infections [9–11]. Members of this genus are widely distributed across numerous ecosystems. *A. veronii* has been isolated from the environment (air, water and soil), food animals (shellfish, poultry, cattle, and pigs), as well as various human infections, making it a potential One Health indicator pathogen [5,12–15]. Faecal carriage rates of *Aeromonas* in normal humans are thought to be between 0% and 4% [16], while carriage rates in patients with symptomatic diarrhea range between 0.8 and 7.4 [17].

The virulence and pathogenesis of *Aeromonas* have been described as multifaceted and linked to the expression of genes that encode different metalloproteins, secretion systems, structural components, and toxins [4,18–20]. Studies have also reported the expression of different immune-related genes in the host, following an *Aeromonas* infection, including those involved in apoptosis, cell signaling, and pathogen recognition [3,21]. Although these virulence factors may confer variable abilities to establish infection, knowledge of *A. veronii* pathogenicity is currently incomplete. Also, despite the apparent increase in the incidence of this emerging pathogen globally, information on *A. veronii* in humans, animals, and the environment in Africa is limited. Thus, in this study, we describe for the first time in Africa, the comparative genomics of *A. veronii* recovered from pigs in KwaZulu-Natal, South Africa.

2. Materials and Methods

2.1. Ethical Approval

This study was approved by the Animal Research Ethics Committee (AREC/079/018D), University of KwaZulu-Natal (UKZN, Durban, South Africa). Permission for animal sampling was obtained from the Department of Agriculture, Forestry and Fisheries, Republic of South Africa (Ref 12/11/15). The State Veterinarian and the Health and Safety Officers at the abattoir were duly consulted. All samplers took a compulsory competency to ensure that all health and safety standards were met, and the necessary precautions were taken during animal sampling.

2.2. Sampling

This study was part of a larger point-prevalence study undertaken to determine the molecular epidemiology of carbapenem-resistant bacteria in humans (from public hospitals), food animals (a food processing plant with an abattoir), and the environment (a wastewater treatment plant) in uMgungundlovu, KwaZulu-Natal, South Africa. The current study focused on pigs. A total of 345 rectal swabs were collected from pigs, post-slaughter at an abattoir, using nylon-flocked swabs. Swabs were transported in 5 mL of Amies gel transport medium on ice to a reference laboratory authorized to process animal samples and processed within 6 h of sample collection.

2.3. Isolation and Identification

2.3.1. Screening of Carbapenemase-Producing Isolates

All rectal swabs were cultured on ChromID CARBA chromogenic agar (BioMérieux, Marcy l'Étoile, France) as described previously [22,23], with an incubation period of 18 to 24 h at 37 °C. Carbapenemase-negative *Klebsiella pneumoniae* ATCC 700603 and carbapenemase-positive *Klebsiella pneumoniae* ATCC BAA-1705 served as controls.

2.3.2. Confirmation of Isolates and Determination of Antibiotic Susceptibility Profiles

The carbapenem-resistant isolates obtained were sub-cultured onto MacConkey plates and subjected to phenotypic identification and susceptibility testing using the VITEK 2 (BioMérieux, Marcy l'Ëtoile, France) automated platform. The Clinical and Laboratory Standards Institute (CLSI) interpretative criteria were used to categorize isolates as susceptible or resistant [24]. The following β-lactam antibiotics were tested: ampicillin, amoxicillin, amoxicillin-clavulanate, ceftriaxone, ceftriaxone, cefepime, cefuroxime, cefoxitin, ceftazidime, imipenem, meropenem and piperacillin-tazobactam.

2.4. DNA Purification, Genome Sequencing and Pre-Processing of Sequence Data

Isolates that were confirmed as *Aeromonas* were sub-cultured on nutrient agar (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 24 h. Genomic DNA was then extracted from these pure cultures using the Quick-DNA™ Bacterial Miniprep Kit (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). The extracted DNA was quantified using a Nanodrop 8000, Qubit (Thermo Scientific, Waltham, MA, USA) and verified on an agarose gel electrophoresis. A paired-end library (2 × 300 bp) was prepared using Illumina Nextera XT DNA Sample Preparation Kit and sequenced on a MiSeq machine (Illumina, San Diego, CA, USA). The generated sequenced reads were quality assessed, trimmed, and de novo assembled using the SKESA Assembler (version 2.3; https://github.com/ncbi/SKESA), with default parameters for all software, unless otherwise specified.

2.5. Genome Visualization and Annotation

The genomes of the isolates were visualized using the GView Server [25]. The National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP; version 4.3) [26], and Rapid Annotation using Subsystem Technology (RAST) Server (version 2.0) [27] were used for annotation of the size, GC content, number of contigs, N50, L50, number of RNAs, protein-coding sequences for subsystem categorization, and comparison of the isolates. The OrthoVenn2 web server [28] was used to identify orthologous gene clusters that were either unique or shared among *A. veronii* strains. The analysis was performed with default parameters for the protein-coding genes of the strains.

2.6. Whole Genome Sequencing-Based Confirmation and Molecular Typing of Aeromonas Veronii

The generated contigs from the WGS data were used to confirm the identity of the isolates on the Pathogenwatch platform [29]. The assembled genomes were submitted to the *Aeromonas* MLST database, which assessed the allelic profiles of six housekeeping genes to assign the new sequence type (ST) [30]. An eBURST [31] algorithmic analysis was performed in the MLST database (https://pubmlst.org/aeromonas/) to ascertain whether the novel ST was a single-locus variant (SLV) or double-locus variant (DLV) of the known STs. The STs of globally deposited *A. veronii* genomes were obtained from the PATRIC database (https://www.patricbrc.org/view/Taxonomy/2).

2.7. Antibiotic Resistance Genes, Efflux Genes, and Mobile Elements Identification

The bacterial analysis pipeline of GoSeqIt via ResFinder [32], Antibiotic Resistance Gene-Annotation database (ARG-ANNOT) [33], and the Comprehensive Antibiotic Resistance Database (CARD) [34] tools were also used to annotate and identify resistance and efflux genes. PHAge Search Tool [35] server was used for the identification, annotation, and visualization of prophage sequences. The presence of insertion sequences and transposons was determined by blasting contigs on the ISFinder database [36], while the presence of integrons was ascertained from the PGAP and RAST subsystems and blasted on the IntegronFinder database. The distribution of CRISPR-Cas systems in *A. veronii* genomes was determined by CRISPRfinder [37]. Annotations from the Restriction-Modification Finder predicted the R-M system in the isolates [38].

2.8. Assessment of Pathogenic Potential and Prediction of Putative Virulence Factors

The pathogenic potential of the *A. veronii* isolates was assessed using the PathogenFinder web service under the automated mode [39]. All isolates were subjected to the pathogenicity prediction using Fasta formatted genome data. Virulence determinants (sequences and functions), corresponding to four major bacterial virulence factors (adherence, motility, secretion system and toxin) associated with *A. veronii*, were searched for in the pathogenic bacteria database, VFanalyzer [40] and validated by blasting assembled genomes to a pseudomolecule generated by concatenating a set of target genes using the NCBI in-house BLASTN tool. The known *A. veronii* B565 (4,551,783 bp, Accession number: NC_015424) was used as the reference genome.

2.9. Global Phylogenomic Relationship and Metadata Analysis

A phylogenomic analysis was undertaken to compare the genomes of the study isolates with all available *A. veronii* genomes downloaded from GenBank and PATRIC via CSI Phylogeny-1.4 [41]. The genome of *Tolumonas auensis* DSM 9187 (GenBank accession number: CP001616) of the Aeromonadaceae family was used to root the tree, facilitating the configuration of the phylogenetic distance between the isolates on the branches. A bootstrapped indicator with 100 replicates was applied to identify recombined regions and provide the phylogenetic accuracy in groups with little homoplasy. Figtree (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize and edit the phylogenetic tree. The phylogeny was visualized alongside annotations for isolate metadata (WGS in silico molecular typing, source, and country) using Phandango [42], to provide a comprehensive analysis of the generated phylogenomic tree.

2.10. Data Availability

The raw read sequences and assembled whole-genome contigs have been deposited in GenBank. The data is available under project number PRJNA564235.

3. Results

3.1. Prevalence and Phenotypic Resistance Profiles of A. Veronii Isolates

Five (5) carbapenem-resistant (CR) *A. veronii* isolates were obtained from 5 different pig samples, giving a carriage rate of 1.5% (5/345) among the sampled pig population. The isolates exhibited similar resistance phenotypes, all of them displaying 100% resistance to ampicillin, amoxicillin and imipenem, except for piperacillin-tazobactam to which only one isolate showed resistance (Table 1).

Table 1. Susceptibility pattern of *A. veronii* isolates.

Antibiotics	Resistance Phenotypes [1]				
	A5	A31	A34	A86	A136
Ampicillin (AMP)	R	R	R	R	R
Amoxicillin (AMX)	R	R	R	R	R
Amoxicillin-clavulanate (AMC)	I	S	S	S	I
Piperacillin-tazobactam (TZP)	S	S	S	S	R
Cefuroxime (CXM)	S	S	S	S	S
Cefotaxime (CTX)	S	S	S	S	S
Ceftriaxone (CRO)	S	S	S	S	S
Ceftazidime (CAZ)	S	S	S	S	S
Cefepime (FEP)	S	S	S	S	S
Cefoxitin (FOX)	S	S	S	S	S
Imipenem (IMI)	R	R	R	R	R
Meropenem (MER)	S	S	S	S	S

[1] S = susceptible; I = intermediate; R = resistant.

3.2. Genome Confirmation, Statistics, Annotation, and Visualization

The global Pathogenwatch platform confirmed the phenotypic identity of the *A. veronii* genomes. The genomic features of the sequences, in terms of size, GC content, number of contigs, N50, L50, number of RNAs and number of protein-coding sequences are listed in Supplementary Materials Table S1. The genome size of the *A. veronii* isolates ranged from 4.64 Mb to 4.77 Mb, with GC content of 58.2–58.5 and a coverage range between 99% to 102%. Comparative visualization analysis, via the GView server (Figure 1), showed a similarity of DNA synteny with >98% coverage and identity from the AVNIH2 reference in all the *A. veronii* genomes.

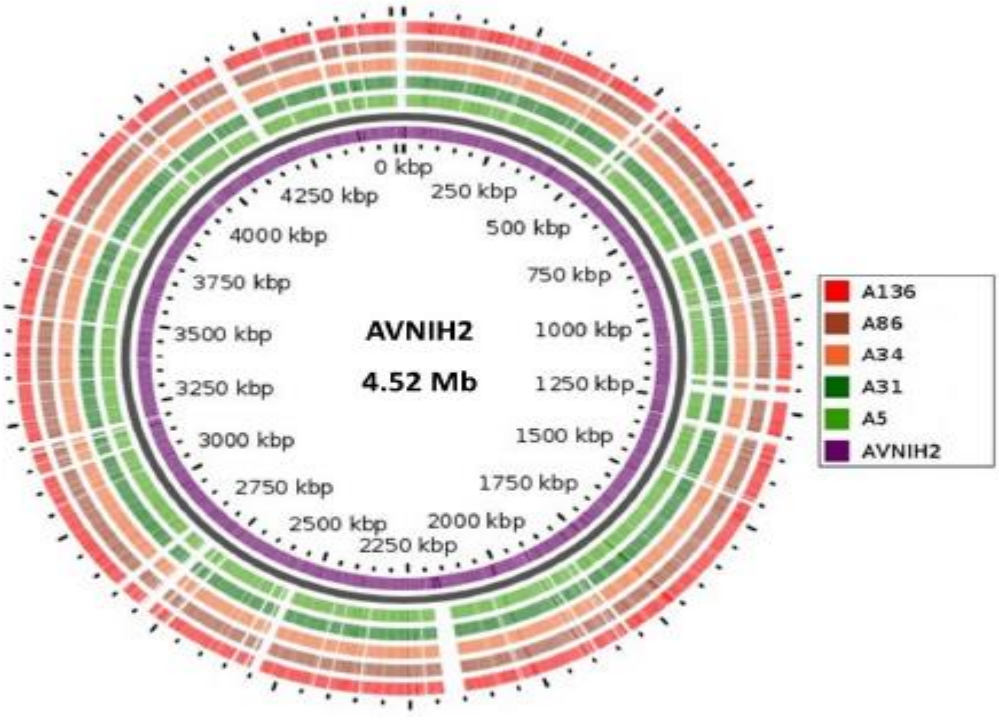

Figure 1. Comparative visualization of the isolates (n = 5) with the *A. veronii* reference strain (AVNIH2, Accession number: LRBO00000000). The map was constructed using the GView online server (https://server.gview.ca/). The concentric circles represent comparisons between AVNIH2 and, starting with the inner circle, genome assemblies from *A. veronii* genomes (isolate ID: A5, A31, A34, A86 and A136). Colour codes are given for each isolate with a synteny identity, ranging from >98%.

Pan-genome and ortholog analysis revealed that all the isolates formed a total of 4349 clusters and shared 4296 orthologous clusters (98.8%) (Figure 2). The singletons ranged from 10 to 42 gene clusters. The isolates shared a similar range of orthologous clusters (n_{range} = 26–30) with each other; however, no unique orthologue gene cluster (n = 0) was found (Figure 3).

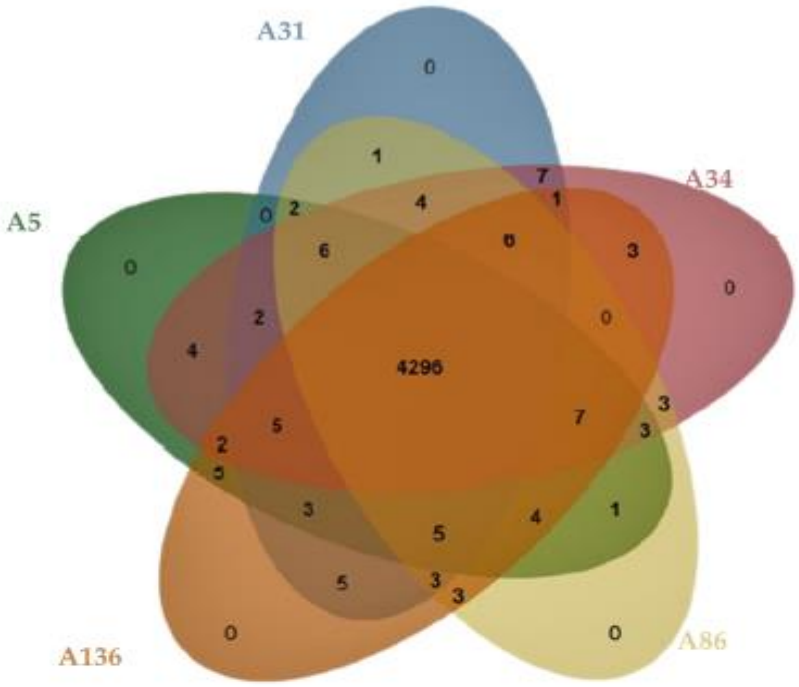

Figure 2. Venn diagram of the unique and shared number of orthologous gene clusters among the genomes of the five *A. veronii* isolates.

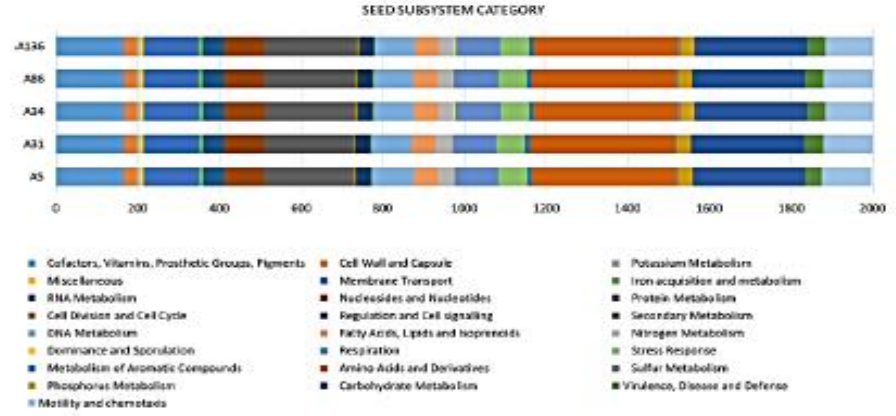

Figure 3. SEED subsystem category for *A. veronii* genomes. Comparison of functional categories in 5 *A. veronii* genomes based on SEED. The functional categorization is based on the roles of annotated and assigned genes. Each coloured bar represents the number of genes assigned to each category.

3.3. Defence Systems (CRISPRCas Cluster and Restriction-Modification (R-M) System), Antibiotic Resistome, Mobilome and Genetic Environment Analysis

The isolates shared a similar resistome (same resistance and efflux genes), with the mobilome comprised of variable insertion sequence families (Table 2 and Figure 4), while plasmids, integrons and intact prophages were absent. However, isolates contained the same incomplete prophage (PHAGE_Escher_PA28 [Accession number: NC_041935]). The bla_{CPHA3} and bla_{OXA-12} genes were located on the chromosome, with >97% homology to *Aeromonas veronii* strain AVNIH1 (Accession no.

CP047155.1) and *Aeromonas veronii* strain 17ISAe (Accession no. CP028133.1) (Table S3). The isolates contained 4–5 clustered, regularly interspaced, short palindromic repeat (CRISPR) arrays with no Cas element (Table 2). None of the strains harboured the restriction-modification (R-M) system.

Table 2. Summary of specimen source, sample type, and genotypic characteristics of the isolates.

Isolate	In Silico Typing	Defence Systems	Resistome	Mobilome [2]	Pathogenicity [3]
ID	MLST [1]	CRISPR (Cas)		No. of Insertion Sequences	Score (Pathogenic Families)
A5	ST657	4 (0)	CfxA-12, cphA3,	45	0.607 (30)
A31	ST657	5 (0)	CfxA-12, cphA3,	68	0.607 (30)
A34	ST657	5 (0)	CfxA-12, cphA3,	73	0.607 (30)
A86	ST657	4 (0)	CfxA-12, cphA3,	43	0.603 (29)
A136	ST657	5 (0)	CfxA-12, cphA3,	45	0.607 (30)

[1] Isolates belonged to the same clone with sequence type (ST657). [2] None of the isolates possessed a restriction-modification (R-M) system, plasmids, integrons and intact prophage. The isolates contained the same incomplete prophage (PHAGE_Escher_PA28 [Accession number: NC_041935]. [3] Pathogenicity score predicted potential pathogenicity in humans.

IS Family	A5	A31	A34	A86	A136
IS1	0	0	1	0	0
IS3	12	6	13	4	12
IS4	1	6	2	0	1
Tn3	3	6	3	4	3
IS256	5	6	0	1	5
IS66	3	3	8	3	3
IS30	2	0	1	0	2
IS5	2	10	9	6	2
IS1634	1	0	0	0	1
IS21	1	9	6	7	1
IS701	1	1	1	0	1
IS110	5	6	1	4	5
ISNCY	2	1	2	0	2
IS200/IS605	5	2	3	2	5
ISAzo13	1	1	1	2	1
IS630	1	0	3	0	1
IS481	0	4	8	3	0
IS1595	0	2	5	4	0
IS1380	0	1	3	0	0
ISKra4	0	1	0	1	0
ISL3	0	3	0	0	0
IS607	0	0	2	0	0
ISAs1	0	0	1	0	0
IS1182	0	0	0	1	0
IS91	0	0	0	0	0
IS6	0	0	0	0	0
IS1182	0	0	0	1	0
TOTAL	45	68	73	43	45

Scale
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Figure 4. Distribution of insertion elements in *A. veronii* genomes. The total number of insertion sequences includes complete, partial, and unknown regions.

3.4. Molecular Typing, Global Epidemiological and Phylogenomic Analysis

In silico determination of the sequence types (STs) of the isolates, using the *Aeromonas* MLST scheme resulted in an undescribed ST comprising new alleles for *gltA*_473, *groL*_445, *gyrB*_465, *metG*_470, *ppsA*_512 and *recA*_519. Allele sequences were submitted for curation and assigned to the

new ST657 (Table 2). BURST (Based Upon Related Sequence Types) analysis identified the novel ST657 as a satellite clone (more distantly) with no single-, double nor triple-locus variants of global curated *A. veronii* STs.

The metadata analyses of the five isolates, together with the 49 global strains, showed the diversity of *A. veronii* sequence types (n = 29), country of origin (n = 13) and sources. The most prevalent sequence types were ST23 (n = 9), ST657 (n = 5, study isolates), ST91 (n = 4), ST166 (n = 3), ST485 (n = 3) and ST50 (n = 2) (Figure 5 and Table S4). The *A. veronii* isolates were from different sources, predominantly animal hosts (n = 38; mostly from fish sources [n = 25], pigs [n = 6] and cow [n = 4]), followed by humans (n = 10) and the environment (n = 6). The USA (n = 17), China (n = 10), Greece (n = 9), South Africa (n = 5) and India were the countries with the highest number of isolates deposited on the databases.

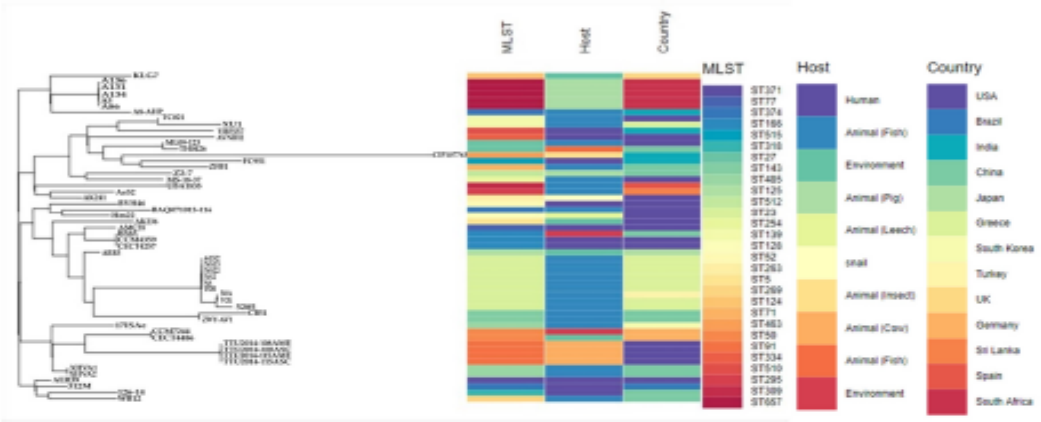
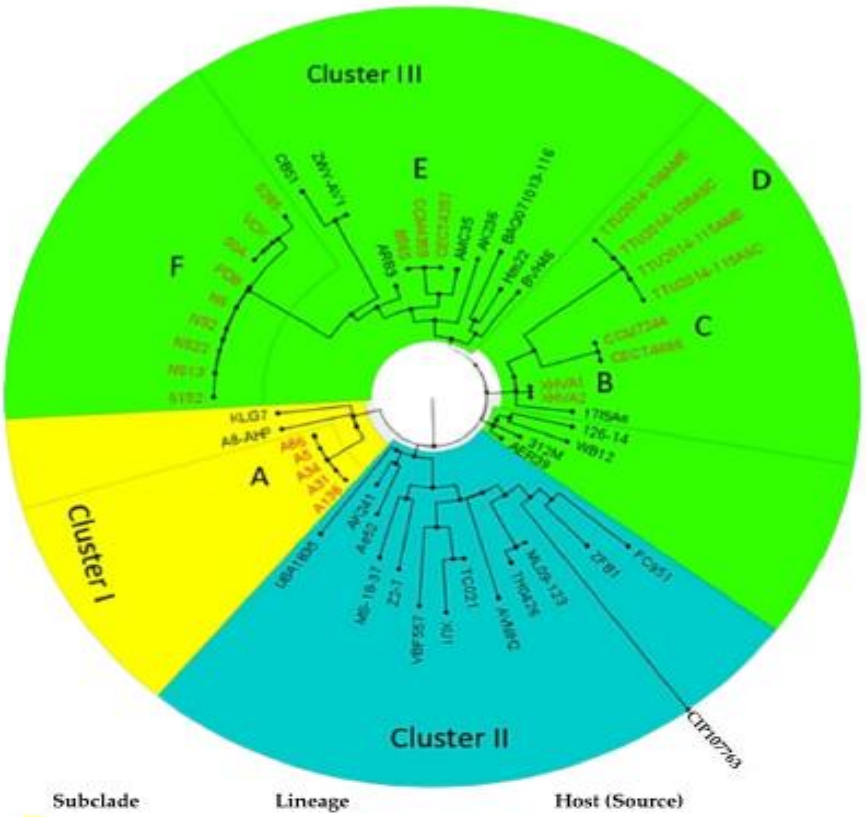


Figure 5. A global phylogenomic branch coupled with metadata of isolate (country; host (source) and WGS in silico sequence typing (STs)) showing the relationship between the study isolates (n = 5) and all deposited *A. veronii* strains (n = 49) from PATRIC database using Phandango. The colour codes differentiate the different countries, hosts, and sequence types.

The phylogenomic analyses via the WGS SNP tree differentiated the global strains into three clusters (I, II and III) (Figures 5 and 6). The study isolates were found in Cluster I with a 100% branch conservation and close lineage to two international stains, KLG7 (UK) and A8-AHP (India) from the environment and fish source, respectively. Cluster II contained strains (n = 14) which were mostly of animal origin (n = 11), except for three strains which were from humans (AVNIH2, VBF557 and FC951). Cluster III was the largest group and contained strains from diverse sources (humans, animals, and environment) (Figures 5 and 6). Six highly conserved genetic subclades (A-E) were identified, which depicted a clustering of isolates mainly according to their sequence types (clonal lineages) and sources (Figure 6). Moreover, the results of the global phylogenetic tree demonstrated the complexity and diversity of *A. veronii* regarding geography, source, and clonality, with many unresolved clusters.



Subclade	Lineage	Host (Source)
A	ST657	Animal (Pig)
B	ST485	Animal (Fish)
C	ST50	Environment (Water)
D	ST91	Animal (Cow)
E	ST166	Human/Environment
F	ST23	Animal (Fish)

Figure 6. A circular cladogram of global *A. veronii* genomes depicting the association between isolates in three clusters (I, II and III). The KwaZulu-Natal (South Africa) isolates belonged to the smallest cluster I and were mainly related to international strains KLG7 (UK) and A8-AHP (India). Sub-clades (A-E) depicted a clustering of isolates, mainly according to sequence types/sources.

3.5. Pathogenic Potential and Putative Virulence Factors

The mean pathogenicity score of 0.60 indicated the potential pathogenicity of *A. veronii* in humans and was found to match 30 pathogenic families. The whole virulome analysis predicted a total of 200 putative virulence-encoding genes belonging to six major virulence factor classes of *Aeromonas*, namely, adherence factor (lateral flagella, mannose-sensitive hemagglutinin pilus, polar flagella, tap type IV pili and type I fimbriae), secretion system (T2SS and T3SS), toxins (aerolysin/cytotoxic enterotoxin and hemolysin), anaerobic respiration (nitrate reductase), antiphagoctyosis (capsular polysaccharide) and immune evasion (capsule and LOS) with minor differences (Figure 7 and Table S5). A total of 195 conserved virulence factors were observed across the isolates.

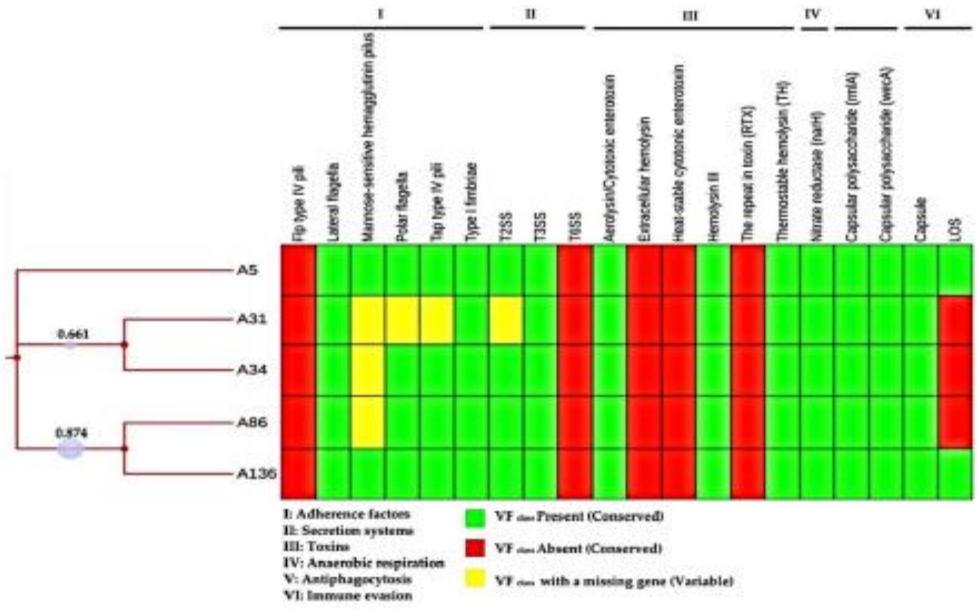


Figure 7. Heatmap generated with phylogenetic and distribution of virulence factors across the *A. veronii* isolates ($n = 5$). The colour coding depicts the presence (green), absence (red) and variability (yellow) of the virulence gene sets. The virulence factors are represented by Roman numerals I: adherence factors; II: secretion systems III toxins; IV; anaerobic respiration; V anti-phagocytosis and VI: immune evasion.

4. Discussion

Aeromonas spp. are important human pathogens and colonizers and have also been increasingly isolated in animals (food-animals, wildlife, and companion animals), and the environment (soil, water, and air) globally. They, thus, have potential as One Health indicator bacteria for monitoring the spread of antibiotic-resistant bacteria between humans, animals and the environment [4,43–47]. However, information on this pathogen in food animals, using high-throughput technologies such as whole genome sequencing in Africa, is lacking. In this study, we describe for the first time in Africa, the comparative genomics of five *A. veronii* isolates recovered from pigs in South Africa. We also show the phylogenomic relationship between this novel strain and all globally deposited *A. veronii* genomes with complete metadata (country, sources and sequence types), as their incidence and geographic spread is vital to understand the evolution of this emerging pathogen which is on a global rise.

Analyses of the genomic data revealed a high degree of genomic synteny (>98%), suggesting a close association between the study isolates (Figure 1). All the isolates shared orthologous clusters (98.8%) with no unique gene cluster (Figure 2), indicating a relatively large set of core functions with low variable sections as well as a vast array of similar central networks (Figure 3) which are crucial for their survival in the microbial community [48]. The high degree of similarity between the isolates predicted by the different analyses also corroborated the novel clonal lineage (ST657), where the isolates possessed the same genetic make-up with low variation. For instance, mobilome analysis of the ST657 highlighted the lack of plasmids, integrons and intact prophages in this lineage. However, variability in chromosome-borne insertion sequence (IS) families was observed (Table 2 and Figure 4). More so, the isolates contained the same incomplete prophage (Escher_PA28) which did not harbor any resistance and virulence genes. A similar scattered IS pattern was previous reported in *A. veronii* by Tekedar et al. [20]. Further analyses of this clonal lineage (ST657) depicted a unique satellite-variant, implying that it was distantly related to global sequence types, hence does not have any ancestral linkage with the STs found in the *Aeromonas* MLST database.

Bacteria often accrue defence systems which offer protection against foreign DNA invasion and viral predation [49,50]. Regarding the CRISPR-Cas defence systems consisting of two main components, CRISPR array and associated genes (Cas), the *A. veronii* genomes encoded the CRISPR elements with no Cas system (Table 2). This shows that the genomes of the isolates contained short repeat clusters which have been implicated in bacterial adaptation strategies, ranging from immune evasion and tissue tropism to the modulation of environmental stress tolerance. This finding was similar to a study by Tekedar et al. [20], which confirmed the presence of CRISPR elements in all compared *A. veronii* isolates ($n = 41$), with only a few ($n = 4$) harbouring the Cas elements. This implies that the complete CRISPR-Cas systems are less prevalent in *A. veronii*, probably because of the lack of nucleotide biosynthesis capacity [51]. Moreover, the Cas systems were predominantly found in human isolates, suggesting the need for further studies to understand the CRISPR-Cas-mediated host interactions.

A. veronii has been reported in different food animals and products including pigs, chicken, cattle, sheep, buffalo and fish [10,46,52]. The consumption of undercooked/raw meat or meat products is an important route for human infection with *Aeromonas* spp. [4,10]. The carriage rate of carbapenem-resistant *A. veronii* isolates was 1.5%, which was comparable to the prevalence rate in food-producing animals in Europe (<1%) and in the lower range of the resistance reported in both Africa (2–26%) and Asia (1–15%) [53]. Although the overall prevalence of carbapenem-resistant *A. veronii* in food animals appears to be low, the transmission of these pathogens from food animals to their derived products could be a threat to consumers, supporting the transmission of resistant bacteria and their determinants between commensal and pathogenic microorganisms with unknown, but potentially severe, consequences for human health [53,54].

The resistance phenotypes corroborated the presence of bla_{CPHA3} and bla_{OXA-12} conferring resistance to imipenem and penicillin (ampicillin and amoxicillin) (Tables 1 and 2). The Aeromonads, including *veronii* and *A. hydrophilia*, have been reported to harbour conserved resistance genes on their chromosome, conferring intrinsic resistance against these antibiotics [20,55,56]. More so, they often harbour genes that code for the production of β-lactamases such as class B metallo-β-lactamase, class C cephalosporinase, and class D penicillinases [3,5,41,42]. However, *Aeromonas* spp. are reported to be susceptible to monobactams, third-and fourth-generation cephalosporins, aminoglycosides, and fluoroquinolones, as found in this study [3].

The pathogenic potential (P_{score}), with the probability ranging from 0 to 1, is used to predict the ability of bacteria to cause infection in humans [39,48]. This theoretical estimation of the pathogenic potential, using trained algorithms to differentiate between pathogenic or commensal strains, predicted a relatively higher average probability ($P_{score} \approx 0.60$), suggesting that the clone (ST657) (Table 2) could be potentially pathogenic to humans. However, it originated from a non-human source, highlighting the role of *Aeromonas* spp. from food animal sources as potential human pathogens [4]. The global emergence of *Aeromonas* spp. in all One Health settings (human-animal-environment), makes it a potential One Health indicator organism.

Several insertion sequences were found in our isolates (Table 2 and Figure 4). Insertion elements are significant in the evolution of *Aeromonas* genome [57], contributing to its resistome and virulome through the incorporation of additional genes, genome reduction and rearrangement, gene decay and inactivation, and expansion of flanking regions [58,59]. The virulome analysis revealed the possession of a battery of determinants which play a significant role in their survival and pathogenesis, comparable to previously reported studies on *Aeromonas* spp. [5,20,55,60] and supporting the pathogenic potential of this pathogen. The ST657 clone contained an array of six putative virulence factor classes, which were mostly conserved across the isolates, suggesting that *A. veronii* relies heavily on these factors for host invasion, immune evasion, tissue damage, and competition in diverse ecological niches (Figure 7 and Table S5). Adherence factors were the most prevalent putative virulence determinants followed by secretion systems and toxins (Figure 7 and Table S5) in contrast to virulence factors possessed by *A. veronii* isolated from fish samples, where the secretion system and its components were found to be the most predominant [20]. This observation could imply that predominating virulence factors may be

host-specific. Interestingly, differences in the virulome of the ST657 lineage were evident. Some of the isolates lacked specific genes within the sub-components of adherence factors, secretion systems and immune evasion virulence factors. For example, the T2SS sub-component of the secretion system, which is known for exporting hydrolytic enzymes and aids in the gut colonization [61,62], lacked the *exe*H gene in isolate A31. The expression of this putative virulome probably confers a competitive advantage, contributing to its success as a pathogen [48]. Moreover, the genomic detection of these virulence genes could aid in identifying targets for the development of novel vaccines for this emerging pathogen [63,64].

Global epidemiological comparison of deposited *A. veronii* genomes revealed the diverse nature of this pathogen regarding its host, clonal lineages, and geographical distribution (Figure 5 and Table S4). This diversity implies that the *A. veronii* can serve as good One Health indicator pathogen to understand and track the geographical spread of antibiotic resistance. Phylogenomic analysis depicted the clustering of group members from disparate geographies. Interestingly, the study isolates were closely related to strains from the UK and India, from different lineages (Figure 7), but not close enough to suggest import into South Africa from other countries. The small number of global deposited strains with insufficient metadata made it challenging to make much inference from the tree analysis on the transmission dynamics of this species, as there were many unresolved clusters. It is thus recommended that more studies be carried out in all the sectors (human, animal and environment) to harness the ability of genomics and bioinformatic analysis in making useful predictions about the dynamics of emerging pathogens in the One Health context.

5. Conclusions

The comparative genomics of *A. veronii* revealed the clonal dominance of the novel strain, ST657, isolated from South Africa. The genomic data presented lends useful insights into the pan-genome, resistome, defense system, virulome, pathogenic potential, clonal lineages, global dissemination, and phylogenetic relationship of this pathogen. To the best of our knowledge, this is the first comprehensive genomic analysis of *A. veronii* isolates in Africa and presents this species as a potential One Health indicator.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/12/2008/s1, Table S1: Genomic features of the *A. veronii* isolates, Table S2: Distribution of selected functional categories in the *A. veronii* isolates, Table S3: Genetic environment of resistance genes found in the isolates, Table S4: Table showing the *A. veronii* isolates with full metadata (sequence type, host (source) and country of origin) retrieved from the PATRIC database, Table S5: Table of the virulence factor distribution in the 5 *A. veronii* genomes.

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Table S1. Genomic features of the *A. veronii* strains.

No.	Strain names	Size (Mb)	GC%	No. of contigs	N50(bp)	L50	No. of RNAs	Protein-coding sequences	Coverage%	Accession Num
1	A5	4.77	58.2	33	230041	7	92	4511	99	VWTX0000000
2	A31	4.64	58.5	84	114767	14	82	4380	102	VWTW0000000
3	A34	4.64	58.5	86	139859	13	86	4396	102	VWTV0000000
4	A86	4.64	58.5	42	206004	8	95	4359	102	VWTT0000000
5	A136	4.67	58.4	41	213730	7	94	4396	102	VWTS0000000

Table S2. Distribution of selected functional categories in the *A. veronii* strains.

No.	Functional category of in silico predicted proteins	*A. veronii* strains				
		A5	A31	A34	A86	A136
1	Cofactors, Vitamins, Prosthetic Groups, Pigments	167	167	168	167	167
2	Cell Wall and Capsule	28	28	28	28	28
3	Potassium Metabolism	9	9	9	9	9
4	Miscellaneous	9	9	9	9	9
5	Membrane Transport	137	137	136	136	136
6	Iron acquisition and metabolism	11	11	11	11	11
7	RNA Metabolism	51	51	51	51	51
8	Nucleosides and Nucleotides	97	97	98	97	98
9	Protein Metabolism	220	219	222	224	227
10	Cell Division and Cell Cycle	6	6	6	6	6
11	Regulation and Cell signalling	34	34	34	34	34
12	Secondary Metabolism	4	4	4	4	4
13	DNA Metabolism	102	98	99	98	98
14	Fatty Acids, Lipids and Isoprenoids	59	59	60	59	59
15	Nitrogen Metabolism	39	39	40	39	39
16	Dominance and Sporulation	3	3	3	3	3
17	Respiration	108	108	110	108	108
18	Stress Response	70	71	70	70	71
19	Metabolism of Aromatic Compounds	10	10	10	10	10
20	Amino Acids and Derivatives	353	353	353	353	353
21	Sulfur Metabolism	7	7	7	7	7
22	Phosphorus Metabolism	35	35	35	35	35
23	Carbohydrate Metabolism	276	277	276	276	276
24	Virulence, Disease and Defense	40	46	45	44	45
25	Motility and chemotaxis	122	122	122	122	122

Table S3. Genetic environment of resistance genes found in the isolates.

Strain (MLST)	Contig	Resistance gene/s (position on contig)	Mobile genetic elements	Plasmid/Chromosomal sequence with the closet nu homology (accession number)
A5 (ST657)	14	bla$_{CPHA3}$ (12605..13369)	None	*Aeromonas veronii* strain AVNIH1 chromosome (CP0
	17	bla$_{OXA-12}$ (11009..11781)	None	*Aeromonas veronii* strain 17ISAe chromosome (CP02
A31 (ST657)	9	bla$_{CPHA3}$ (12605..13369)	None	*Aeromonas veronii* strain AVNIH1 chromosome (CP0
	29	bla$_{OXA-12}$ (11009..11781)	None	*Aeromonas veronii* strain 17ISAe chromosome (CP02
A34 (ST657)	9	bla$_{CPHA3}$ (12605..13369)	None	*Aeromonas veronii* strain AVNIH1 chromosome (CP0
	21	bla$_{OXA-12}$ (11009..11781)	None	*Aeromonas veronii* strain 17ISAe chromosome (CP02
A86 (ST657)	5	bla$_{CPHA3}$ (274887..275651)	None	*Aeromonas veronii* strain AVNIH1 chromosome (CP0
	18	bla$_{OXA-12}$ (11009..11781)	None	*Aeromonas veronii* strain 17ISAe chromosome (CP02
A136 (ST657)	15	bla$_{CPHA3}$ (12605..13369)	None	*Aeromonas veronii* strain AVNIH1 chromosome (CP0
	16	bla$_{OXA-12}$ (11009..11781)	None	*Aeromonas veronii* strain 17ISAe chromosome (CP02

Table S4. Table showing the *A. veronii* isolates with full metadata (sequence type, host (source) and country of origin) retrieved from the PATRIC database.

Strain	Country	Host	MLST
AER39	USA	Human	ST371
AMC35	USA	Human	ST77
312M	Brazil	Human	ST374
CECT4257	USA	Human	ST166
CCM4359	USA	Human	ST166
FC951	India	Human	ST515
126-14	China	Human	ST318
ML09-123	USA	Animal (Fish)	ST27
ZWY-AV1	China	Animal (Fish)	ST143
XHVA1	China	Animal (Fish)	ST485
ARB3	Japan	Environment (Pond)	ST125
Z2-7	China	Animal (Pig)	ST512
XHVA2	China	Animal (Fish)	ST485
VCK	Greece	Animal (Fish)	ST23
PDB	Greece	Animal (Fish)	ST23
5285	Greece	Animal (Fish)	ST23
NS	Greece	Animal (Fish)	ST23
17ISAe	South Korea	Animal (Fish)	ST485
MS-18-37	USA	Animal (Fish)	ST254
NS2	Greece	Animal (Fish)	ST23
50A	Turkey	Animal (Fish)	ST23
6152	Greece	Animal (Fish)	ST23
XU1	Greece	Animal (Fish)	ST139
TC021	USA	Animal (Fish)	ST139
Hm22	USA	Animal (Leech)	ST128
BVH46	USA	Human	ST52
AK241	USA	Animal (snail)	ST263
BAQ071013-116	USA	Animal (Fish)	ST374
AK236	USA	Environment (Lake)	ST5
WB12	China	Animal (Fish)	ST269
NS22	Greece	Animal (Fish)	ST23
NS13	Greece	Animal (Fish)	ST23
KLG7	UK	Environment (River)	ST124
A8-AHP	India	Animal (Fish)	ST374
ZFB1	China	Animal (Fish)	ST71
CIP107763	India	Animal (Insect)	ST463
CECT4486	Germany	Environment	ST50
TTU2014-108AME	USA	Animal (Cow)	ST91
TTU2014-108ASC	USA	Animal (Cow)	ST91
TTU2014-115AME	USA	Animal (Cow)	ST91
TTU2014-115ASC	USA	Animal (Cow)	ST91
TH0426	China	Animal (Fish)	ST27
CB51	China	Animal (Fish)	ST143
AVNIH2	USA	Human	ST334
VBF557	India	Human	ST510
Ae52	Sri Lanka	Animal (Fish)	ST295
CCM7244	Germany	Environment (Water)	ST50
UBA1835	Spain	Animal (Fish)	ST309
B565	China	Environment (Pond)	ST166
A5 *	South Africa	Animal (Pig)	ST657
A31 *	South Africa	Animal (Pig)	ST657
A34 *	South Africa	Animal (Pig)	ST657
A86 *	South Africa	Animal (Pig)	ST657
A136 *	South Africa	Animal (Pig)	ST657

NB- *-→ Study isolates.

Table S5a. Virulence factors associated with adherence and secretory function**.**

VFclass	Virulence factors	Related genes
Adherence	Flp type IV pili	*flp1, flpA, flpB, flpC, flpD, flpE, flpF, flpG, flpH, flpI, flpJ, flp, flpL, flgC, flgE, flgI, flgJ, fliF, fliG, flip, lafB, lafC, lafE, lafF, lafK, lafS, lafT, lafU, lafX, lfgA, lfgB, lfgF, lfgG, lfgH, lfgK, lfgL, lfgM, lfgN, lfhA, lfhB, life, fiH, lfiI, lfiJ, lfiM, lfiN, lfiQ, lfiR, maf-5*
	Lateral flagella	
	Mannose-sensitive hemagglutinin (Msh) pilus	*mshA, mshB, mshC, mshD, mshE, mshF, mshG, mshI1, mshI, mshJ, mshK, mshL, mshM, mshN, mshO, mshP, mshQ, cheA-2, cheB-2, cheR-3, cheV, cheW, cheY, cheZ, flaA, flab, flag, flaH, flaJ, flgA, flgB, flgC, flgD, flgE, flgF, flgG, flgH, flgI, flgJ, flgK, flgL, flgM, flgN, flhA, flhB, flhF, flhG, fliA, fliE, fliF, fliG, fliH, fliI, fliJ, fliK, fliL, fliM, fliN, fliO, flip, fliQ, fliR, flmD, flmH, flrA, flrB, flrC, maf-1, maf-2, motX, motY, nueA, nueB, pomA2, pomA, pomB2, pomB, tapA, tapB, tapC, tapD, tapF, tapM, tapN, tapO, tapP, tapQ, tapT, tapU, tapV, tapW, tapY1, tapY2, tppA, tppB, tppC, tppD, tppE, tppF, fimA, fimC, fimD, fimE, fimF, exeA, exeB, exeC, exeD, exeE, exeF, exeG, exeH, exeI, exeJ, exeK, exeL, exeM, exeN, tapD, acr1, acr2, acrG, acrH, acrR, acrV, aexT, aopB, aopD, aopH, aopN, aopO, aopX, ascB, ascC, ascD, ascE, ascF, ascG, ascH, asci, ascJ, ascK, ascL, ascN, ascO, ascP, ascQ, ascR, ascS, ascT, ascU, ascV, ascX, ascY, ati1, ati2, exsA, exsB, exsC, exsD, exsE, sycH, sycO, sycX, atsA,, atsB, atsC, atsD, atsG, atsH, atsI, atsJ, atsK, atsL, atsP, atsQ, atsS, clpV1, dotU, hcp1, hcp, vasH, vasK/atsR, vgrG1, vgrG2, vgrG3, vipA, vipB*
	Polar flagella	
	Tap type IV pili	
	Type I fimbriae	
Secretion system	T2SS	
	T3SS	
	T6SS	

Table S5b. Virulence factors associated with toxin production, antiphagocytosis, immune evasion, and others.

Toxin	Aerolysin AerA/Cytotoxic enterotoxin Act	*aerA/act*
	Extracellular hemolysin	*ahh1*
	Heat-stable cytotonic enterotoxin	*ast*
	Hemolysin III	Undetermined
	Hemolysin	*hlyA*
	The repeat in toxin (RTX)	*rtxA, rtxB, rtxC, rtxD, rtxE, rtxH*
	Thermostable hemolysin (TH)	Undetermined
Anaerobic respiration	Nitrate reductase (Mycobacterium)	*narH*
Antiphagocytosis	Capsular polysaccharide (Vibrio)	*rmlA, wecA*
Immune evasion	Capsule (Acinetobacter)	
	LOS(Campylobacter)	
Others	O-antigen (Yersinia)	*cpsB, wcaG*

CHAPTER 5 – CONCLUSION

This study describes the molecular epidemiology and genomic profiles of Carbapenemase-producing Enterobacterales isolated from humans, food animals and the environment, in KwaZulu-Natal, South Africa based on a point prevalence study amongst hospitalized patients, pigs slaughtered at an abattoir and water (influent and effluent from a waste-water treatment plant and upstream and downstream surface water samples).

5.1. Conclusions and key findings

The aim of this study was to highlight the importance of an integrated and holistic multi-sectoral One Health approach in combating antibiotic resistance specifically CRE which is classified as a critical pathogen according to the severity of threat on the WHO Global Priority Pathogens List. The study informs the need for surveillance of AMR involving the human, animal, and the environmental sector in a One Health context to combat AMR and its dissemination. This point prevalence study conducted in KwaZulu-Natal, South Africa brings to light the potential threat of dissemination of antibiotic resistance genes and relevant MGEs between the human and environmental sectors. The isolation of common carbapenemase-encoding genes located on the same MGEs from environmental and human isolates provides evidence of potential movement of antibiotic resistance determinants between bacterial species as well as between humans and and the environment. This exchange of antibiotic resistance determinants between clinical and environmental isolates represents a serious threat to public health. With potential movement of pathogens and associated resistance genes between sectors, there is a need for a cross-sector One Health approach to monitor the spread of AMR that occurs at the environment-animal-human interface.

Rectal swabs obtained from humans, food producing animals (pigs) and the environment were screened for CPE using ChromID CARBA chromogenic agar medium (bioMérieux, Marcy l'Étoile, France). Environmental samples consisting of influent, effluent, upstream and downstream surface water from a wastewater processing plant were similarly screened for CRE harboring carbapenemases. Screening of samples was followed by phenotypic identification and antimicrobial susceptibility testing using the VITEK 2 platform (bioMérieux, Marcy l'Étoile, France). All CPE isolates were subjected to whole genome sequencing (WGS) and bioinformatics analysis in a One Health context. The key findings are listed below:

- Of 587 rectal swab samples screened for CRE, 230 (39.1%) were from humans, and 345 (58.7%) were from pigs. Twelve (2%) water samples obtained from a WWTP were also included.

- Overall, total of 19/587 (3.2%) isolates i.e., 15 from humans and 4 from the environment were CRE. All the environmental isolates (4) and 12/15 human isolates were carbapenemase producers. The three non-carbapenemase-producing isolates human isolates were resistant to ertapenem but susceptible to meropenem and imipenem.
- There were five isolates of *Aeromonas* spp. obtained from pigs during the screening process. These isolates were part of a novel sequence type – ST657 and harboured *bla*CPHA3 and *blaOXA-12* conferring resistance to imipenem and penicillin. No MGEs identified in these isolates.
- The most prevalent CPE identified was *Klebsiella pneumoniae* 9/16 (56%), followed by *Enterobacter hormaechei* 3/16 (19%), *Klebsiella quasi-pneumoniae* 2/16 (13%), *Citrobacter freundii* 1/16 (6%) and *Serratia marcescens* 1/16 (6%).
- All isolates were resistant to ertapenem, 10 isolates were resistant to meropenem, and nine isolates were resistant to imipenem.
- Eight isolates (*K. pneumoniae* (3), *E. hormaechei* (1), *K. quasi-pneumoniae* (2), *C. freundii* (1) and *S. marcescens* (1)) were resistant to all three carbapenems tested.
- Six and three isolates showed intermediate susceptibility to imipenem and meropenem, respectively.
- All isolates were resistant to amoxicillin-clavulanate, cefuroxime, ceftazadime, piperacillin-tazobactam and trimethoprim/sulfamethoxazole with 84% isolates resistant to cefepime and cefoxitin, gentamycin and ciprofloxacin and tigecycline resistance was observed in 36% of the isolates. Eleven isolates were extensively drug resistant (XDR) and eight were MDR.

Whole genome sequencing was performed on the 19 isolates to delineate clonality, the resistome, virulome and mobilome, including MGEs associated with resistance and virulence. The results in summary are as follows:

- Isolates belonged to different MLSTs:
    - *K. pneumoniae* (9) – (ST17 (5), ST15 (2), ST152 (1), ST607 (1)).
    - *E. hormaechei* (6) - (ST90 (3), ST88 (1), ST78 (1), ST231 (1)).
    - *K. quasipneumoniae* (2) – (ST1889 (1), ST3318 (1)).
    - *S. marcesens* (1) – (ST90 (1)).
    - *C. freundii* (1) – which was a novel isolate belonging to ST498.

- A diverse range of genes conferring resistance to β-lactams, aminoglycosides, fluroquinolones, trimethoprim, sulphonamides, rifampicin and phenicols were identified across human and animal isolates.

- Genes encoding for β-lactam resistance included those encoding for carbapenem resistance: *bla*$_{\text{OXA-181}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-484}}$ and *bla*$_{\text{GES-5}}$, those encoding for ESBLs: *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{SHV-94}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{OKP-B-3}}$, and those encoding for AmpC β-lactamases: *bla*$_{\text{ACT-15}}$, *bla*$_{\text{ACT-7}}$, *bla*$_{\text{CMY-48}}$ and *bla*$_{\text{SRT-1}}$
- Genes encoding for fluoroquinolones resistance included *aac(6')-Ib-cr, qnrB1, oqxB, oqxA, qnrS1*
- Genes encoding for aminoglycoside resistance included *aac(6')Ib-cr, aac(3)-lId, aadA1, aac(6')Ib-cr, aph(3')-Ia, aph(3'')-Ib, aac(3)-Iia, aph(6)-Id, and rmtC, aadA16*
- Genes encoding for fosfomycin resistance: *fosA*
- Genes encoding for phenicol resistance: *catB3, cmlA1, catA2, cmlA1, floR*
- Genes encoding for rifampicin resistance: *ARR-2, ARR-3*
- Genes encoding for sulphonamide resistance: *sul1* and *sul2*
- Genes encoding for macrolide resistance: *mph(A), ere(A),*
- Genes encoding for trimethoprim resistance: *dfrA23, dfrA7, dfrA14, dfrA15, dfrA19, dfrA12, dfrA27*
- Genes encoding for tetracycline resistance*: tet(A), tet(B)*

- Aspects of the mobilome and genetic environment of the ARGs (plasmids, insertion sequences and integrons) common to human and environmental isolates are listed below:
  - The most frequently isolated plasmid replicon, Col440I was identified in 11 (26.82%) isolates across the human and the environmental sectors. These plasmids were also found in different species of bacteria.
  - The ColKP3 (24.38%) and Col440II (19.51%) plasmid replicons were detected in similar isolates as Col440I.
  - The Col440II was found in all isolates of *E. hormaechei* in humans and the environment, and all *K. quasipneumoniae* from the environment.
  - Similarly, the Col440I plasmid replicon was found in all six isolates of *bla*$_{\text{OXA-181}}$-producing *E. hormaechei*, two isolates of *K. quasipneumoniae*, three *bla*$_{\text{OXA-181}}$-producing *K. pneumoniae*, two belonging to ST17 and common to human and environmental isolates.
  - The IncFIB(K) plasmid replicon was found in all isolates of *K. quasipneumoniae* (2) from the environment and the majority of the *K. pneumoniae* strains (7/9) from humans with majority of the *K. pneumoniae* isolates being OXA-181 (5/9) producers.
  - The IncHI2, IncHI2A, IncN plasmid replicons were identified in selected *E. hormaechei* and *K. quasipneumoniae* strains across human and environmental sectors.
  - The *bla*$_{\text{OXA-181}}$ gene was found alongside the *ereA* gene in all OXA-181 producing isolates from humans and the environment.

- ISKpn19 was associated with human and environmental isolates.
- The *bla*$_{NDM-1}$ bearing isolates from humans and the environment were associated with ble and IS91 in addition to IS30 and IS3 were present in isolates across both sectors.
- Other β-lactamase genes such as *bla*$_{TEM-1b}$ and *bla*$_{CTX-M-15}$ gene were associated with recombinase/integrase, IS91 and *Tn*3 respectively.

- Phylogenetic analysis revealed the following:
  - The *C. freundii* and *S. marcescens* isolated from this study were phylogenetically closely related when compared to aligned to other human isolates from South Africa. The *S. marcescens* isolate in particular, was part of a similar clone circulating in South Africa.
  - The *E. hormaechei* isolates showed diverse phylogenetic relationships with clustering and environmental isolates like other environmental isolates submitted from South Africa.
  - Isolates of *K. pneumoniae* clustered together with other clinical isolates from South Africa of the same sequence type.
  -

5.2. Limitations and Recommendations

5.2.1 Limitation – Point prevalence study

- This was a point prevalence study and is therefore not a true reflection of the burden of CRE and CPE in the human, animal, and environmental sector in South Africa.

Recommendation

- The establishment of enhanced surveillance systems for resistant infections in humans, animals and the environement from multiple sites would result in a better One Health approach in understanding and addressing the prevalence and spread of AMR. Monitoring of AMR should be an ongoing regionally and nationally, with data reporting uniformity.
- A comprehensive One Health surveillance system will enable a comparison of AMR burden at local and international level translating to information that is evidence based to guide policy related to monitoring of AMR.
- Ongoing surveillance of MDROs such as CRE will assist in the establishment of databases, which will serve as monitoring tools locally and internationally as recommended by the WHO (World Health Organisation) amongst others.

5.2.2 Limitation – Screening of CPR/CRE islolates.

- Screening methodology using ChromID (bioMérieux, Marcy l'Étoile, France) chromogenic agar was used to screen for CPE. The screening chromogenic agar isolated *Aeromonas spp*. from the animal samples. *Aeromonas spp*. is not classified as part of the Enterobacterales family. There were carbapenem resistant isolates from the Enterobacterales family that were isolated on the screening agar but were not carbapenemase producers. Resistance to carbapenems in this instance may have been due to other mechanisms such as the production of ESBLs or AmpC β-lactamases coupled with porin loss and efflux pumps.

Recommendation

Whilst screening for CPE using chromogenic agar is the most cost effective and efficient method to isolate these organisms for monitoring of AMR, consideration should be given to the use of other innovative technologies such as WGS. The ideal would be the establishment of local and national AMR surveillance initiatives that support the use of WGS in conducting molecular surveillance to provide high quality data identifying the early emergence and spread of resistant bacteria and resistant determining genes.

5.2.3 Limitation – Wastewater samples

- Small number of samples from the wastewater treatment plants representative of environmental sampling .
- The sample was not processed by filtration to concentrate the bacterial isolates, and this may thus have been an under-estimation of CPE in the environmental component of this study.

Recommendation

- Future studies looking at AMR, ARGs and MGEs should include a larger number of water samples from various environmental sites, effluent and sewage samples from hospitals and abbatoirs. The additional sampling will be a more representative of this component of the One Health triad resulting in more robust data. Filtering of water samples would increase the yield of organisms.

5.2.4 Limitation – Human sampling to include ICU patients and the paediatric population.

- Sampling in this study was confined to hospital patients who were able to consent to a rectal swab. Most patients admitted to ICU and the paediatric population were not sampled for lack of ability to provide consent, despite increased risk of CPE colonization, carriage and infection in ICU patients. There could thus also be an underestimation of the CPE carriage data in this study as the ICU population was not sampled.

Recommendation

Future CPE screening studies should include ICU patients. This will provide a more accurate reflection of the patients carrying CPE within a hospital setting.

5.3. Significance of research

AMR is a global problem, and successfully addressing it requires a multi-disciplinary approach incorporating many stakeholders across various sectors. The public health challenge posed by AMR is aggravated by increasing reports on resistance to carbapenems, antibiotics of last resort. The loss of carbapenems' efficacy leaves very few therapeutic options to treat infectious diseases, compromising the of care millions of people the world over. Historically, the problem of AMR has always been linked to human health with consideration seldom given to animal and environmental health. The existing dynamic relationship between human health, animal health, and environmental health must be considered when addressing AMR. The One Health approach to AMR, therefore, requires a collaborative effort from the human health sector, animal health sector and the environmental sector to combat AMR. Confining the problem of AMR, and in this case CPE, exclusively to human health would be short-sighted. Thus, this study investigated AMR, specifically CRE, across One Health domains identifying potential routes of transmission between humans, animals, and the environment.

Pivotal to addressing AMR is understanding its nature and spread using innovative diagnostic technology like WGS paired with bioinformatics. Amongst the results were two novel findings, a novel extensively-drug-resistant *C. freundii*, ST 498 isolate carring the $bla_{\text{NDM-1}}$ gene from a human sample and the discovery of a novel ST 657 clone of *Aeromonas veronii* from pigs in South Africa. Thus, following sampling and analysis of samples from the humans, animals and the environment, this study identified common carbapenem resistance genes in different isolates from humans and the environment. These genes, along with other genes conferring resistance to different antibiotic classes, were closely associated with various MGEs common to human and environmental isolates. The mobile nature of these genetic elements and their location in different species of bacteria from humans and the environment suggests the possible movement of resistance determining elements between sectors. This One Health study's finding sheds light on the possibility of potential circulation of ARGs between humans, animals, and the environment. The environment could serve as a potentially rich source of antimicrobial resistance determinants for humans and animals.

The use of advanced diagnostic tools in AMR surveillance and spread in a One Health context as conducted in this study, provides a strong base for recommending such approaches in large-scale studies. If implemented locally and nationally, such surveillance would provide a platform for developing evidence-based strategies to mitigate the spread of AMR. The cost would undoubtedly play a role in the implementation of molecular diagnostic surveillance. However, AMR's long-term economic burden on

a low- and middle-income countries is far-reaching. The concurrent One Health sampling study design presented here provides an overarching screening tool to base further efforts to elucidate transmission routes, characterize ARG and ARB reservoirs, and inform policy for promoting good stewardship and combating the spread of resistance among and between animals, humans, and the environment.

APPENDICES

Appendix I – Ethical approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

16 February 2017

Dr Y Ramsamy (209541603)
Discipline of Medical Microbiology
School of Laboratory Medicine and Medical Sciences
yogandree@gmail.com

Dear Dr Ramsamy

Protocol: Triangulating the molecular epidemiology of carbapenem resistant enterobacteriaceae from humans, food animals and environment.
Degree: PhD
BREC reference number: BE599/16 (sub-study of BCA444/16)

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 07 November 2016.

The conditions have now been met and the study is given **full ethics approval** and may begin as from 16 February 2017.

This approval is valid for one year from **16 February 2017.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **14 March 2017.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor V Rambiritch
Deputy Chair: Biomedical Research Ethics Committee

cc supervisor: essacks@ukzn.ac.za
cc postgraduate administrator: dudhrajhp@ukzn.ac.za

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
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UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

17 March 2017

Prof SY Essack
Department of Pharmaceutical Sciences
School of Health Sciences
essacks@ukzn.ac.za

Dear Prof Essack

Title: One Health approach to the containment of antibiotic resistance.
Degree: Non-degree
BREC Ref No: BCA444/16

CLASS APPROVAL

The Biomedical Research Ethics Committee (BREC) has considered the abovementioned application at a meeting held on 13 September 2016.

The study was provisionally approved by BREC pending appropriate responses to queries raised. Your responses dated 28 February 2017 to queries raised on 19 September 2016 have been noted and approved by the Biomedical Research Committee at a meeting held on 14 March 2017.

This approval is valid for one year from **17 March 2017.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

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forestry & fisheries

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Reference: 12/11/1/5

Dr Yogandree Ramsamy
Prince Mshiyeni Memorial Hospital
National Health Laboratory Services
Mangusuthu Highway 2 Umlazi
Durban

Dear Dr Yogandree Ramsamy,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your application, submitted on 02 November 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;

3. All samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act. No. 93 of 1996);
4. Samples must be analysed in the NHLS Public Health Laboratory KZN, Durban;
5. Only a registered waste disposal company may be utilized for the removal of waste generated during the study;
6. Bacterial cultures may be stored at the Micro bank Facility at the Antimicrobial Research Unit, Pharmaceutical Sciences, University of KwaZulu-Natal;
7. The stored bacterial cultures may only be used for further research after having obtained new Section 20 approval;
8. The stored bacterial cultures may not be outsourced without prior written approval from DAFF;
9. This section 20 expires on the 30 of June 2019.

Title of research/study: "Triangulating the Molecular Epidemiology of Carbapenem-resistant Enterobacteriaceae from Humans, Food Animals and the Environment."
Researcher (s): Dr Yogandree Ramsamy
Institution: Prince Mshiyeni Memorial Hospital, National Health Laboratory Services, Mangusuthu Highway 2 Umlazi, Durban
Your Ref./ Project Number:
Our ref Number: 12/11/1/5

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date:
2018 -11- 2 8

CLASSIFICATION: CONFIDENTIAL

SUBJECT: xxxxxxxxxxxxxxxxxxxxxxxxx

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Physical Address: 330 Langalibalele Street, Pietermaritburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3189/ 3123 Fax: 033 394 3782
Email: [illegible]
www.kznhealth.gov.za

DIRECTORATE:
Health Research & Knowledge Management

NHRD Ref: KZ_201808_049

Dear Dr Y Ramsamy
UKZN

Approval of research

1. The research proposal titled **'Triangulating the molecular molecular epidemiology of Carbapenem-Resistant Enterobacteriaceae from Human, Food, Animals and the environment'** was reviewed by the KwaZulu-Natal Department of Health.

   The proposal is hereby **approved** for research to be undertaken at Appelsbosch, Edendale, Grey's and Northdale Hospital

2. You are requested to take note of the following:
   a. Kindly liaise with the facility manager BEFORE your research begins in order to ensure that conditions in the facility are conducive to the conduct of your research. These include, but are not limited to, an assurance that the numbers of patients attending the facility are sufficient to support your sample size requirements, and that the space and physical infrastructure of the facility can accommodate the research team and any additional equipment required for the research.
   b. Please ensure that you provide your letter of ethics re-certification to this unit, when the current approval expires.
   c. Provide an interim progress report and final report (electronic and hard copies) when your research is complete to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Mr X. Xaba on 033-395 2805.

Yours Sincerely

E Lutge

Dr E Lutge
Chairperson, Health Research Committee
Date: 11/09/18

Fighting Disease, Fighting Poverty, Giving Hope

Appendix II – Awards

ICAN-Institut Merieux Young Investigator Award 2018

7th ICAN Congress 2018
Infection Control Africa Network

8 - 11 July 2018
Century City Conference Centre
Cape Town | South Africa
www.icancongress.org

2 July 2018

7th Infection Control Africa Network Congress 2018 | 08 – 11 July 2018
Century City Hotel & Conference Centre, Century City, Cape Town, South Africa

Dear Dr Yogandree Ramsamy

On behalf of Bio Merieux and ICAN we are pleased to inform you that you are the laureate of the joint ICAN – Institut Merieux Young Investigator Award 2018.

On behalf of the Scientific Committee of the Infection Control Africa Network, it is our great pleasure to invite you to the 7th ICAN International Congress to receive your award at the closing ceremony on the 11th July 2018 at 4:00 pm by a Bio Merieux representative and ICAN. It is an achievement indeed and wish you all the best in your research in the future.

As part of attending the conference, you would need to register as a delegate: Registration cost to the conference will be R4750 for doctors, attending the BSAC workshop on 12/7/2018 after the closing of the conference, registration fee of R505.00 would also need to be paid.
As it is very close to the conference date, online registrations are closed, but Chipo Minawu from EOA will be able to assist you to pay via EFT.

If you are in need of accommodation, she would also be able to assist you. Please contact Chipo with all your details to assist you with the registration at: Chipo Minawu chipo@eoafrica.co.za

We look forward to welcoming you to Cape Town!

Warmest Regards,

Professor Shaheen Mehtar
Chair ICAN

Professor Val Robertson
Chair Scientific Committee

Congress Office: Europa Organisation Africa
Telephone: + 27 (0)11 325 0020/2/3 • Fax: +27 (0)11 325 0028
Email: chipo@eoafrica.co.za
PO Box 782243 • Sandton • 2146 • Johannesburg • South Africa
www.eoafrica.co.za

SEDRIC-Wellcome Trust Travel Award - 2019

E m.watson@wellcome.ac.uk
T +44 (0)20 7611 8229

4 July 2019

Dr Yogandree Ramsamy
National Health Laboratory Services -
Prince Mshiyeni Memorial Hospital,
KwaZulu-Natal Academic Complex,
University of KwaZulu-Natal
Griffiths Mxenge Highway, Umlazi V
4060
South Africa

Dear Dr Ramsamy

You are invited to the Surveillance and Epidemiology of Drug Resistant Infections Consortium (SEDRIC) Global Meeting. The theme of the meeting is 'mobilising surveillance and epidemiology data to inform patient care pathways'.

The meeting will take place on Wednesday 9 October, beginning at 09:00 and ending at 18:00 at Wellcome, 215 Euston Road, London, United Kingdom. The final agenda will be circulated to all participants shortly before the meeting.

Travel and accommodation
Wellcome will book and cover your travel and accommodation for these events. This includes restricted economy/standard class travel, and accommodation for 8th and/or 9th October at the hotel we have reserved for this group (Ambassadors Bloomsbury hotel). We will book your travel on the Heathrow Express and London Underground once your flights are confirmed. You should book your own transfers between your home and departure airport, using public transport where possible. Please keep all your receipts for your claim following the meeting. Please indicate your travel requirements and accommodation requirements during the registration process. It is possible for you to book your own travel to attend this meeting, however we will require a quote and itinerary in order to guarantee reimbursement following the meeting. Please send your quote and itinerary to Mary Watson before you make any bookings.

There are no formal dinner arrangements for the evenings, therefore please keep your receipts and we will be be able to reimburse up to £30 for each night of your stay in London, excluding alcohol (maximum of 2 nights). Please note we are unable to reimburse alternative hotel costs if you choose not to stay in the hotel we have reserved for participants of this meeting.

We look forward to hearing from you

The SEDRIC Secretariat

Wellcome Trust
215 Euston Road
London NW1 2BE
United Kingdom

wellcome.ac.uk

The Wellcome Trust is a charity registered in England and Wales, no. 210183. Its sole trustee is The Wellcome Trust Limited, a company registered in England and Wales, no. 2711000 (whose registered office is at 215 Euston Road, London NW1 2BE, UK).

L'Oreal-UNESCO for Women in Science Award 2019

18 September 2019

Dear Yogandree

Re: L'Oreal-UNESCO for Women in Science South Africa Young Talents Programme

We would like to take this opportunity to congratulate you on your selection as one of five Doctoral research grant recipients for the 2019 **L'Oreal-UNESCO For Women In Science South African Young Talents programme.**

As you know in 2010, the L'Oréal Corporate Foundation and UNESCO have begun a new stage in their For Women in Science international programme with the launch of the L'OREAL-UNESCO For Women in Science South African Young Talents Programme in 2019.

Intended for young women finishing their doctorates or at post-doctorate level, the research grants will enable them to make themselves known, showcase their work, and drive their projects forward. More generally they are intended to encourage women to take up scientific careers. The research grants will enable them to make themselves known, showcase their work, and drive their project forward. More generally they are intended to encourage women to take up scientific careers.

In this respect, we wish to thank you once again for the interest shown in our programme by submitting your application, and for the time and energy you have put into preparing your file. We look forward to hosting at the South African ceremony in Johannesburg from 6th – 8th November 2019 as well as in Senegal for leadership training from 17th -21st November 2019.

A reminder that this announcement is under embargo until the ceremony and must not be shared with anyone or published in the media.

Yours Sincerely

Helen Da Fonseca

Corporate Communications Manager

L'Oréal South Africa

C1 - Internal use

Appendix III - Congress attendance and presentations

SAPA and SAAPS Congress 2016 – Durban, South Africa

SAPA & SAAPS CONGRESS 2016
ELANGENI & MAHARANI HOTEL, DURBAN
PROVISIONAL PROGRAMME (subject to change)

	WEDNESDAY 31 AUGUST 2016	
	Pre-Congress Workshops	
07:00 - 08:30	REGISTRATION FOR WORKSHOPS OPENS	
08:30 - 16:15	**SASPID Workshop**	Venue: Tugela (Maharani)
	INFECTION CONTROL AND ANTIBIOTIC STEWARDSHIP SYMPOSIUM	
08:30 – 08:45	SESSION 1	Chair: Prof N Du Plessis
	INTRODUCTION AND CASE SCENARIO	
08:45 – 09: 15	Approach to a patient with suspected Hospital- acquired infections	Dr F Naby
09:15 – 09:45	Strategies to prevent spread of multi-drug resistant organisms	Dr A Dramowski
09:45 – 10:15	Antimicrobial resistance in suspected hospital acquired infection	Dr C Govind
10:15 – 10:30	QUESTIONS	
10:30 – 11:00	TEA/COFFEE	
	SESSION 2	Chair: Dr M Lawler
11:00 – 11:30	The A to Z of outbreak investigation	Dr A Dramowski
11:30 – 12:00	Ethics of outbreak reporting	Prof N Du Plessis
12:00 – 12:30	Duration of antibiotic therapy and Therapeutic Drug Monitoring in Paediatri	Dr U Hallbauer
12:30 – 13:30	LUNCH	
	SESSION 3	Chair: Dr M Archary
13:30 – 14:00	The role of the microbiology laboratory in hospital-acquired infection	Dr Y Ramsamy
14:30 – 15:00	Challenges in Stewardship: Allergies, Interactions and Adverse Events	Prof N Du Plessis
15:00 – 15:30	Antibiotic Stewardship in the South African context- Where to start	Dr M Lawler
15:30 – 16:00	Pediatric Case: Intervention to Improve Antimicrobial Use in Paediatrics and	Dr A Dramowski
16:00 – 16:10	QUESTIONS	
16:10 - 16:15	CLOSURE	Dr M Archary
08:00 - 17:00	**GIT Workshop**	Venue: Congela & Umgeni (Maharani)
	Post Graduate Course in Paediatric Gastroenterology	Chair: Prof E Nel
08:15 - 08:30	Welcome	Prof E Nel/ Dr L Goddard
08:30 - 09:00	Disease related malnutrition	Prof E Nel
09:00 - 09:30	Approach to constipation	Prof S Kolacek
09:30 - 10:00	Approach to the child with abdominal pain	Prof J Taminiau
10:00 - 10:30	TEA/COFFEE	Dr S Bisetty
		Chair: Dr S Lala
10:30 - 11:00	Gut Flora and Gut Immunity	Prof S Kolacek
11:00 - 11:30	Chronic diarrhoea in childhood	Dr M Lentze
11:30 - 12:00	Appropriate management of GORD in childhood	Prof E Nel
1200 - 12:30	Eosinophilic diseases of the intestine	Dr M Lentze
12:30 - 13:30	LUNCH	
		Chair: Dr L Cooke
13:30 - 14:00	The Approach Jaundiced Infant	Dr A Terblanche
1400 - 14:30	Persistently abnormal liver biochemistry	Dr P Walabh
14:30 - 15:00	Managing portal hypertension	Prof R de Lacy
15:00 - 15:30	Autoimmune Hepatitis	Dr Liz Goddard
15:30 - 16:00	TEA	
		Chair: Prof C Hajanicalou
16:00 - 16:30	Nutritional support of chronic liver disease	Dr R de Lacy
16:30 - 17:00	Nutritional support of CF/Improving the outcome of SAM	Prof E Nel

FIDSSA 2019 – Johannesburg, South Africa

09 April 2019

Dear Dr Ramsamy

8th FIDSSA Congress 2019 | 07 – 09 November
Indaba Hotel & Conference Centre, Fourways, Johannesburg, South Africa

On behalf of the **8th FIDSSA Congress 2019**, we thank you for accepting the invitation to present at our upcoming congress as part of the esteemed invited faculty.
The following role(s) have been allocated.

Proposed Programme Involvement:

Fri 08 November
Parallel Session 2: AMS (Antimicrobial Stewardship)
Time: 10h30 - 12h30
Chair Session

Sat 09 November
Plenary Session 3
Time: 08h30 - 09h00
Presentation Title: UKZA – AMS

Final time allocation to be advised. Kindly confirm acceptance by **return.**

In lieu of your participation, the organising committee are pleased to offer the following benefits:

- Full congress registration fee.
- 1 night single accommodation at the Indaba Hotel, Johannesburg (extra nights for own account) For more information visit www.indabahotel.co.za.
- One economy return flight e-ticket. Please note due to the rising cost of flights, we will source the most cost-effective flight option. Ticket to be booked through the congress office.
- Airport transfers from Lanseria Airport to Indaba Hotel & Conference Centre.
- Invitation to the faculty dinner

We invite you to finalise your travel and accommodation arrangements by return email to chipo@eoafrica.co.za. May we also kindly request that you forward an abbreviated professional profile, (maximum 300 words, Word doc format) and photograph to be featured on the FIDSSA Congress 2019 website and final Congress App.

We look forward to assisting with your arrangements and welcoming you to the congress.

Professor Nicolette du Plessis
Congress Chairperson

8th FIDSSA Congress 2019
Congress Office: Europa Organisation Africa
Telephone: +27(0)11 325 0020 • **Fax:** +27(0)11 325 0028 • **Email:** chipo@eoafrica.co.za
Address: PO Box 782243 Sandton 2146 Johannesburg South Africa
Webpage: www.fidssacongress.co.za

Whole-Genome Sequencing and Bioinformatics provides a better understanding of the molecular mechanisms and spread of drug-resistant strains such as carbapenem resistant *Klebsiella pneumoniae* within the healthcare setting.

Yogandree Ramsamy Ramsamyy@ukzn.ac.za +27 833 082710

Molecular Characterization of Carbapenem Resistant *Klebsiella pneumoniae* Isolates Collected from a Central Public Hospital in Durban, South Africa between 2016 and 2017

Ramsamy Y[a,b,d], Mlisana KP[a,b], Allam M[c], Amoako DG[d], Singh R [a,b], Ismail A[c], Abia Akebe, LK[d], Essack SY[d]

Introduction

The ongoing dissemination of carbapenemase-producing *Enterobacteriaceae* (CPE) represents a significant public health issue. Early detection and infection prevention and control strategies are key in limiting further spread of CPE within healthcare systems. The molecular epidemiology of antibiotic resistance is important in evaluating the effectiveness of infection prevention and control programs and provides information for the development of new antimicrobial targets. This study investigated genomic epidemiology and clonal relationships among the carbapenem-resistant *K. pneumoniae* isolated from patients at a public hospital in South Africa.

Methods

The study was approved by the Biomedical Research Ethics Committee (BE453/15), University of KwaZulu-Natal, South Africa. Between May 2016 and May 2017, 10 isolates were obtained from patients admitted to the intensive care unit at a public hospital in Durban, South Africa. Five isolates were from rectal swabs of colonized patients and five from blood cultures in critically ill patients. Following the phenotypic microbial identification and antibiotic susceptibility tests, the isolates were subjected for whole genome sequencing (WGS). The resultant contigs were annotated using bioinformatic platforms (PGAP, RAST, ResFinder, GoSeqIt, CARD, PlasmidFinder, ISFINDER and PHAST) to delineate resistome and acquisition of MGEs in the isolates. *In-silico* MLST and capsular serotyping was performed using the PubMLST and Kaptive online tool to determine the clonal relatedness of the isolates. Phylogenomic analyses coupled with available metadata via Phandango was performed to provide deeper insights into the clonal linages of the isolates.

Results

All isolates were extensively drug-resistant (XDR) with detectable phenotypic and genotypic resistance to tested β-lactams. Resistance to carbapenems was conferred by the NDM-1 β-lactamase mediated by the acquisition of the p18-43_01 plasmid with multi-replicon types [ColRNAI, IncFIB(pB171), Col440I, IncFII, IncFIB(K) and IncFII(Yp)]. All ten isolates had the same plasmid multilocus sequence type (IncF[K12:A-:B36]) and capsular serotype KL149, affirming the epidemiological linkage between the *K. pneumoniae* isolates. All but one isolate belonged to ST152 sequence type. A novel sequence type, ST3136 that differed from the primary clone by a single-locus variant in the *rpob* allele was detected. These findings suggest an independent plasmid acquisition followed by local dissemination indicating horizontal spread of this blaNDM-1-bearing plasmid structure.

Conclusion

The acquisition of resistance-encoding plasmids, horizontal transfer, and clonal dissemination facilitated the spread of carbapenemase-producing *K. pneumoniae* in a public hospital. WGS and bioinformatics analysis provides a better understanding of the molecular mechanisms and spread of drug-resistant strains within the healthcare setting.

No	MIC (mg/L)* IMP	MER	FEP	CXM	CTX	CAZ	CRO	FOX	AMP	AMC	TZP	AMX	GEN	AMK	CIP	ERT	SXT	TGC
B1	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	≤0.5
B2	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	2
B3	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	1
B4	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	2
B5	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	2
R1	≥16	≥16	32	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	1
R2	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	≤0.5
R3	≥16	≥16	32	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	2
R4	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	1
R5	≥16	≥16	32	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	1

TABLE 1: Antibiotic susceptibility characteristics of the *Klebsiella pneumoniae* collection (n=10)
* EUCAST resistant breakpoints (v 7.1) are used. Abbreviations are used for all antibacterial agents as follows: **β-lactams** (IMI imipenem (R>8mg/L); MEM=meropenem (R>8mg/L); FEP=cefepime (R>4mg/L); CXM= cefuroxime (R>8mg/L); CTX=cefotaxime (R>2mg/L); CAZ=ceftazidime (R>4mg/L); CRO=ceftriaxone (R>2mg/L); FOX=cefoxitin (R>8mg/L); AMP=ampicillin (R>8); AMC=amoxicillin-clavulanic acid (R>8mg/L); TZP=piperacillin-tazobactam (R>16mg/L)); AMX=amoxicillin (R>8mg/L)); **Aminoglycosides** (GEN=gentamicin (R>4mg/L); AMK=amikacin (R>16mg/L)); **Macrolide** (ERT=erythromycin (R>4)); **Fluoroquinolone** (CIP=ciprofloxacin (R>0.5 mg/L)); **Sulfonamides** (SXT=trimethoprim-sulfamethoxazole (R>4mg/L)); **Glycylcyclines** (TGC=tigecycline (R>2mg/L)). † Categorized as MDR, XDR or PDR according to standard criteria (Magiorakos et al. 2011).

No	MLST	K Typing	Allelic types	β-lactamase genes	Genes conferring resistance to Aminoglycosides	Genes conferring resistance to Phenicols	Genes conferring resistance to Trimethoprim	Genes conferring resistance to Sulfonamides	Genes conferring resistance to Fluoroquinolones
B1	ST152	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, ……, strA, strB, aac(6')Ib-cr	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, ……
B2	ST152	KL149	wzc:928, wzi:110	NDM-1, ……, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, …… rmtC, ……, strA, strB, aac(6')Ib-cr	catB4, catA1	……	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, ……
B3	ST152	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, aph(3)-Ia, strA, strB ……	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, ……, ……
B4	ST152	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, aph(3)-Ia, strA, strB, aac(6')Ib-cr	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, QnrB6
B5	ST152	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, aph(3)-Ia, strA, strB, aac(6')Ib-cr	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, QnrB6
R1	ST3136	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, aph(3)-Ia, strA, strB, aac(6')Ib-cr	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, QnrB6
R2	ST152	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, aph(3)-Ia, strA, strB, aac(6')Ib-cr	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, ……
R3	ST152	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, ……, strA, strB, aac(6')Ib-cr	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, QnrB6
R4	ST152	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, ……, strA, strB, aac(6')Ib-cr	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, QnrB6
R5	ST152	KL149	wzc:928, wzi:110	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	aac(3)-IIa, aadA16, rmtC, ……, strA, strB, aac(6')Ib-cr	catB4, catA1	dfrA27	sul1, sul2	oqxB, oqxA, aac(6')-Ib-cr, QnrB6

TABLE 2: Genotypic characteristics of the CR *Klebsiella pneumoniae* isolates from Durban, South Africa. MLST multi locus sequence typing. *K typing* Klebsiella surface polysaccharide capsule characterization, wzc and wzi type- allelic typing scheme. Genomic analysis of the resistome in *Klebsiella pneumoniae* from WGS data

FIGURE 1

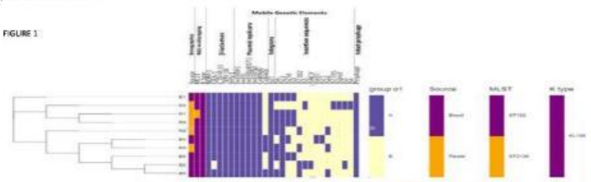

FIGURE 2

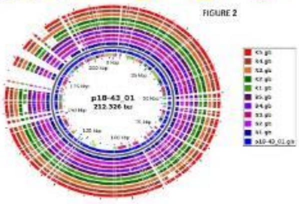

FIGURE 1: The core genome phylogenetic branch and metadata (demographics; WGS *in silico* typing; β-lactamases, plasmid replicons, integrons, insertion sequences and intact prophages) coupled with the use of Phandango (https://github.com/jameshadfield/phandango/wiki) in isolated CP-*K. pneumoniae* strains (n=10) from the public hospital in South Africa. The colour codes for β lactamases, plasmid replicons, integrons, insertion sequences and intact prophages showed presence (light blue) and absence (yellow) in the isolates.

FIGURE 2: Tracking of **plasmid p18-43_01** in NDM-1-encoding CP-*K. pneumoniae* isolates (n =10). The map was constructed using GView online server (https://server.gview.ca/). The concentric circles represent comparisons between p18-43_01 and, starting with the inner circle, genome assemblies from *Klebsiella pneumoniae* species (strain ID B1, B2, B3, B4, B5, R1, R2, R3, R4 and R5). Colour codes are given for each species and for DNA identity, ranging from 80 to 100%.

NATIONAL HEALTH LABORATORY SERVICE

[a]School of Laboratory Medicine and Medical Science, Department of Medical Microbiology, University of KwaZulu-Natal, Durban, South Africa
[b]National Health Laboratory Service, Sandringham, South Africa
[c]Sequencing Core Facility, National Institute for Communicable Diseases, National Health Laboratory Service, Sandringham, South Africa
[d]Antimicrobial Research Unit, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa

Acknowledgements & Disclosures
We wish to acknowledge Professor David J. Muckart, Dr Khine Swe Swe Han, Dr Timothy C Hardcastle, Dr T Kistan, Dr VI Suleman and the team of curators at Klebsiella MLST database in Institute Pasteur, Paris, France. The Institute Merieux is acknowledged for financial support.
Professor Essack is Chairperson of the Global Respiratory Infection Partnership sponsored by an unrestricted educational grant from Reckitt & Benckiser.

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Antibiotic Stewardship and Conservancy in Africa Conference, Durban, South Africa, 2019

Date: 25 March 2020

Email: ramsamyy@ukzn.ac.za

Dear Ramsamy, Y

Abstract Decision for the Antibiotic Stewardship and Conservancy in Africa Conference 20-23 October 2019, Westville Campus, University of KwaZulu-Natal, Durban, South Africa

Your abstract: MOLECULAR CHARACTERIZATION OF CARBAPENEM RESISTANT Klebsiella pneumoniae ISOLATES COLLECTED FROM A CENTRAL PUBLIC HOSPITAL IN DURBAN, SOUTH AFRICA BETWEEN 2016 AND 2017

Your abstract has been **ACCEPTED** for a/an **ORAL** presentation pending you registering for the conference at https://norhed.ukzn.ac.za/registration/.

Please send confirmation of your intent to present at this conference by the **12th August 2019** to norhed@ukzn.ac.za.

You will be able to access the date and time of your **10-minute oral presentation** on the conference website, when the conference programme is finalised.

Please note, the conference is free, and limited to 250 participants. Priority will be given to those who have submitted abstracts and completed the registration.

Registration for non-presenters will be confirmed from the 15th August onwards.

Thank you for submitting your abstract to the conference and we look forward to welcoming you to Durban, South Africa.

Yours sincerely

Professor Arnfinn Sundjsford (Arctic University of the North, Trompso, Norway)

Dr Lyn Middleton (University of KwaZulu-Natal, Durban, South Africa)

For the Scientific Committee


PIONEERING DIAGNOSTICS
BIOMÉRIEUX
INTEGRATED SYMPOSIUM • SUNDAY APRIL 19, 13:30-15:30 • HALL C
ANTIMICROBIAL RESISTANCE
MOVING FROM
ACTION PLANS TO
IMPLEMENTATION
A GLOBAL PERSPECTIVE
30th ECCMID EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES
Paris, France
18–21 April 2020

BIOMÉRIEUX

INTEGRATED SYMPOSIUM • SUNDAY APRIL 19, 13:30-15:30 • HALL C

ANTIMICROBIAL RESISTANCE: MOVING FROM ACTION PLANS TO IMPLEMENTATION, A GLOBAL PERSPECTIVE

ESCMID CHAIR: Jeroen Schouten, Radboudumc - Intensive Care, Nijmegen, The Netherlands
CHAIR: Mark Miller, Chief Medical Officer, bioMérieux, France

PROGRAMME

13:30 - 13:40 Introduction

13:40 - 14:05 Light in the face of Darkness: the critical role of a Clinical Microbiologist in AMS in the South African Landscape
Yogandree Ramsamy, Clinical Microbiologist, Prince Mshiyeni Memorial Hospital, National Health Laboratory services, University of KwaZulu-Natal, Durban, South Africa

14:05 - 14:30 Using Diagnostics to Advance Antimicrobial Stewardship in Canada
Victor Leung, Medical Director, Infection Prevention and Control and Medical Lead, Antimicrobial Stewardship Program, Providence Health Care (PHC), Vancouver, Canada

14:30 - 14:55 The pivotal role of the clinical microbiologist in implementing an AMR National Action Plan: an Italian experience
Stefania Stefani, Professor of Microbiology, Department of Biomedical and Biotechnological Sciences, University of Catania, Italy

14:55 - 15:20 Antimicrobial Stewardship and rapid diagnostics for better prescribing: a UK perspective
Vanya Gant, Divisional Clinical Director for Infection at University College London Hospitals, United Kingdom

15:20 - 15:30 Closing Remarks

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Appendix lV – Scientific Meeting

INSTITUT MERIEUX

Prince Mshiyeni Memorial Hospital
National Health Laboratory Service
University of KwaZulu-Natal
Department of Medical Microbiology
Griffiths Mxenge Hwy,
Umlazi, Durban
KwaZulu-Natal
South Africa 4060

December 2, 2019

Subject: Invitation Letter - Dr Yogandree Ramsany

Dear Madam/Sir,

As a former laureate of the Institut Merieux Young Investigator Award program, we officially invite Dr. Yogandree Ramsany to attend a two-day meeting during which a selected set of former award recipients will present their work to a broad audience involving primarily employees of the Merieux Group.

This meeting will be held at the Fondation Mérieux conference center, close to Annecy (France) and Geneva (Switzerland), from March 16th to 17th, 2020.

Travel expenses (train (1st class) or flight (economy or premium)) and accommodation for the whole duration of the meeting would be covered by the Institut Mérieux.

Looking forward to hearing from you soon,

With my kindest regards

Marc Bonneville
VP Medical and Scientific Affairs

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Appendix V – Training Workshops

AENOR

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20179615AMRNEU@aenor.com

Dr. Yogandree RAMSAMY

National Health Laboratory Services, University of Kwazulu-Natal
Prince Mshiyeni Memorail Hospital
2 Mongusthu Highway, Umlazi
Umlazi, Durban
SOUTH AFRICA

Ref.: AMR Invitation letter
Subject: CHAFEA 2017 96 15 BTSF ANTIMICROBIAL RESISTANCE
Date: 2019-01-14

Dear Dr. Yogandree RAMSAMY,

I have the pleasure to confirm that your nomination to attend to the training programme on **"PREVENTION AND CONTROL ON ANTIMICROBIAL RESISTANCE (AMR) IN THE CONTEXT OF AN OVERALL 'ONE HEALTH' APPROACH INVOLVING BOTH VETERINARY AND HUMAN FIELDS"** financed by the Directorate General for Health and Consumers from the European Commission, under the Better Training for Safer Food initiative has been accepted.

The training session will take place at the **Premier Hotel Cape Town** - 1 Marais Rd, Sea Point, Cape Town, 8001, South Africa, from the 5th to the 8th of February 2019.

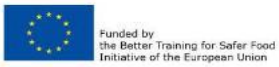
Having been chosen to participate representing your country, **SOUTH AFRICA**, the project will fully finance your attendance, that includes international flight or transport to Cape Town, transfers from the airport to the hotel and way back, travel insurance, visa costs, airport taxes, accommodation, documentation, all meals and training certificate.

For this course, no "perdiem" or "pocket money" will be distributed to participants. Personal expenses such as, mini-bar, laundry, room services, and extra drinks/meals will be charged to the participants by the hotel.

Any petition or request additional information, please contact the Event Manager, Nuria GUARDO (20179615AMRNEU@aenor.com)

Yours sincerely,

Nuria GUARDO
Deputy Project Manager
AENOR



European Commission | Food Safety

Better Training for Safer Food

CERTIFICATE

Dr Yogandree RAMSAMY (SOUTH AFRICA)

has attended the Training on **Prevention and control on Antimicrobial Resistance (AMR) in the context of an overall 'one health' approach involving both veterinary and human fields (Contract number 2017 96 15)**

with **7** hours of lectures and **13** hours of practical work *(60 minutes hours)*

Cape Town, South Africa, 5th- 8th February 2019

Ms Nuria GUARDO

Project Management Unit
AENOR

AENOR JVL CONSULTING

Mrs Anne BUCHER

Signed

Director General
European Commission
DG Health and Food Safety

Ms Véronique WASBAUER

Signed

Director
Consumers, Health, Agriculture and Food Executive Agency

Appendix VI
CRE-Related manuscript 1

microorganisms

MDPI

Article

Genomic Analysis of Carbapenemase-Producing Extensively Drug-Resistant *Klebsiella pneumoniae* Isolates Reveals the Horizontal Spread of p18-43_01 Plasmid Encoding bla_{NDM-1} in South Africa

Yogandree Ramsamy [1,2,3,*], Koleka P. Mlisana [2,3], Mushal Allam [4], Daniel G. Amoako [1,*], Akebe L. K. Abia [1], Arshad Ismail [4], Ravesh Singh [2,3], Theroshnie Kisten [5,6], Khine Swe Swe Han [2,3], David J. Jackson Muckart [7], Timothy Hardcastle [7], Moosa Suleman [8] and Sabiha Y. Essack [1]

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check for updates

Abstract: Whole-genome sequence (WGS) analyses were employed to investigate the genomic epidemiology of extensively drug-resistant *Klebsiella pneumoniae* strains, focusing on the carbapenem resistance-encoding determinants, mobile genetic support, clonal and epidemiological relationships. A total of ten isolates were obtained from patients admitted to the intensive care unit (ICU) in a public hospital in South Africa. Five isolates were from rectal swabs of colonized patients and five from blood cultures of patients with invasive carbapenem-resistant infections. Following microbial identification and antibiotic susceptibility tests, the isolates were subjected to WGS on the Illumina MiSeq platform. All the isolates showed genotypic resistance to tested β-lactams (NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1) and other antibiotics. All but one isolate belonged to the ST152 with a novel sequence type, ST3136, differing by a single-locus variant. The isolates had the same plasmid multilocus sequence type (IncF[K12:A-:B36]) and capsular serotype (*KL149*), supporting the epidemiological linkage between the clones. Resistance to carbapenems in the 10 isolates was conferred by the bla_{NDM-1} mediated by the acquisition of multi-replicon [ColRNAI, IncFIB(pB171), Col440I, IncFII, IncFIB(K) and IncFII(Yp)] p18-43_01 plasmid. These findings suggest that the acquisition of bla_{NDM-1}-bearing plasmid structure (p18-43_01), horizontal transfer and clonal dissemination facilitate the spread of carbapenemases in South Africa. This emphasizes the importance of targeted infection control measures to prevent dissemination.

Keywords: genomics; carbapenemase; *Klebsiella pneumoniae*; extensively drug-resistant; mobile genetic elements; epidemiology; phylogenomic; South Africa

1. Introduction

The last decade has witnessed a dramatic increase both in the proportion and absolute number of multi-drug resistant bacterial pathogens [1]. Infections caused by extensively drug-resistant (XDR) Gram-negative pathogens have emerged as one of the world's greatest threats [2], not the least of which are the carbapenem-resistant bacteria that are on the rise globally [3–5]. There is a continuous rise in bacterial resistance, and unfortunately, there are potentially no new drugs on the horizon to replace the existing antibiotics against which resistance has developed [6,7]. This necessitates the urgent search and development of potential candidates in the drug pipeline to help manage this global threat of antibiotic resistance [8–11].

Carbapenems are a potent class of β-lactam antibiotics that are often used as "last-line agents" or "antibiotics of last resort" when infected patients become severely ill or are suspected of harboring resistant bacteria [12]. They are considered first-line agents in the treatment of infections caused by extended-spectrum β-lactamase (ESBL)-producing organisms [13]. The widespread use of carbapenems for empiric and directed treatment of severe infections has resulted in the emergence of carbapenem-hydrolyzing β-lactamases, also known as carbapenemases, as the most well-recognized mechanism of resistance to carbapenems. These enzymes inactivate all known β-lactams and represent the most versatile family of β-lactamases, with a breadth of spectrum unrivaled by other β-lactam-hydrolyzing enzymes [14]. The production of carbapenemases by *Enterobacterales* such a *K. pneumoniae* results in limited treatment options with an inevitable high mortality rate caused by carbapenemase-producing *Enterobacterales* (CPE) [15,16]. Infection with carbapenem-resistant Enterobacterales has emerged as an important problem that threatens the health and wellbeing of patients in health-care settings [17,18]. The alarming global spread of CPE isolates has reached African countries including in Angola, Algeria, Gabon, Mali, Nigeria, and South Africa with NDM-1 and OXA-48 been the most commonly reported carbapenemases [19–24]. This rapid dissemination of CPE is supported by intra- and interspecies plasmid-mediated transfer of carbapenemase-encoding genes detected on a diversity of plasmid backbones [25,26]. Plasmids of several incompatibility groups (Inc) can mediate the spread of carbapenem resistance, mostly resulting in XDR *K. pneumoniae* [27,28]. Till date, a total of 760 *K. pneumoniae* plasmid annotation reports are available at the Pathosystems Resource Integration Center (PATRIC) database (https://www.patricbrc.org/).

Fecal carriage of CPE isolates has been investigated rarely compared with carriage of isolates producing ESBLs [29], particularly in a "non-outbreak" setting. Rapid identification of patients colonized with CPE is an integral part of intervention strategies and infection control measures required to contain hospital infections due to CPE [30]. There is a growing body of evidence that suggests that early detection of patients colonized with CPE on admission to health-care facilities may assist in the prevention of outbreaks limiting the regional spread of this emerging threat [6]. Additionally, understanding the molecular mechanisms of resistance could provide valuable insights into the management of drug-resistant in *K. pneumoniae* infections. Herein, whole-genome sequence (WGS) analysis was employed to investigate the molecular epidemiology of ten XDR *K. pneumoniae* strains (from both colonized and infected patients), focusing on the carbapenem resistance-encoding determinants, their mobile genetic support, clonal and epidemiological relationships in a public hospital in KwaZulu-Natal, South Africa.

2. Materials and Methods

2.1. Ethical Approval

Ethical clearance was granted by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (approval no: **BE: 453/15,** approval date: 29 February 2016).

2.2. Study Site and Sample Collection

This prospective study was performed at the Inkosi Albert Luthuli Central Hospital (IALCH). IALCH is a centralized healthcare facility located in Durban, KwaZulu-Natal, South Africa. This Level 4 hospital has a Medical and Surgical ICU comprising of 6 beds each. The ten-bed trauma ICU is exclusively for trauma patients who are admitted either directly from an injury scene or transferred from another district hospital within 24 h of sustained injuries unless otherwise specified. Rectal swab specimens to identify CPE were obtained from patients admitted to the Medical, Surgical and Trauma ICU between May 2016 and May 2017. A total of 263 patients were screened. Additionally, clinical isolates of CR *K. pneumoniae* implicated in bloodstream infections were obtained from the same wards in the same period.

2.3. Isolation and Identification of Carbapenemase-Producing Klebsiella Pneumoniae Isolates

2.3.1. Culture Screening Methods

Rectal swabs were obtained using a nylon flocked swab system with 5 mL of Amies gel transport medium. The swabs were immediately streaked on ChromID CARBA SMART chromogenic agar medium (BioMérieux, Marcy l'Étoile, France) containing antibiotics that enable selective isolation of and identification of carbapenemase-producing *Enterobacterales*. This media provides rapid and reliable identification of all CPE/CRE, particularly KPC, NDM-1, and OXA-48—producing isolates [31]. Inoculated plates were incubated for 18 to 24 h at 37 °C in ambient air. All ChromID CARBA SMART (BioMérieux, Marcy l'Étoile, France) agar plates were inoculated with the following control strains: carbapenemase-negative *K. pneumoniae* ATCC 700603, and carbapenemase-positive *K. pneumoniae* ATCC BAA-1705.

2.3.2. Detection and Identification of CPE Colonies

Presumptive CPE colonies from isolated from the ChromID CARBA SMART agar were sub-cultured onto MacConkey plates, and pure colonies were phenotypically identified using the VITEK II system (BioMérieux, Marcy l'Étoile, France). Confirmed CRE were then subjected to the RAPIDEC® CARBA NP (BioMérieux, Marcy l'Étoile, France) test to detect carbapenem hydrolysis by carbapenemase-producing bacteria.

2.4. Antibiotic Susceptibility Testing (AST)

Antibiotic susceptibility testing was performed, and the minimum inhibitory concentrations (MICs) were ascertained using the VITEK II (BioMérieux Marcy l'Étoile, France) platform. The results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines [32]. The VITEK II AST-N255 card was used to perform antibiotic susceptibility testing. The universal antibiotic test panel included: penicillin, ampicillin, amoxicillin-clavulanate, ceftriaxone, cefepime, cefuroxime, cefoxitin, ceftazidime, imipenem, meropenem, ertapenem, piperacillin-tazobactam, amikacin, gentamicin, nitrofurantoin, trimethoprim/sulfamethoxazole, ciprofloxacin, and tigecycline. Colistin susceptibility testing was not performed as currently all available laboratory methods are unreliable and may not predict clinical outcome [33]. Isolates were characterized as susceptible or resistant using CLSI breakpoints [32].

2.5. DNA Extraction Genome Sequencing and Analysis

The isolates were grown on nutrient agar (Oxoid, UK) and incubated overnight at 37 °C prior to genomic DNA extraction. Genomic DNA (gDNA) was extracted using the GenElute® bacterial genomic DNA kit (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The quantification of extracted gDNA was determined on a Nanodrop spectrophotometer, Qubit, and verified on an agarose gel electrophoresis. Multiplexed paired-end libraries (2 × 300 bp) were

prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) and sequences were determined on an Illumina MiSeq platform with 100× coverage at the National Institute of Communicable Diseases Sequencing Core Facility, South Africa. The resulting raw reads were checked for quality, trimmed, and *de novo* assembled into contigs using the CLC Genomics Workbench version 10 (CLC, Bio-QIAGEN, Aarhus, Denmark) [34]. The *de novo* assembled reads were uploaded in GenBank and annotated using NCBI prokaryotic genome annotation pipeline and RAST 2.0 server [35], which identified encoding proteins, rRNA and tRNA, assigned functions to the genes and predicted subsystems represented in the genome.

2.6. WGS-Based Confirmation and Molecular Typing of K. Pneumoniae Isolates

The generated contigs from the WGS data were used to confirm the *Klebsiella pnuemoniae* isolates using the SpeciesFinder 2.0 platform (https://cge.cbs.dtu.dk/services/SpeciesFinder/) which predicts the genus and species the strains in-silico. Multilocus sequence typing (MLST) was performed in-silico using the WGS data online platform tool from the assembled genomes (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) which also predicted the allelic profiles of the seven housekeeping genes, *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* of *K. pneumoniae*. The reference *Klebsiella* WGS data online platform tool, Kaptive-web (http://kaptive.holtlab.net/) was used to infer the serotypes (*K* types, *wzc* and *wzi* allelic types) of the isolates.

2.7. WGS Identification of the Acquired and Chromosomal Mutations in the Isolates

The bacterial analysis pipeline GoSeqIt (https://www.goseqit.com/web-services/) via ResFinder [36] and the comprehensive antibiotic resistance database (CARD; https://card.mcmaster.ca) [37] were used to annotate and identify antibiotic resistance genes. To detect the molecular basis of resistance (developing by chromosomal SNPs) against quinolones (*gyrA* and *parC*), the nucleotide allele sequences were translated with tBLASTn to call SNPs in these genes. The fluoroquinolone susceptible *K. pneumoniae* ATCC 13883 (PRJNA244567) was used as the reference/wild-type strain. The detected mutations were confirmed using the CARD platform which can equally predict chromosomal mutations.

2.8. WGS Identification of Mobile Genetic Elements (MGEs)/Genetic Support

Plasmid replicons were predicted through PlasmidFinder [38]. PHAge Search Tool (PHAST) server was used for the identification, annotation, and visualization of prophage sequences [39]. Insertion sequences (IS) resident in genomes were predicted by uploading contigs on the ISFinder database (https://www-is.biotoul.fr/) [40]. The carbapenemase genes and their flanking sequences obtained from the RAST SEED viewer were searched on the NCBI microbial nucleotide BLAST. Fully sequenced plasmids, with the closet synteny obtained from the BLAST search, were used as a reference input to GView Server (https://server.gview.ca/), together with the 10 annotated Illumina sequence reads of the XDR-*Klebsiella pneumoniae* isolates to visualize the presence/absence of specific plasmid DNA.

2.9. Phylogenomic Analyses of the K. Pneumoniae Isolates (n = 10)

The *de-novo* assembled contigs were submitted to CSI Phylogeny-1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny-1.2), an online service which identifies SNPs from WGS data, filters and validates the SNP positions, and then infers phylogeny based on concatenated SNP profiles [41]. The genome of *K. quasi-pneumoniae* strain P27-02 (accession number: NXHG00000000.1) served as the outgroup to root the tree enabling the easy configuration of the phylogenetic distance between the strains on the branches. The pipeline was run with default parameters: a minimal depth at SNP positions of 10 reads, a minimal relative depth at SNP positions of 10%, a minimal distance between SNPs of 10 bp, a minimal *Z-score* of 1.96, a minimal SNP quality of 30 and a minimal read mapping quality of 25. The obtained phylogenomic tree was downloaded in Newick format, annotated and visualized or edited using an interactive tree of life (ITOL) (https://itol.embl.de/).

Additionally, a genome-wide gene-by-gene comparison approach was used to assess the phylogenetic relatedness between isolates using Rapid large-scale prokaryote pangenome analysis (Roary; https://sanger-pathogens.github.io/Roary/) to estimate the tree for the core genome. The annotated genome assemblies were used to determine the core genes and predicted coding regions were extracted and converted into protein sequences. A total of 4605 core genes were extracted with an alignment length of 4,294,572 bp shared by the ten *K. pneumoniae* genomes.

The allelic distance from the cgMLST was edited and visualized using Figtree v1.4.3 (https://tree.bio.ed.ac.uk/sofware/figtree/) in a maximum likelihood phylogenetic tree using optimized parameters: nucleotide substitution model, Jukes-Cantor; transition/transversion ratio, 2; estimate substitution rate, yes; number of substitution rate, 4; perform bootstrap analysis, yes; replicates, 1000. The phylogeny was visualized with annotations for isolate demographics, WGS in-silico typing (ST, *K type*), β-lactamases, and mobile genetic elements metadata using Phandango [42] to provide a comprehensive analysis of the generated phylogenomic tree.

2.10. Accession Numbers

The raw read sequences and the assembled whole-genome contigs have been deposited in GenBank. The data is available under project number PRJNA411997.

3. Results

3.1. Identification, Confirmation and Phenotypic Analysis

Five out of the 263 rectal swabs (colonization rate of 1.9%) as well as the five blood culture samples obtained from infected patients for comparison, were confirmed as carbapenem-resistant K. pneumoniae (CRKP). Antibiotic susceptibility testing (AST) revealed that all the isolates were extensively drug-resistant (XDR) (Table 1). The relevant patient data, source of the specimen, and relevant phenotypic features (AST and Carba NP test) for the ten collected CRKP isolates are summarized in Tables 1 and 2.

3.2. Genomic Confirmation and Resistance Profiling of β-Lactamases

The SpeciesFinder platform confirmed all the with generated genomic data as *K. pneumoniae*. The genomic attributes of the 10 sequenced CRKP isolates are shown in Table S1. Resistance to antibiotics was attributed to multiple genes mediating resistance to different antibiotic classes (Table 2 and Table S2).

Table 1. Antibiotic susceptibility of the *Klebsiella pneumoniae*.

Bacterial Isolate *			MIC (mg/L) †																
No.	Strain ID	Category	IMP	MEM	FEP	CXM	CTX	CAZ	CRO	FOX	AMP	AMC	TZP	AMX	GEN	AMK	CIP	ERT	SXT	TGC
1	B1	XDR	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	≤0.5
2	B2	XDR	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	2
3	B3	XDR	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	1
4	B4	XDR	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	2
5	B5	XDR	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	2
6	R1	XDR	≥16	≥16	32	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	1
7	R2	XDR	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	≤0.5
8	R3	XDR	≥16	≥16	32	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	2
9	R4	XDR	≥16	≥16	≥64	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	1
10	R5	XDR	≥16	≥16	32	≥64	≥64	≥64	≥64	≥64	≥32	≥32	≥128	≥32	≥16	≥64	≥4	≥8	≥320	1

† CLSI resistant breakpoints are used. Abbreviations are used for all antibacterial agents as follows: β-lactams {IMI = imipenem (R > 8 mg/L); MEM = meropenem (R > 8 mg/L); FEP = cefepime (R > 4 mg/L); CXM= cefuroxime (R > 8 mg/L); CTX = cefotaxime (R > 2 mg/L); CAZ = ceftazidime (R > 4 mg/L); CRO = ceftriaxone= (R > 2 mg/L); FOX = cefoxitin (R > 8 mg/L); AMP = ampicillin (R > 8); AMC = amoxicillin-clavulanic acid (R > 8 mg/L); TZP = piperacillin-tazobactam (R > 16 mg/L)}; AMX = amoxicillin (R > 8 mg/L)}; Aminoglycosides {GEN = gentamicin (R > 4 mg/L); AMK = amikacin (R > 16 mg/L)}; Macrolide {ERI = erythromycin (R > 4)}; Fluoroquinolone {CIP = ciprofloxacin (R > 0.5 mg/L)}; Sulfonamides {SXT = trimethoprim-sulfamethoxazole (R > 4 mg/L)}; Glycylcyclines {TGC = tigecycline (R > 2 mg/L)}. * Categorized as MDR, XDR or PDR according to standard criteria [43].

Table 2. Relevant patient data, source of specimens, phenotypic, and genotypic characteristics of CRKP isolates.

Isolate *		Patient's		Isolate's		Carba NP †	β-Lactamase Genes	In-Silico Typing		
No.	Strain ID	Sex	Age (Years)	Date	Source			MLST	*K Typing*	Allelic Types
1	B1	M ‡	24	15/04/2017	Blood	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110
2	B2	F §	14	29/01/2017	Blood	+	NDM-1, ———, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110
3	B3	M	30	03/01/2017	Blood	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110
4	B4	M	15 days	21/03/2017	Blood	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110
5	B5	-	8 months	25/04/2017	Blood	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110
6	R1	F	61	20/05/2016	Rectal	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST3136	KL149	*wzc*:928, *wzi*:110
7	R2	F	72	18/07/2016	Rectal	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110
8	R3	M	25	13/06/2016	Rectal	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110
9	R4	F	21	11/07/2016	Rectal	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110
10	R5	F	66	27/07/2016	Rectal	+	NDM-1, OXA-1, CTX-M-15, TEM-1B, SHV-1	ST152	KL149	*wzc*:928, *wzi*:110

* Taxonomy determined by NCBI by comparing to proxytype strains in GenBank using the average nucleotide identity (ANI) test [44]. † Carba NP test for the detection of carbapenemase activity (+). ‡ Male. § Female.—Missing data. MLST—multilocus sequence typing, *K typing*—Klebsiella surface polysaccharide capsule characterization, *wzc* and *wzi* type—allelic typing scheme.

3.3. WGS-Based Capsular Serotyping and Multilocus Sequence Typing (MLST)

The epidemiological typing scheme via the Kaptive database predicted the same capsular polysaccharide serotype [KL149-*wzc*:928, *wzi*:110] for all the isolates. Further MLST-analyses revealed that the 9 of the CRKP strains belonged to ST152 (same clonal lineage) with the allelic profiles (*gapE-2*, *infB-3*, *mdh-2*, *pgi-1*, *phoE-1*, *rpoB-4*, *tnoB-56*) except for the novel ST3136 (n = 1) [45] which differed by a single-locus variant (SLV) in the *rpob* allelic gene_85 (Table 2 and Table S2).

3.4. WGS Detection of Carbapenemase-Encoding Bla_{NDM-1} Plasmid Involved in Horizontal Spread

All the bla_{NDM-1} genes always occurred with bleomycin resistance determinants (ble_{MBL}). The NCBI microbial nucleotide BLAST search of the carbapenemase (NDM-1) and its flanking sequences in all the isolates revealed that the bla_{NDM-1} was located on a 212.3 Kbp multi-replicon plasmid (p18-43_01; accession no. CP023554.1) (Table 2). Comparative analyses via the GView server (Figure 1) tracked and confirmed the presence of similar DNA synteny with 99–100% coverage and identity to the p18-43_01 reference in all the bla_{NDM-1} positive CRKP isolates (Tables 2 and 3).

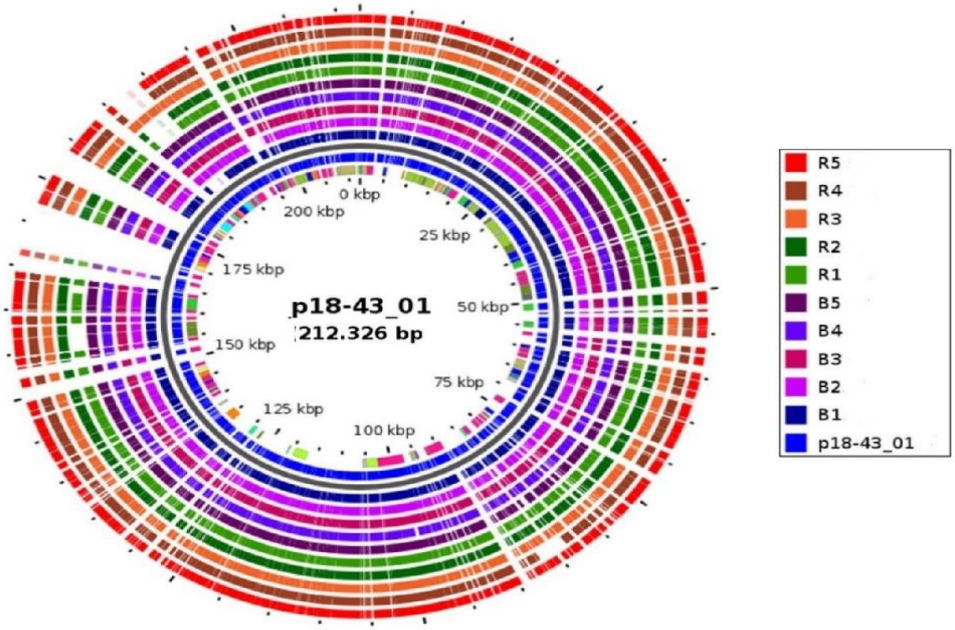

Figure 1. Tracking of plasmid p18-43_01 in NDM-1-encoding CP-*K. pneumoniae* isolates (n = 10). The map was constructed using the GView online server (https://server.gview.ca/). The concentric circles represent comparisons between p18-43_01 and, starting with the inner circle, genome assemblies from *Klebsiella pneumoniae* species (strain ID: B1, B2, B3, B4, B5, R1, R2, R3, R4, and R5). Color codes are given for each strain with a plasmid synteny identity, ranging from 99–100%.

WGS analysis via the PlasmidFinder online platform revealed different plasmid replicon types (Inc FIB(K), Inc FII, Inc FIB (pB171), Inc FII(Yp), ColRNAI, and Col440I) grouped into two different combinations in the CRKP isolates (Table 3). The ISFinder predicted 14 insertion sequences in 5 varied permutations (Figure S1 and Table 3).

Table 3. Genetic environment of carbapenemase-encoding bla_{NDM-1} genetic structure borne on a plasmid with other mobile genetic elements.

Bacterial Strain		Carbapenemase [1]	Plasmids Structure [2] (% Identity) [3]	Plasmid Replicon Types	Plasmid MLST (Pmlsts)	Insertion Sequences	Intact Prophage
No.	ID						
1	B1	NDM-1:bleMBL	p18-43_01-like [100%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI	IncF[K12:A-:B36]	IS6, ISL3, IS256, IS3	10
2	B2	NDM-1:bleMBL	p18-43_01-like [99%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI	IncF[K12:A-:B36]	IS1182, IS5, ISNCY, ISL3	10
3	B3	NDM-1:bleMBL	p18-43_01-like [99%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI	IncF[K12:A-:B36]	IS6, ISL3, IS256, IS3	10
4	B4	NDM-1:bleMBL	p18-43_01-like [100%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI, Col440I	IncF[K12:A-:B36]	ISL3, IS256, IS481, IS21	10
5	B5	NDM-1:bleMBL	p18-43_01-like [100%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI, Col440I	IncF[K12:A-:B36]	IS6, IS66, IS1182, ISL3	10
6	R1	NDM-1:bleMBL	p18-43_01-like [100%]	IncFIB(K), IncFII, IncFIB(pB171),IncFII(Yp), ColRNAI	IncF[K12:A-:B36]	IS6, ISL3, IS256, IS3	10
7	R2	NDM-1:bleMBL	p18-43_01-like [99%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp),ColRNAI	IncF[K12:A-:B36]	IS6, IS66, IS1182, ISL3	10
8	R3	NDM-1:bleMBL	p18-43_01-like [99%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI, Col440I	IncF[K12:A-:B36]	IS6, IS66, IS1182, ISL3	10
9	R4	NDM-1:bleMBL	p18-43_01-like [100%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI	IncF[K12:A-:B36]	IS1595, ISLre2, IS5, IS4	10
10	R5	NDM-1:bleMBL	p18-43_01-like [100%]	IncFIB(K), IncFII, IncFIB(pB171), IncFII(Yp), ColRNAI	IncF[K12:A-:B36]	IS6, ISL3, IS256, IS3	10

[1] All the *bla*NDM-1 genes always occurred with bleomycin resistance determinants (bleMBL). [2] Referred to as "-like" when plasmid sequence is not circularized, but the carbapenemase-encoding contig revealed 99–100% nucleotide identity or synteny to the given plasmid. [3] Unless otherwise stated, all queries are of 100% coverage to subject/reference sequences.

3.5. Phylogenomic Insights

Phylogenomic tree analysis based on the single nucleotide polymorphism (SNPs) differences from whole genomes grouped the isolates into a single clade confirming the high genetic similarity depicted in their epidemiological profiles (capsular serotypes and sequence types) (Figure 2a). The tree depicted a major clade with the nodes (bootstrap in blue circular dots) showing a slight differentiation between the CRKP isolates from both colonized and infected patients on the phylogenomic branch (Figure 2a).

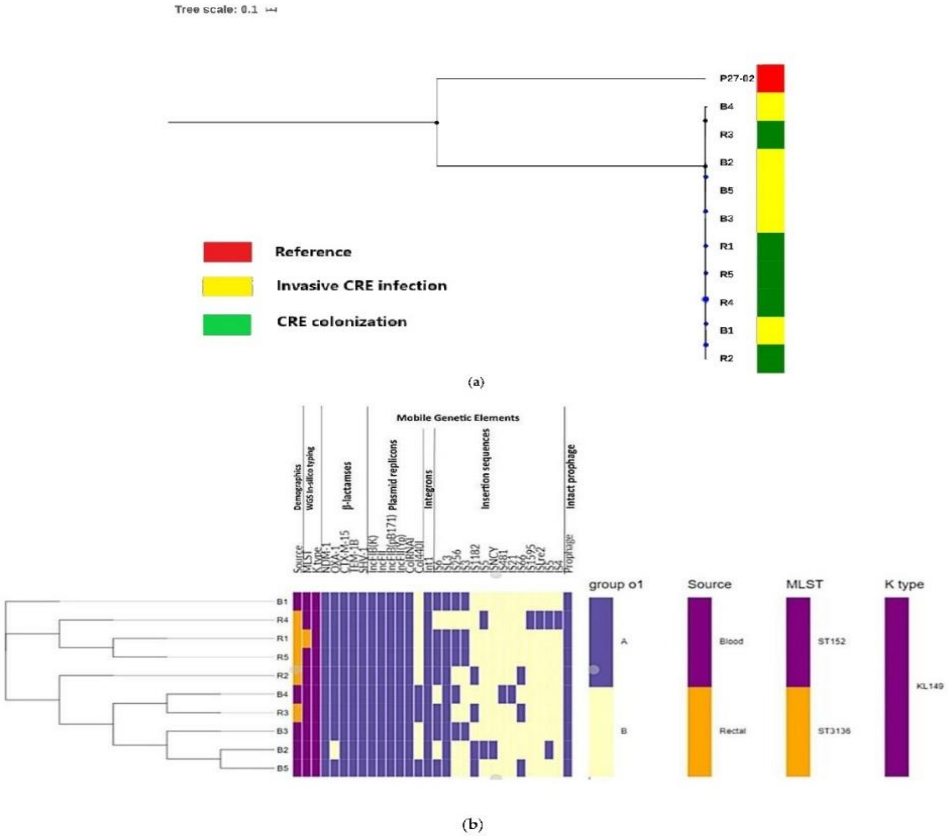

Figure 2. (**a**): A phylogenomic tree based on the single nucleotide polymorphism (SNPs) differences from whole genomes of the 10 carbapenem resistant-K. pneumoniae isolates. The K. quasi-pneumoniae strain P27-02 (accession number: NXHG00000000.1) was rooted and used as the outgroup in the tree. The bootstrap values (in blue dots) for the nodes have been indicated on the tree. The tree depicted a major clade with the node showing the slight differentiation of the isolates in the phylogenetic tree. The scale bar represents one nucleotide substitution per 1000 sequence positions. (**b**): The core genome phylogenetic branch and metadata (demographics; WGS in-silico typing; β-lactamases, plasmid replicons, integrons, insertion sequences, and intact prophages) coupled by the use of Phandango (https://github.com/jameshadfield/phandango/wiki) in isolated carbapenem resistant-*K. pneumoniae* strains (n = 10) from the public hospital in South Africa. The color codes for β-lactamases, plasmid replicons, integrons, insertion sequences and intact prophages (10) showed presence (light blue; A) and absence (yellow; B) in the isolates.

Core genome phylogenetics via Roary coupled with metadata analysis, however, provided useful insights into the slight distinctions between the CRKP isolates (Figure 2b). Specifically, there were differences in the plasmid replicons and insertion sequences in the genomes that were possibly associated with variations in the common genetic backbone of the $bla_{\text{NDM-1}}$.

4. Discussion

The global dissemination of carbapenemase-producing *Enterobacterales* (CPE) poses a serious threat to public health and clinical practice as these bacteria are resistant to the last-resort antibiotics (carbapenems) and cause high mortality [46–48]. The rapid emergence and widespread dissemination of XDR *K. pneumoniae* over recent years are of great concern [49]. As eluded by Yang et al., 2011 antibiotic resistance mediated by plasmids has been increasing at a remarkable rate, especially through genes encoding carbapenemases [50]. Therefore, a thorough understanding of their resistance mechanisms and spread will offer valuable insights into their management. Herein, whole-genome sequence (WGS) analyses were employed to investigate the molecular epidemiology of carbapenem-resistant *K. pneumoniae* strains, focusing on the carbapenem resistance-encoding determinants, mobile genetic support, clonal and epidemiological relationships.

The CRE colonization (or carriage) rate of 1.9% obtained, which was much higher than the 4.2% faecal colonisation with CRKP isolates in a paediatric hospital in South Africa [51]. However, the finding was comparable to a study in the tertiary hospital in Korea by Kang et al. [52], where a CRE carriage rate on admission in 833 adults was 2.8%. Similarly, less than 2% CRKP was recorded in 7- year surveillance study in a primary health care centre in China [53]. However, in Brazil, a 6.8% CRE colonization rate has been reported on admission [54]. The different rates across different settings are not peculiar to the carbapenem-resistant *K. pneumoniae* isolates were extensively drug-resistant (XDR) and harbored the New Delhi Metallo-β-lactamase ($bla_{\text{NDM-1}}$) that mediates resistance to carbapenems (meropenem and imipenem) [55,56]. The detection of $bla_{\text{NDM-1}}$ carbapenemases in both infected and colonized patients has been reported in South Africa [51,57–59], Africa [19,21,56,60] and globally [61]. Furthermore, all the isolates possessed chromosomal mutations, plasmid-mediated quinolone resistance genes, and efflux genes, whose combined effect mediates high-level quinolone resistance [62–64].

In-silico *Klebsiella* typing scheme that represents useful epidemiological markers for *Klebsiella* strain serotyping predicted the same capsular serotypes (KL149-*wzc*:928, *wzi*:110). This finding suggests a possible epidemiological linkage between the isolates [65]. Interestingly, the KL149 serotype has been linked with ESBL producing and carbapenemase positive invasive *K. pneumoniae* isolates from South and Southeast Asia (Hong Kong, India, and Vietnam) [66]. Furthermore, MLST analyses revealed the same clone (ST152) for the CRKP isolates except for one which belonged to the novel ST3136 [45] and differed by a single allelic gene affirming the high epidemiological linkage in the isolates and a possible clonal expansion of ST152. While there were no studies on *K. pneumoniae* capsular serotypes in the country for comparison, a study by Agyapong et al. [63] on *K. pneumoniae* isolates from Ghana showed a 100% concordance between 2 typing schemes and reported that ST152 isolates contained a similar capsular serotype to that shown in this study. The slight differences in the two typing results indicate that the MLST is more resolute than capsular polysaccharide serotyping.

Analysis of the genetic backbone of the carbapenemase and its flanking sequences linked $bla_{\text{NDM-1}}$ to a mobile element p18-43_01 (multi-replicon plasmid) [58] in all CRKP isolates (Figure 1, Tables 2 and 3). This p18-43_01 plasmid has been reported for the spread of $bla_{\text{NDM-1}}$ in CRE (including *K. pneumoniae, K. michiganensis, Serratia marcescens, Citrobacter freundii, and Enterobacter* spp.) in South Africa [58]. This implicates $bla_{\text{NDM-1}}$ acquisition as well as nosocomial spread and development of an XDR genetic lineage in different species. This is of concern in terms of our last-resort antibiotic arsenals for the treatment of drug-resistant bacteria in the country.

The different combinations of genetic support such as the varied plasmid replicons (Inc FIB(K), Inc FII, Inc FIB(pB171), Inc FII(Yp), ColRNAI, and Col440I) and insertion sequences support the assertion of the versatility in this $bla_{\text{NDM-1}}$ encoding plasmid backbone structure enabling a local horizontal

transfer between isolates (Figure S1 and Table 3). Wenzi Bi. et al., in 2017, reported the dissemination and epidemicity of clinical XDR *K. pneumoniae* strains result from horizontal transmission of multiple resistance determinants via IncF plasmids [67]. More so, the multi replicon nature of the p18-43_01 plasmid was not peculiar, as a diversity of plasmid incompatibility groups (Inc), including IncX, IncR, IncN, IncL/M, IncA/C, and IncF have been linked with NDM variants [26,27,68,69]. The intra- and inter-clonal spread of the $bla_{\text{NDM-1}}$ plasmid-bearing structure in both ST152 and ST3136 support the findings that NDM-positive *K. pneumoniae* strains of African origin have been multi-clonal [60]. Further insight into the host adaptation and evolution of the p18-43_01 plasmid in the CRKP isolates would require DNA sequence circularization.

Comparative phylogenomic analysis of the 10 CRKP isolates with WGS SNPs analysis corroborated their close epidemiological profiles (capsular serotypes and sequence types), which showed less genetic variation in isolates recovered from the colonized and infected patients (Figure 2a). This has been reported in many species, including *Acinetobacter baumannii* [70]. Furthermore, core genome phylogenetics, combined with metadata analysis, provided useful insights into the slight distinctions (replicons and insertion sequences) between the CRKP isolates (Figure 2b) [71]. This is possibly associated with variations in the common genetic backbone of the $bla_{\text{NDM-1}}$, supporting its versatility via a local horizontal transfer and subsequent evolution in their host by recombination events [72,73]. This reiterates the need for further circularization and annotation of the plasmid DNA using sequencing techniques that provide long-read sequences to offer insights into its evolution and spread [74]. Moreover, further larger epidemiological studies should be conducted in the province to trace the primary source(s) of their spread, possibly through frequent contact with healthcare workers and the movement of colonized patients among different healthcare settings [75,76].

5. Conclusions

The acquisition of resistance-encoding plasmids, horizontal transfer and clonal dissemination facilitate the spread of carbapenemases in KZN, South Africa, which is very worrisome for infectious disease management and highlights the importance of early detection of CRE and targeted infection control measures to prevent dissemination. Further studies would elucidate the extent of CPE dissemination in this region and identify the primary source(s) of their spread. Such knowledge will enable the development of effective countermeasures against the spread of CPE.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/1/137/s1, Table S1: Genomic attributes of the 10 sequenced CRKP isolates. Table S2: Genomic analysis of other resistomes in Klebsiella pneumoniae ($n = 10$) from WGS data. Table S3: A table showing the diversity of MLST (ST types) and allelic profiles of the 7 housekeeping genes in the *XDR K. pneumoniae* isolates ($n = 10$). Figure S1: The total number of each predicted insertion sequence (IS) families via the ISFINDER database platform (https://isfinder.biotoul.fr/) in the isolates.

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Appendix VII

CRE-Related manuscript 2

AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Resource Announcements

GENOME SEQUENCES

Whole-Genome Sequence of a Novel Sequence Type 3136 Carbapenem-Resistant *Klebsiella pneumoniae* Strain Isolated from a Hospitalized Patient in Durban, South Africa

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ABSTRACT Here, we describe the genome sequence of a novel sequence type 3136 (ST3136) *Klebsiella pneumoniae* strain isolated in South Africa. The 5,574,236-bp genome harbored 23 resistance determinants and 12 virulence factors that are of cardinal importance to infections. The genomics of *Klebsiella pneumoniae* offer valuable insights into its pathogenicity.

Klebsiella pneumoniae is an encapsulated, nonmotile, Gram-negative pathogen of great medical significance that is implicated in a wide variety of infections in humans, including urinary tract infections, pneumonia, bacteremia, meningitis, and liver abscesses (1, 2). Carbapenem-resistant *Enterobacteriaceae* species, such as *K. pneumoniae*, are included in the World Health Organization global priority pathogen list, and carbapenems are usually the last-resort antibiotic by virtue of their broad spectrum of activity (3, 4). Here, we present the emergence of sequence type 3136 (ST3136), a novel carbapenem-resistant sequence type isolated from a rectal swab of an adult female patient in an intensive care unit of a public hospital in Durban, South Africa.

A chromogenic screening medium, Chromid Carba Smart (bioMérieux, France), was used for the isolation of the strain. Phenotypic microbial identification and antibiotic susceptibility testing were performed using the Vitek 2 system (bioMérieux, France). The Rapidec Carba NP (bioMérieux, France) test was used to detect carbapenem hydrolysis by carbapenemase-producing bacteria. The strain was grown on nutrient agar (Oxoid, England) and incubated overnight at 37°C prior to genomic DNA extraction. The QIAamp DNA minikit (Qiagen, Germany) was used to extract the total genomic DNA. A paired-end library (2 × 300 bp) was prepared using a Nextera XT DNA sample preparation kit, and whole-genome sequencing (WGS) was carried out on a MiSeq machine (Illumina, USA). The sequenced reads (3,325,060 reads) were quality trimmed using Sickle version 1.33 (https://github.com/najoshi/sickle) and *de novo* assembled using SPAdes version 3.11 (5). All resultant contiguous sequences were then submitted to GenBank, where gene annotation was implemented using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (6). Furthermore, the assembled genome was submitted to the *Klebsiella* multilocus sequence type (MLST) database at Institut Pasteur (Paris, France) (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) to assign the new sequence type (7). The resistance genes and virulence factors were predicted using ResFinder (8) through the GoSeqIt tools Web platform (https://www.goseqit.com/web-services/) and the VirulenceFinder database (http://www.mgc.ac.cn/VFs/) (9), respectively.

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The assembled genome yielded 180 contiguous sequences of longer than 200 bp, covering 5,574,236 bp, with a G+C content of 57.37%, an N_{50} value of 103,476 bp, and a longest contig size of 283,489 bp. The total number of 5,771 genes predicted by PGAP includes 5,427 protein-coding genes, 235 pseudogenes, and 109 RNA genes. The novel sequence type was defined as ST3136 by the *Klebsiella* MLST database (identifier 6431). Acquired antibiotic resistance genes to aminoglycosides [*aac(6')Ib-cr, aac(3)-IIa, aac(6')Ib-cr, aadA16, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id*, and *rmtC*), β-lactams ($bla_{CTX-M-15}$, bla_{NDM-1}, bla_{OXA-1}, bla_{SHV-1}, and bla_{TEM-1B}), fluoroquinolones [*aac(6')-Ib-cr, oqxA, oqxB*, and *qnrB6*], fosfomycin (*fosA*), macrolides [*mph(A)*], phenicol (*catA1* and *catB4*), rifampin (*ARR-3*), sulfonamides (*sul1* and *sul2*), and trimethoprim (*dfrA27*) were found, which confirmed the ability of WGS to make accurate resistome predictions. The VirulenceFinder database determined the following virulence factors: type I fimbriae (*fimG*) and type III fimbriae (*mrkA, mrkB, mrkC, mrkD*, and *mrkF*), lipoproteins (*pulG, pulO*, and *pulS*), and capsular polysaccharides (*wza, wzc*, and *wzi*), which contribute to the bacterium's ability to adhere to, lyse, and invade host tissues, respectively (10). The valuable information offered by genomics provides a good step toward a better understanding of the pathogenicity of multidrug-resistant strains.

This study was approved by the Biomedical Research Ethics Committee (approval BE453/15) of the College of Health Sciences, University of KwaZulu-Natal (UKZN).

Data availability. This whole-genome shotgun project has been deposited in DDBJ/ENA/GenBank under the accession no. QMCA00000000. The version described in this paper is version QMCA01000000. The raw sequencing reads have been submitted to the SRA under the accession no. SRR8079358.

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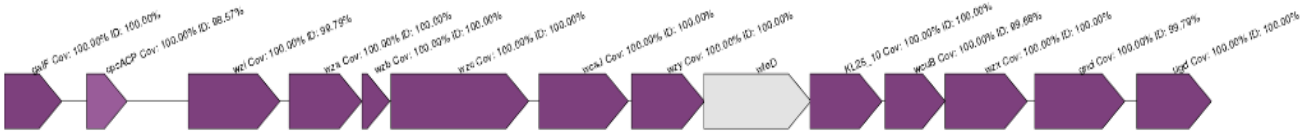
Appendix IX – CIDRAP ASP WEBINAR

Appendix – X – INDVIDUAL IMAGES FOR FIGURE S2

H 2737

H2737 **Best locus:** KL25 **Match confidence :** High **Cov :** 100.00% **ID :** 99.17% **Genes:** 13 / 14

KL25 reference :



Other genes found in locus : 0 **Other genes found outside locus :** 3

Allelic type : wzc: 26 wzi: 141

Assembly pieces : Download as FASTA

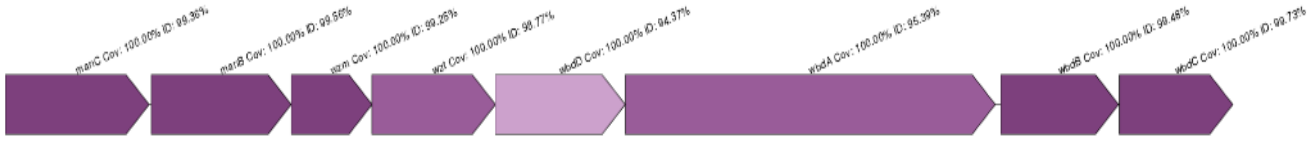
KL25 reference size : 18881

Length discrepancy : +3 bp

Contig name	Start position	End position	Length
contig00001	447623	466506	18884

H2737 **Best locus:** O5 **Match confidence :** High **Cov :** 100.00% **ID :** 94.21% **Genes:** 8 / 8

O5 reference :



Other genes found in locus : 0 **Other genes found outside locus :** 0

Assembly pieces : Download as FASTA

O5 reference size : 12084

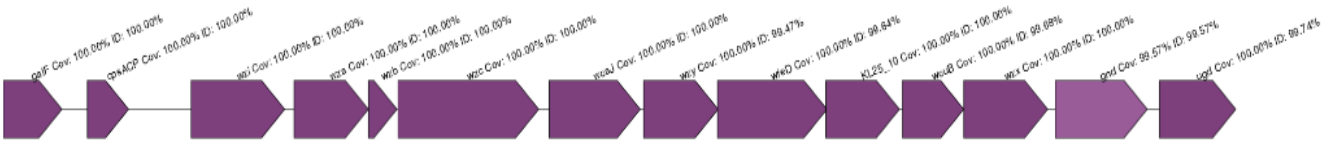
Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00001	469536	481619	12084

H 2745

H2745 **Best locus:** KL25 **Match confidence** : Very high **Cov** : 100.00% **ID** : 98.53% **Genes:** 14 / 14

KL25 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 2

Allelic type : wzc: 26 wzi: 133

Assembly pieces : Download as FASTA

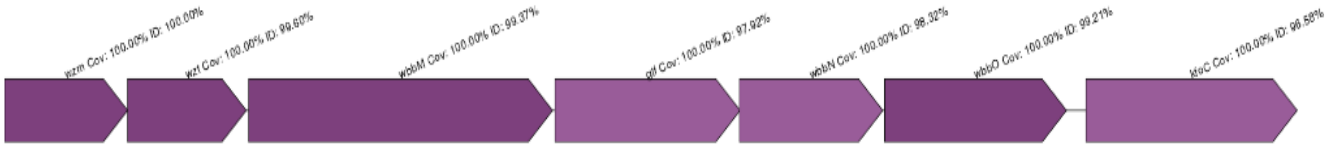
KL25 reference size : 18881

Length discrepancy : +3 bp

Contig name	Start position	End position	Length
contig00008	107354	126237	18884

H2745 **Best locus:** O1v1 **Match confidence** : Very high **Cov** : 100.00% **ID** : 98.03% **Genes:** 7 / 7

O1v1 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 2

Assembly pieces : Download as FASTA

O1v1 reference size : 8064

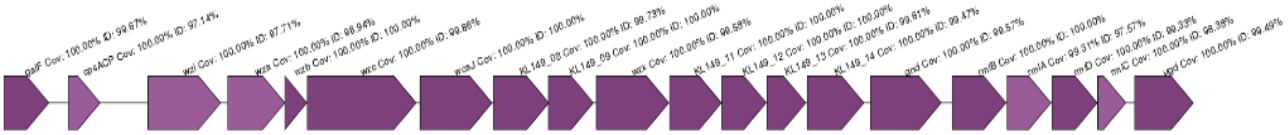
Length discrepancy : -2 bp

Contig name	Start position	End position	Length
contig00008	95144	103205	8062

H 4767

H4767 **Best locus:** KL149 **Match confidence** : Very high **Cov** : 100.00% **ID** : 97.68% **Genes:** 20 / 20

KL149 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 4

Allelic type : wzc: 928 wzi: 452

Assembly pieces : Download as FASTA

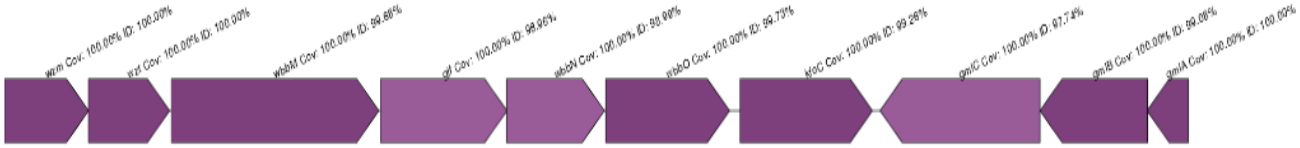
KL149 reference size : 23691

Length discrepancy : +1 bp

Contig name	Start position	End position	Length
contig00028	27771	51462	23692

H4767 **Best locus:** O1v2 **Match confidence** : Very high **Cov** : 100.00% **ID** : 99.01% **Genes:** 10 / 10

O1v2 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 6

Assembly pieces : Download as FASTA

O1v2 reference size : 10812

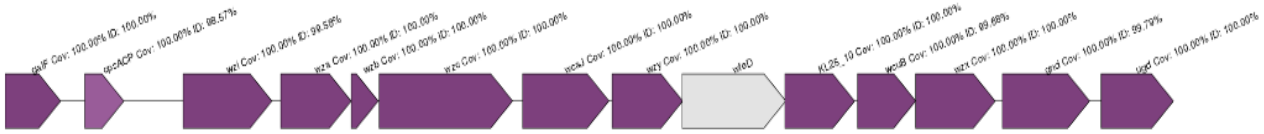
Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00028	13964	24775	10812

H 8770

H8770 **Best locus:** KL25 **Match confidence :** High **Cov :** 100.00% **ID :** 99.16% **Genes:** 13 / 14

KL25 reference :



Other genes found in locus : 0 **Other genes found outside locus :** 3

Allelic type : wzc: 26 wzi: 141

Assembly pieces : Download as FASTA

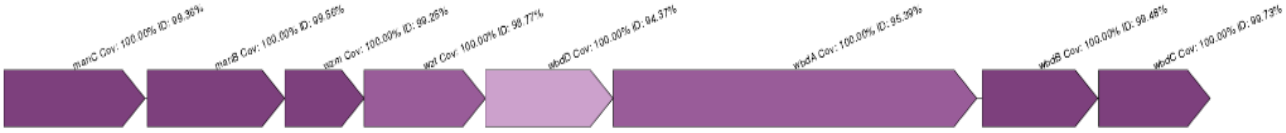
KL25 reference size : 18881

Length discrepancy : +3 bp

Contig name	Start position	End position	Length
contig00013	48167	67050	18884

H8770 **Best locus:** O5 **Match confidence :** High **Cov :** 100.00% **ID :** 94.21% **Genes:** 8 / 8

O5 reference :



Other genes found in locus : 0 **Other genes found outside locus :** 0

Assembly pieces : Download as FASTA

O5 reference size : 12084

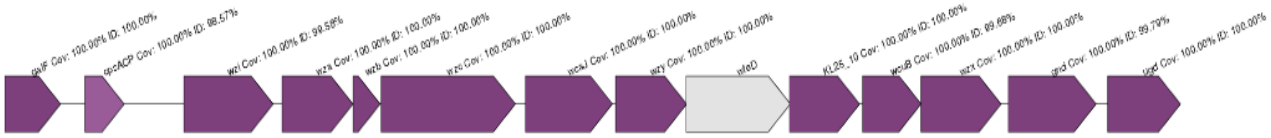
Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00013	70080	82163	12084

H 8773

H8773R **Best locus:** KL25 **Match confidence** : High **Cov** : 100.00% **ID** : 99.16% **Genes:** 13 / 14

KL25 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 3

Allelic type : wzc: 26 wzi: 141

Assembly pieces : Download as FASTA

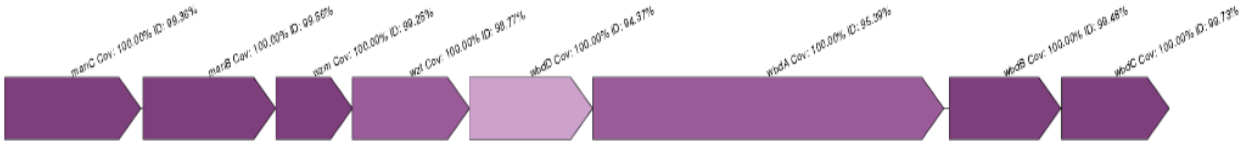
KL25 reference size : 18881

Length discrepancy : +3 bp

Contig name	Start position	End position	Length
contig00002	447860	466743	18884

H8773R **Best locus:** O5 **Match confidence** : High **Cov** : 100.00% **ID** : 94.21% **Genes:** 8 / 8

O5 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 0

Assembly pieces : Download as FASTA

O5 reference size : 12084

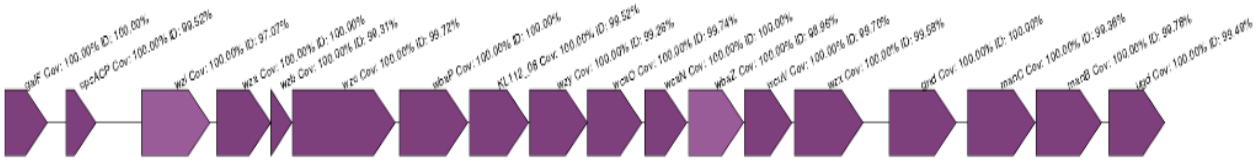
Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00002	469773	481856	12084

H2850 K

H2850 **Best locus:** KL112 **Match confidence** : Very high **Cov** : 100.00% **ID** : 98.64% **Genes:** 18 / 18

KL112 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 2

Allelic type : wzc: 923 wzi: 93

Assembly pieces : Download as FASTA

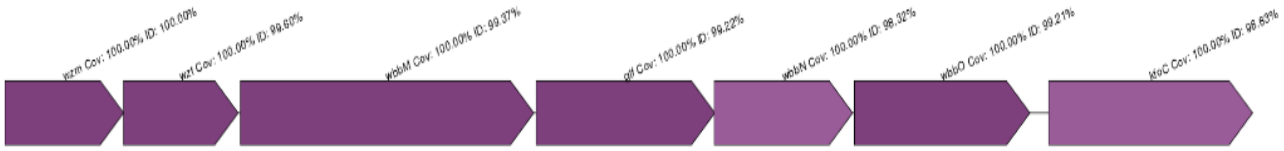
KL112 reference size : 24370

Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00001	363448	387817	24370

H2850 **Best locus:** O1v1 **Match confidence** : Very high **Cov** : 100.00% **ID** : 98.52% **Genes:** 7 / 7

O1v1 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 4

Assembly pieces : Download as FASTA

O1v1 reference size : 8064

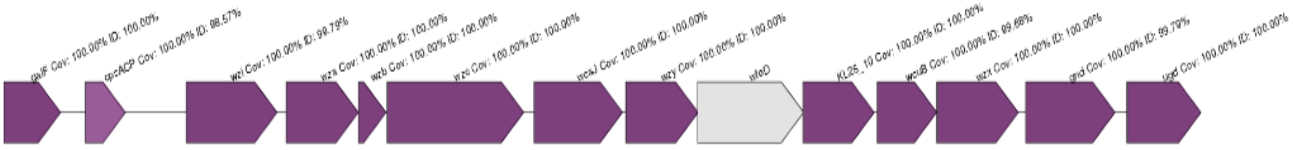
Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00001	390816	398879	8064

H8844R

H8844R **Best locus:** KL25 **Match confidence** : High **Cov** : 100.00% **ID** : 99.17% **Genes:** 13 / 14

KL25 reference :



Other genes found in locus : 0 ▼ **Other genes found outside locus** : 3 ▼

Allelic type : wzc: 26 wzi: 141

Assembly pieces : Download as FASTA

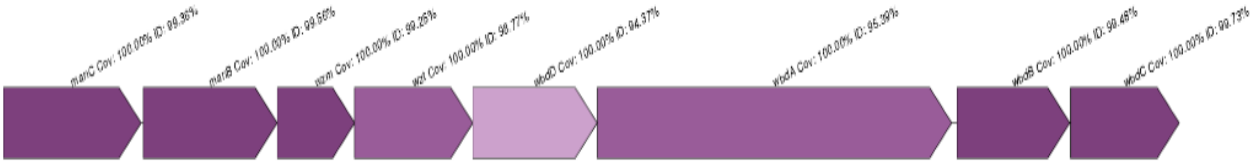
KL25 reference size : 18881

Length discrepancy : +3 bp

Contig name	Start position	End position	Length
contig00004	243162	262045	18884

H8844R **Best locus:** O5 **Match confidence** : High **Cov** : 100.00% **ID** : 94.21% **Genes:** 8 / 8

O5 reference :



Other genes found in locus : 0 ▼ **Other genes found outside locus** : 0 ▼

Assembly pieces : Download as FASTA

O5 reference size : 12084

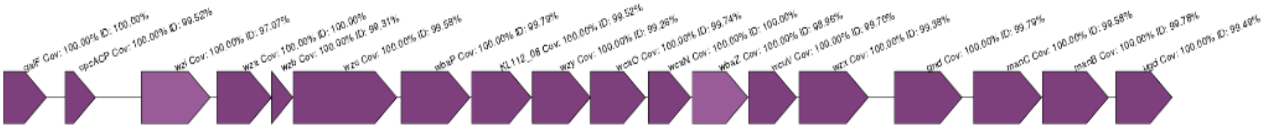
Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00004	265075	277158	12084

H8860

H8860 **Best locus:** KL112 **Match confidence** : Very high **Cov** : 100.00% **ID** : 98.63% **Genes:** 18 / 18

KL112 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 2

Allelic type : wzc: 923 wzi: 93

Assembly pieces : Download as FASTA

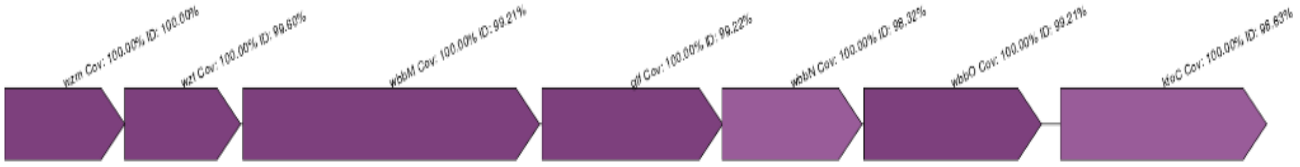
KL112 reference size : 24370

Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00009	41471	65840	24370

H8860 **Best locus:** O1v1 **Match confidence** : Very high **Cov** : 100.00% **ID** : 98.51% **Genes:** 7 / 7

O1v1 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 4

Assembly pieces : Download as FASTA

O1v1 reference size : 8064

Length discrepancy : 0 bp

Contig name	Start position	End position	Length
contig00009	68839	76902	8064

INF 1 a

INF1-a **Best locus:** KL146 **Match confidence** : Good **Cov** : 99.79% **ID** : 99.98% **Genes:** 17 / 20

KL146 reference :

Other genes found in locus : 1 **Other genes found outside locus** : 7

Allelic type : wzc: Not found wzi: 398

Assembly pieces : Download as FASTA

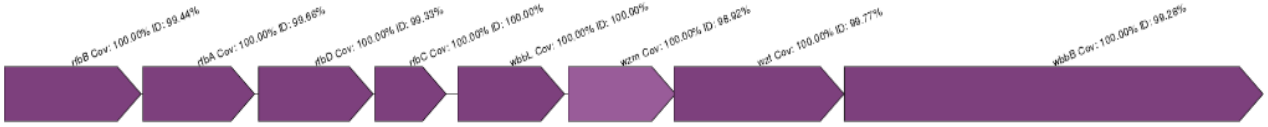
KL146 reference size : 26375

Length discrepancy : n/a

Contig name	Start position	End position	Length
contig00383	1	2702	2702
contig00289	1344	5191	3848
contig00058	7669	25221	17553
contig00414	1	2220	2220

INF1-a **Best locus:** O12 **Match confidence** : Very high **Cov** : 100.00% **ID** : 98.96% **Genes:** 8 / 8

O12 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 1

Assembly pieces : Download as FASTA

O12 reference size : 9797

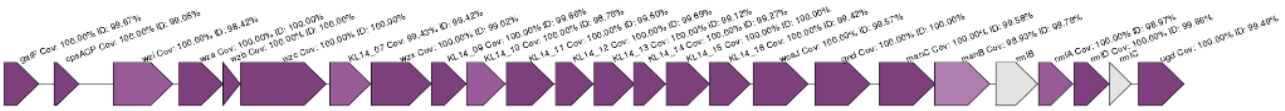
Length discrepancy : +1 bp

Contig name	Start position	End position	Length
contig00037	1438	11235	9798

INF 3 b

INF3b **Best locus:** KL14 **Match confidence** : Low **Cov** : 99.67% **ID** : 96.73% **Genes:** 23 / 25

KL14 reference :



Other genes found in locus : 2 **Other genes found outside locus** : 1

Allelic type : wzc: 15 wzi: 46

Assembly pieces : Download as FASTA

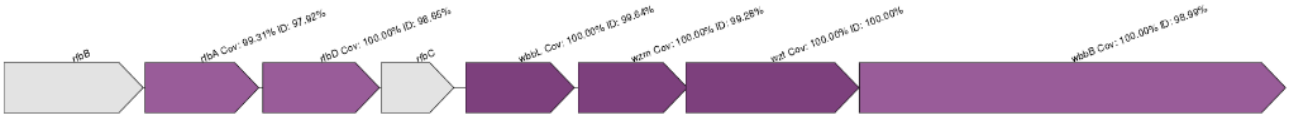
KL14 reference size : 30094

Length discrepancy : n/a

Contig name	Start position	End position	Length
contig00226	1	2352	2352
contig00010	56230	57857	1628
contig00046	9469	35449	25981
contig00224	1	2366	2366
contig00179	1	1506	1506

INF3b **Best locus:** O12 **Match confidence** : Good **Cov** : 98.99% **ID** : 93.29% **Genes:** 6 / 8

O12 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 4

Assembly pieces : Download as FASTA

O12 reference size : 9797

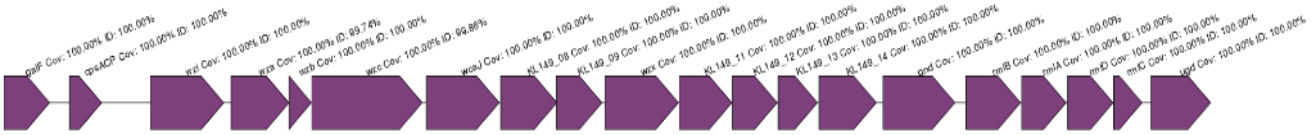
Length discrepancy : n/a

Contig name	Start position	End position	Length
contig00010	56220	58070	1851
contig00224	1	2366	2366
contig00059	25104	31616	6513
contig00179	4302	4987	686

US 2

US2 **Best locus:** KL149 **Match confidence** : Good **Cov** : 100.00% **ID** : 99.97% **Genes:** 20 / 20

KL149 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 1

Allelic type : wzc: 928 wzi: 110

Assembly pieces : Download as FASTA

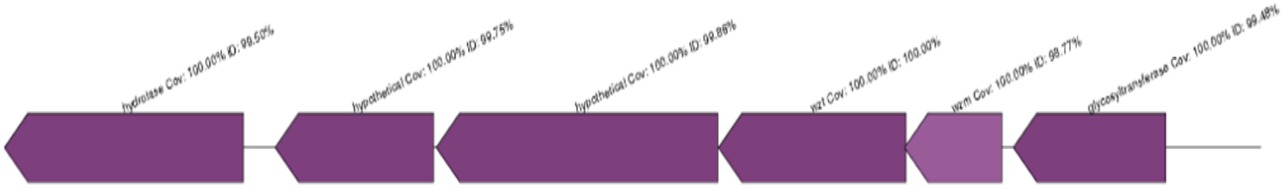
KL149 reference size : 23691

Length discrepancy : n/a

Contig name	Start position	End position	Length
contig00019	32572	51881	19310
contig00185	1	4386	4386

US2 **Best locus:** O4 **Match confidence** : Very high **Cov** : 100.00% **ID** : 99.71% **Genes:** 6 / 6

O4 reference :



Other genes found in locus : 0 **Other genes found outside locus** : 4

Assembly pieces : Download as FASTA

O4 reference size : 9449

Length discrepancy : +1 bp

Contig name	Start position	End position	Length
contig00019	20835	30284	9450