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# MOLECULAR CHARACTERIZATION OF INTEGRONS AND THEIR ASSOCIATED GENE CASSETTES IN MULTIDRUG-RESISTANT ENTEROBACTERIACEAE ISOLATES FROM ENVIRONMENTAL SOURCES AND THE EXPLORATION OF ANTIBIOTIC COMBINATION AGAINST SOME RESISTANT STRAINS

**FOLAKE TEMITOPE FADARE**



**DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY**

**FACULTY OF SCIENCE AND AGRICULTURE**

**UNIVERSITY OF FORT HARE**

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**FOLAKE TEMITOPE FADARE**

**A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIOLOGY**

**DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY**



**FACU/LTY OF SCIENCE AND AGRICULTURE**

**UNIVERSITY OF FORT HARE**

**ALICE, 5700**

**SUPERVISOR: PROF ANTHONY I. OKOH**

**2023**

## CERTIFICATION

This thesis titled ‘Molecular characterization of integrons and their associated gene cassettes in multidrug-resistant Enterobacteriaceae isolates from environmental sources, and the exploration of antibiotic combination against some resistant strains’ meets the regulation governing the award of the degree of Doctor of Philosophy in Microbiology of the University of Fort Hare and is approved for its contribution to scientific knowledge and scholarly presentation.



_____ ___14th March 2023_____

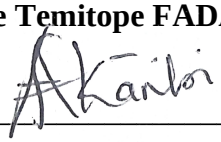
**Prof A.I Okoh** Date

Supervisor

## DECLARATION 1

I, the undersigned, now declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained therein is an original work with the exemptions to the citations. I declare that this work has not been submitted to any other university or institution entirely or partly for awarding any degree or diploma.

Name: **Folake Temitope FADARE**

Signature: 

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I, **Folake Temitope Fadare**, with student number 201608581, now declare that I am fully aware of the University of Fort Hare′s policy on plagiarism, and I have taken every precaution to comply with the regulations.

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## DECLARATION 3- RESEARCH ETHICAL CLEARANCE

I, **Folake Temitope Fadare,** with student number 201608581, now declare that I am fully aware of the University of Fort Hare′s policy on research ethics, and I have taken every precaution to comply with the regulations. I confirm that the Faculty Research Ethics Committee issued an ethical research clearance with project number OKO011SFAD01. The ethical certificate is attached (Appendix 1).

Signature:  Date: _14th March 2023_____

## DECLARATION 4- PUBLICATIONS

Contributions to publications are given in detail, which are parts of the chapters given in this thesis (these involve publications and manuscripts submitted with the detailed contribution of each author to the experimental work and the writing of each manuscript for publication).

**Publication 1**

Okoh, A.I. and Fadare, F.T 2022. Integrons as emerging contaminants facilitating the widespread of antimicrobial resistance in Enterobacteriaceae: a review. (Published in *Advances in Biomedical and Health Sciences*).

I conceived and designed the review under the guidance of my supervisor, Prof A.I. Okoh, who proofread the manuscript through the publication process. The study is presented in this thesis in Chapter 2.



**Publication 2**

Fadare, F.T., Elsheikh, E.A.E., and Okoh, A.I. 2022. *In vitro* assessment of the combination of antibiotics against some integron-harbouring Enterobacteriaceae from environmental sources (Published in *Antibiotics*).

I conceived and designed the work; analyzed and interpreted the data under the guidance of Prof A.I. Okoh. My supervisor and Prof E.A.E Elsheikh proofread the manuscript through the publication process. The study is presented in this thesis in Chapter 8.

**Manuscript 1**

Abundance, molecular characterization of integrons and its associated gene cassettes in *Klebsiella pneumoniae* and *K. oxytoca* recovered from diverse environmental matrices (Under review in *Scientific reports*). I conceived and designed the work; analyzed and

interpreted the data under the guidance of Prof A.I. Okoh. The study is presented in this thesis in Chapter 4.

Manuscript 2

Integron-harbouring *Citrobacter* spp. isolated from rivers, animal dropping, wastewater and hospital effluents: A public health risk (submitted to *Journal of Environmental Sciences*). I conceived and designed the work; anaylzed and interpreted the data under the guidance of Prof A.I. Okoh. The study is presented in this thesis in Chapter 5.

Manuscript 3

Multidrug-resistant *Enterobacter cloacae* harbouring integrons recovered from diverse environmental matrices in South Africa (under review in *International Journal of Environmental Science and Technology*). I conceived and designed the work; analyzed and interpreted the data under the guidance of Prof A.I. Okoh. The study is presented in this thesis in Chapter 6.



Manuscript 4

Classes 1 and 2 integrons in antibiotic-resistant *Escherichia coli* recovered from aquatic and animal origins in the Eastern Cape Province, South Africa (Submitted to *Journal of Exposure Science and Environmental Epidemiology*). I conceived and designed the work; analyzed and interpreted the data under the guidance of Prof A.I. Okoh. The study is presented in this thesis in Chapter 7.

Signature: _____ Date: __14th March 2023_____

## DEDICATION

This thesis is dedicated to the Almighty God, my alpha and omega, for making this dream come true. May your name be forever glorified for strength, guidance, and knowledge. You are indeed Yahweh!

I also dedicate this to my late brother, Akanbi Oluwatoyin Olufunsho. Achieving this feat has always been your dream, and I know you would have been super proud! I miss you bro.

## ACKNOWLEDGEMENTS

Firstly, I acknowledge the Almighty God for his goodness and kindness throughout this journey. For being my very present help in times of challenges, especially when the tides seem high and almost consuming, he steps in just at the nick of time! For never being behind, receive all the glory, honour, and adoration.

I appreciate my supervisor, Prof Anthony I Okoh, for all the academic and financial support he provided during this study. I am thankful for his critical remarks, helpful suggestions, and complete confidence in me and this work. I also acknowledge the South Africa Medical Research Council for the funding of this research.

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I sincerely appreciate Dr Ayangbenro Segun Ayansina, Dr Olusesan Adelabu, and Mr Sulayman Taofiq for always being eager to teach and assist. The times sacrificed for virtual training and troubleshooting made today a reality, for which I am grateful.

I appreciate all friends turned family in South Africa, Prof and Mrs Osunkunle, Dr and Mrs Adefisoye, Dr and Mrs Okaiyeto, Dr and Dr Mrs Bayegunhi, Dr and Mrs Adeniji, Dr and Dr Mrs Olisah, Sis Arinola Olaonipekun, Dr Mrs Justin Fri, Dr Bunmi Popoola, and Dr Chinemerem Ohoro. They keep urging and cheering me on in different ways, making this journey bearable. May God continue to bless and reward you abundantly.

Finally, words fail me to articulate my depth of appreciation to my entire family. My parents, Mr and Mrs J.O Akanbi and Prof. and Mrs S. O Fadare, your encouragement in cash and kind with your prayers are highly appreciated. Oh! How blessed I am with the gifts of such caring parents. To my cute handsome prince, Obafemi Fadare, who was born at the beginning of this

journey, you have always showered me with much love despite having to sacrifice months of our togetherness so mummy could finish her lab work. I cannot forget one of your remarks, ‘mummy does not like sleeping; you are always on your laptop!’, out of a genuine concern for me to have some rest. Thank you, son. To my amiable beautiful princess, Oluwadarasimi Fadare, you have grown much more during the completion of this study. I appreciate your maturity and understanding of my tight study schedule. You always encourage your little brother to ‘allow mummy to do her work’. Special appreciation to my husband of life, Dr Taiwo O. Fadare. Your kind of man is especially rare, and I am eternally grateful for your presence in my life. Sweetheart, allow me to say, your face show, your heart is pure, and I must greet you, especially! With your love and faithfulness throughout this study, when we mainly were apart, I say thank you. For always nudging me ahead even when I wanted to quit! You even took over the complete care of our kids for an entire year so that I could complete the lab work, coupled with your hectic workload. Today would not have been a reality if not for your unwavering support. You were dear and there!

## LIST OF ACRONYMS

| | |
|---|---|
| ARB | Antibiotic-resistant bacteria |
| 59-be | 59-basepair element |
| AEMREG | Applied and Environmental Research Group |
| AMR | Antimicrobial resistance |
| ARGs | Antibiotic resistance genes |
| ATCC | American Type Culture Collection |
| attC | cassette-associated recombination site |
| attI | integron-associated recombination site |
| BHI | Brain Heart Infusion |
| CFU | Colony Forming Unit |
| CLSI | Clinical and Laboratory Standard Institutes |
| CS | Conserved Segment |
| DDST | Double-disk synergy test |
| DDT | Diffusion technique |
| DNA | Deoxyribonucleic acid |
| ECP | Eastern Cape Province |
| EDTA | Ethylenediaminetetraacetic acid |
| ERIC | Enterobacterial Repetitive Intergenic Consensus |
| ESBL | Extended-spectrum β-lactamase |
| FD | Animal faecal droppings |
| FICIs | Fractional inhibitory concentration indices |
| GC | Gene cassette |
| HGT | Horizontal gene transfer |
| HWW | Hospital wastewater |
| ICE | Integrative and conjugative element |
| IMP | Imipenemase (IMP) |
| intI | Integron integrase gene |
| IRs | Imperfect inverted repeat sequences |
| IRi | Integron inverted repeat |
| IRt | Transposition inverted repeat |
| IS | Insertion sequences |
| IVRs | Internal variable regions |
| MAR | Multiple antibiotic resistance |
| MARI | Multiple antibiotic resistance index |
| MARP | Multiple antibiotic resistance phenotype |
| MBL | Metallo β-lactamase |
| MDR | Multidrug-resistant |
| MGE | Mobile genetic elements |
| MHA | Mueller Hinton Agar |
| MHB | Mueller Hinton Broth |
| MIC | Minimum inhibitory concentration |

| | |
|---|---|
| NDM | New Delhi MBL |
| ORF | Open reading frame |
| Pc | Constitutive promoter |
| PCR | Polymerase chain reaction |
| RFLP | Restriction fragment length polymorphism |
| ROK | Rate of kill |
| SC | Sterility control |
| SPSS | Statistical Package for the Social Sciences |
| TKAs | Time-kill assays |
| tni module | Transposition gene module |
| UPGMA | Unweighted pair group method with arithmetic mean |
| UTI | Urinary tract infections |
| VIM | Verona integrin-encoded MBL |
| WHO | World Health Organization |
| WWTP | wastewater treatment plant effluents |

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## GENERAL ABSTRACT

Globally, the increasing rate of antimicrobial resistance against our currently available drugs has been a serious public concern. Due to the selective nature of antibiotics, bacteria are expected to develop resistance against them over time, but the current scourge of antimicrobial resistance is aggravated by factors other than the expected evolutionary trend. The use and overuse of antibiotics in clinical and agricultural contexts have led to the fast rise of multidrug-resistant (MDR) microorganisms. A scenario that necessitates an upsurge in the clinical failures observed with our current drug arsenals is expected to rise if left unchecked. One of the significant drivers implicated in the spread of antimicrobial resistance genes is the integrons. These are mobile genetic elements found on pathogenicity islands, transposons, and plasmids, easing their distribution among various bacteria. They are considered efficient gene expression systems that naturally capture, integrate gene cassettes (GCs) and immediately express the captured antimicrobial resistance genes on the GCs due to the inherent promoters on their structures. Integrons have been known to confer resistance against most classes of antibiotics. These include all known β-lactams, chloramphenicol, trimethoprim, erythromycin, aminoglycosides, quinolones, streptothricin, lincomycin, rifampicin, fosfomycin, and antiseptics of the quaternary ammonium compound family. They have been detected in bacterial populations under direct or indirect antibiotic pressure in clinical, agricultural, and environmental contexts.

The emergence of MDR in Enterobacteriaceae is a critical public health issue that has attracted the World Health Organization (WHO), which classified them as one of the critical priority pathogens urgently requiring new antibiotics. The resistance phenomenon has proven most of the current antibiotics ineffective, further compounded by the slow pace of the discovery of new antibiotics, necessitating the hunt for new, practical remedies. One of such is the exploration of synergy among existing antibiotics. Two medications combined have a

higher impact, thereby allowing current antibiotics to be salvaged for use in treating MDR bacteria, even if the bacteria are resistant against one or both antibiotics separately.

Hence, this research focused on the occurrence and prevalence of multidrug resistance and the characterization of integrons and their associated gene cassettes in members of Enterobacteriaceae, including *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Escherichia coli*, and *Citrobacter* spp. recovered from animal droppings, rivers, and effluents of hospital and wastewater treatment plants in Eastern Cape Province, South Africa. The inhibitory effect of combining two drugs belonging to different antibiotic classes to obtain a possible potentiating effect against some multidrug-resistant Enterobacteriaceae isolates harbouring integrons were examined and studied.

The isolates were identified using the conventional molecular Polymerase Chain Reaction with specific primers. The antimicrobial resistance profile and the production of Extended-spectrum and metallo β-lactamase were detected using disk diffusion technique (DDT), double-disk synergy test (DDST), and ethylenediaminetetraacetic acid (EDTA) tests, respectively. The PCR-based screening method, DNA sequencing analyses, and restriction fragment length polymorphism (RFLP) were used to characterize the integrons and their associated GCs. Furthermore, Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR determined the genotypic relationships between some specific species. The various antibiotics' minimum inhibitory concentration (MIC) was determined using the broth microdilution, while the checkerboard method was used to determine the fractional inhibitory concentration indices (FICIs). The time-kill assays (TKAs) were further used to confirm the synergism observed from the checkerboard assays.



Most of the isolates were resistant against most antibiotics tested and were considered MDR. The least resistance was observed against imipenem, a carbapenem, one of the drugs of last

resort. Also present were the ESBL and MBL producers, with a few isolates co-producing the enzymes. A high prevalence of integrons was observed in the isolates, with class 1 integrons being the most frequently detected. Some isolates co-harboured the *intI1* and *intI2* genes and were classified as class1+2 integrons. Although *Citrobacter* spp. had the least number of isolates among the Enterobacteriaceae studied, it harboured the most diverse gene cassette arrays. The various gene cassette arrays were identified as follows: For *Klebsiella* spp. *aac(6')-Ib, aadA1-dfrA1*, and *dfrA1-sat2*; for *Citrobacter* spp., *dfrA5-aac3-Ib, aac(6')-Ib, aadA1-dfrA1-aadA1, aadA1-dfrA1, aadA5-dfrA17,* and *dfrA21-aac3-Ib*; for *E. coli dfrA21-aac-3-Ib, dfrA5-aac-3-Ib, aadA1-dfrA1*, and *aadA5-dfrA17*; and for *E. cloacae aadA1-dfrA1, dfrA7, dfrA21, dfrA5-aac-3-Ib,* and *dfrA1-sat2*. The GC array *dfrA1-sat2* was the only array detected in class 2 integrons which are analogous to that found in Tn7, *dfrA1-sat2-aadA1,* with the deletion of the last GC (*aadA1*). These detected GCs confer resistance against aminoglycosides, including streptomycin and spectinomycin, and trimethoprim, further increasing the resistance spectrum of the bacterial species harbouring them. The detection of integrons and their associated GC and the presence of these β-lactamases is also associated with co-resistance against other classes of antibiotics by bacterial species harbouring them, further limiting treatment options.



The checkerboard assays combining antibiotics against these drug-resistant integron-harbouring Enterobacteriaceae revealed that 26.3% (10/38) of the interactions were categorized as synergistic, while 73.7% (28/38) were indifferent. None of the combinations was antagonistic. The TKAs revealed all the synergistic interactions as bactericidal. Therefore, the combinations of gentamicin with tetracycline, ciprofloxacin, and ceftazidime against Multidrug-resistant (MDR) *Klebsiella pneumoniae*; tetracycline-ceftazidime combination against MDR *Escherichia coli*, colistin combinations with ceftazidime and

gentamicin, and tetracycline-gentamicin combinations against MDR *Citrobacter freundii* may be future therapeutic alternatives.

# CHAPTER ONE

## GENERAL INTRODUCTION

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## 1.1 Background of the study

Antibiotic misuse in clinical and agricultural settings has accelerated the dissemination of resistance determinants in clinical settings and the environment (Amann et al., 2019; Fletcher, 2015). Although much research has concentrated on the distribution of antibiotic resistance genes (ARGs) in clinical settings, few papers have reported their spread in various environmental settings. (Chaturvedi et al., 2021; Fadare et al., 2020; Fadare & Okoh, 2021b, 2021a; Korzeniewska & Harnisz, 2013; Wu et al., 2020). Many microorganisms, especially Gram-negative bacteria, then develop resistance against an array of antibiotics belonging to different antimicrobial classes used in treating the infections they cause, resulting in multidrug resistance and consequently posing a challenge for treating infections caused by such microorganisms. A variety of factors cause the establishment and spread of antibiotic resistance. It involves a low-frequency genetic mutation and the acquisition of several genes that mediate resistance against these microbes. These resistance-encoding genes can be given to off-springs via vertical gene transfer or, more commonly, horizontal gene transfer mechanisms to other microorganisms in varied niches. The primary vehicles facilitating the horizontal gene transfer are the resistance plasmids, transposons, bacteriophages, and integrons. Since their discovery by Stokes and Hall (Stokes & Hall, 1989), the roles played by integrons in the spread of resistance have been long recognized (Cambray et al., 2010; Ghaly et al., 2021; Gillings, 2014; Kaushik et al., 2018; Mazel, 2006; Singh et al., 2021).



Integrons are genetic elements that allow exogenous genes on gene cassettes (GCs) to be efficiently acquired and subsequently expressed (Cho et al., 2019; Hall et al., 1991, 1999; Sandoval-Quintana et al., 2022; Wright et al., 2008). Integrons are significant in rapidly disseminating antibiotic resistance, especially in Gram-negative bacterial pathogens and are usually present in multidrug-resistant (MDR) bacteria. Apart from clinical contexts, integrons

from environmental niches have been widely reported (An et al., 2018; Chaturvedi et al., 2021; Deng et al., 2015; Gillings, 2014; Kaushik et al., 2018; Koczura et al., 2012; Mokracka et al., 2012; Mukherjee & Chakraborty, 2006; Singh et al., 2021; Zhang et al., 2019).

Integrons have three essential features on a platform known as the 5′ conserved segment (CS). These are the integron integrase gene, *intI*, encoding a site-specific tyrosine recombinase enzyme, which catalyzes gene cassette integration and excision. Others include an integron-associated recombination site, *attI* and a constitutive promoter, $P_c$ (Ghaly et al., 2021; Gillings, 2014; Hall & Collis, 1995; Partridge et al., 2001). The integron system offers two key benefits in terms of genetic innovation in that the newly acquired GC is integrated at the specific *attI* site, hence not disturbing other previously existing genes. Secondly, the presence of the promoter, $P_c$, ensures that the newly acquired gene in a bacterial species is expressed and that genetic traits can be subsequently transferred to others. Therefore, in a population of integron-containing cells, the newly generated variants can immediately express genes likely to confer phenotypic advantages (Gillings, 2014). The level of expression of the genes on the GCs is contingent on its proximity to the promoter, where the maximum level of expression occurs in the GC closest to the promoter (Hall, 2012; Hall & Collis, 1995; Kaushik et al., 2018).



Gene cassettes are variable immobile sequences that exist as free, circular, nonreplicating DNA molecules and are linearized once incorporated into the integron (Bennett, 1999; Cambray et al., 2010; Daly & Fanning, 2004). They confer resistance against most classes of antibiotics. These include chloramphenicol, β-lactams, streptothricin, lincomycin, erythromycin, fosfomycin, rifampicin, trimethoprim, quinolones, aminoglycosides, and quaternary ammonium compounds (Deng et al., 2015; Gillings, 2014; Kaushik et al., 2019; Mazel, 2006). The GCs have simple structures with two components which are an open reading frame (ORF) which encodes ARGs, and a cassette-associated recombination site,

*attC*, recognized by the integrase (*intI)* gene (Cambray et al., 2010; Ghaly et al., 2021; Gillings, 2014; Mazel, 2006; Stokes & Hall, 1989).

Integrons are grouped into various classes depending on the sequences of the *IntI* gene. Integrons with *intI1* are defined as class 1, while those with *intI2* as class 2 and *intI3* as class 3 integrons. Mobile genetic elements were initially linked to the *intI1*, *intI2*, and *intI3* genes, whereas chromosomal integrons are linked to *intI4* and others. (Boucher et al., 2007; Deng et al., 2015). There has long been an association between the *intI1*, *intI2*, and *intI3* genes and multidrug-resistant phenotypes (Cambray et al., 2010; Collis et al., 2002; Mazel, 2006; Sandoval-Quintana et al., 2022). Class 1 integrons have been extensively studied and are the most prevalent due to the ability of the *intI1* to recombine with different *attC* sites on the GCs, even with different nucleotide sequences. They have been involved in the wide distribution of antimicrobial resistance in several clinical Gram-negative bacteria, which includes *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Burkholderia*, *Klebsiella*, *Mycobacterium*, *Providencia*, *Pseudomonas*, *Salmonella*, *Campylobacter*, *Serratia*, *Shigella*, S*tenotrophomonas*, *Aeromonas*, and *Vibrio* (An et al., 2018; Chaturvedi et al., 2021; Deng et al., 2015; Firoozeh et al., 2019; Ghaly et al., 2021; Goldstein et al., 2001; Kaushik et al., 2019; Rosser & Young, 1999). Class 2 integrons are not as widespread among bacteria. Their sequence of the *intI2* gene is interrupted by an early stop codon with a shortened, non-functional 178 amino acid protein product which may be responsible for the low variety of GCs (Gillings, 2014; Hansson et al., 2002). Class 3 integron integrase genes (*intI3*) share 60% of their sequence identity with class 1 integrons. Although rapidly evolving, they are the least prevalent, colonizing new species and acquiring novel GCs. Their GCs mainly confer resistance against aminoglycosides and trimethoprim (Gillings, 2014; Simo Tchuinte et al., 2016; Stalder et al., 2012).

Many genera belonging to the Enterobacteriaceae family have been reported harbouring the *intI1*, *intI2*, and *intI3* genes. Members of the Enterobacteriaceae family are found in various natural environments such as soil, water, and plants. They are also found in the normal microflora of the gastrointestinal tracts of humans and many animals, including mammals, birds, and reptiles (Tärnberg, 2012). The family can be divided into 51 genera, although the taxonomy is continuously updated with new species discoveries. Clinically relevant genera commonly causing infections within this family are *Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *Morganella* spp., *Escherichia coli, Plesiomonas* spp., *Proteus* spp., *Shigella* spp., *Salmonella* spp., *Providencia* spp., *Serratia* spp., and *Yersinia* spp. (Abbott, 2011; Forsythe et al., 2015). These species are commonly implicated in urinary tract infections, while other infections they cause are pneumonia, wound infections, septicaemia, enteritis, diarrhoea, and other infections involving the central nervous system (Tärnberg, 2012). Antimicrobials belonging to the β-lactams are commonly prescribed to treat uncomplicated and severe infections caused by members of the Enterobacteriaceae family. Antibiotics belonging to the aminopenicillins, cephalosporins, and carbapenems were used to treat infections these aforementioned enteric species mediate (CLSI, 2018; WHO, 2019). However, recently, there has been an upsurge in the reported resistance levels against β-lactams in these key infectious agents and many other previously susceptible species necessitating a worrisome trend globally.



Antibiotics are different from other medications for two reasons. They must first fulfil stringent effectiveness and safety standards. Antibiotics are also vulnerable to a stunningly quick loss of potency due to mutation in the organisms or the capacity of the organisms to acquire traits that confer phenotypic advantages on them against our arsenal of drugs. These have been the hurdles in creating antibacterial medications from the commencement of modern antibiotics (Silver, 2007; Tacconelli et al., 2018). A brief retrospective examination

of antibiotic discovery and development reveals that drugs that target a single protein are not as effective as those that target many molecular structures. The latter frequently contain tightly connected, redundantly encoded components that are difficult to alter by a single gene mutation. The importance of antibacterial medications that target many cellular sites cannot be overstated. Another factor worthy of consideration is that most bacteria and fungi that produce these selective antimicrobial agents do not manufacture single compounds but rather complex mixtures of products that work together to accomplish complementarity and synergy (Challis & Hopwood, 2003; Silver, 2007, 2016). This knowledge/principle has the potential to guide knowledge-based antibiotic combination medication development since the most effective antibiotics target many sites.

Recognizing that no single antibiotic molecule is uniformly effective against all infections, one of the primary motivations for combining antibiotics was the potential for greater efficacy than single antibiotics. Antibiotics are combined to achieve a variety of goals. The first is the capacity to broaden the antibacterial range during empirical therapy when the pathogen's identification is still unclear. The second goal is to achieve synergistic effects, boosting treatment effectiveness. Other goals include preventing the formation of resistance and reducing host toxicity (Moellering, 1983; Silver, 2016; Tacconelli et al., 2018). The earliest reported combinations were streptomycin with penicillin in 1950 (Jawetz et al., 1952), while trimethoprim was combined with sulfonamides in 1968 (Bushby & Hitchings, 1968). These combinations improved efficacy and antibacterial spectrum. Clindamycin, fusidic acid, and linezolid are protein synthesis inhibitor antibiotics that had no impact against Gram-negative bacteria when used alone but were effective when combined with colistin against colistin-resistant Enterobacteriaceae (Brennan-Krohn et al., 2018). Such combinations are still used today, validated by thorough mechanistic, clinical, and epidemiological evidence. Though formed fixed-dose antibiotic combinations are uncommon

in drug formularies, except β-lactam–β-lactamase inhibitor combos (a fixed-dose regimen is preferred for out-of-hospital usage to guarantee patient compliance). However, empirical antibiotic combinations covering the pathogens spectrum and combating resistance in clinical practice are frequent (Moellering, 1983; Tyers & Wright, 2019).

Pathogens are becoming progressively resistant against all available medicines, frequently by redundant processes. The slow pace of innovation in developing novel molecules that fit the demanding requirements for effective antibiotic medicines has aggravated the problem (Laxminarayan et al., 2013; Tacconelli et al., 2018; Tyers & Wright, 2019). Given the current situation, it is crucial to rethink monotherapy and aggressively investigate combination medications to actualize multi-target engagement and prevent the emergence of spontaneous antibiotic resistance.



**1.2 Justification of the study**

The emergence and spread of antimicrobial resistance in Enterobacteriaceae throughout the earth make it the third most significant threat to global public health in the 21st century (WHO, 2014). The antimicrobial resistance phenomenon already imposes a weighty burden on healthcare, with estimated annual deaths of 23,000 and 25,000 reported in the United States and Europe, respectively. Also, it has been estimated that this will result in over 10 million annual deaths worldwide by 2050 (CDC, 2013). The genes mediating antimicrobial resistance in bacteria are found on various genetic elements such as integrons. These integrons have a functional platform that enables them to capture exogenous genes located on gene cassettes within their environment and subsequently express the resistance traits on them, thereby conferring phenotypic advantages on the organisms harbouring them (Boucher et al., 2007; Chaturvedi et al., 2021; Gillings, 2014).

The presence of gene cassettes has been known to confer resistance against most classes of antibiotics. These include all known β-lactams, chloramphenicol, trimethoprim, erythromycin, aminoglycosides, quinolones, streptothricin, lincomycin, rifampicin, fosfomycin, and antiseptics of the quaternary ammonium compound family (Cambray et al., 2010; Deng et al., 2015; Ghaly et al., 2021; Mazel, 2006). The integrons are distinguished into various classes based on the sequences of the integron integrase gene, *intI*. The integrons with *intI1*, *intI2*, and *intI3* genes can acquire the same gene cassettes via a similar recombination platform (Hall et al., 1999; Kaushik et al., 2018). These classes of integrons are the most prevalent and most found in the members of Enterobacteriaceae. The remaining integrons classes, including classes 4 and 5, have not been extensively studied. Although these classes also contain gene cassettes, they are yet to be detected within the Enterobacteriaceae family and are generally associated with the Vibrionaceae (Hochhut et al., 2001; Kaushik et al., 2019; Nield et al., 2001; Sørum et al., 1992).



Globally, there have been many published reports on the prevalence of integrons with clinical origin (Firoozeh et al., 2019; Malek et al., 2015; Odetoyin et al., 2017; Zarei-Yazdeli et al., 2018) and some reports from environmental bacteria (Chaturvedi et al., 2021; Machado et al., 2009; McMillan et al., 2019; Mokracka et al., 2012; Zhang et al., 2019). However, there is a dearth of information on the characterization of integrons in Enterobacteriaceae recovered from environmental sources in the Eastern Cape Province, South Africa.

Antimicrobials belonging to the β-lactams are used to treat uncomplicated and severe infections caused by members of the Enterobacteriaceae family (CLSI, 2018; WHO, 2019). However, recently, there has been an upsurge in the reported resistance levels against β-lactams in these key infectious agents and many other previously susceptible species necessitating a worrisome trend globally. Antimicrobial resistance evolved directly from the extensive use of antibiotics, causing bacterial pathogens to be increasingly resistant against

all available antibiotic drugs (Tacconelli et al., 2018). A significant percentage of the traditional pharmaceutical business has withdrawn from developing drugs that fit the demanding requirements for successful antibiotic medications (Laxminarayan et al., 2013; Tyers & Wright, 2019). Antibiotic medication development is currently limited, and the development of resistance against all known antibiotics is rapidly outpacing it. The lack of innovation has exacerbated the development of novel compounds that fit the demanding requirements for effective antibiotics (Laxminarayan et al., 2013; Tacconelli et al., 2018; Tyers & Wright, 2019).

Due to the severity of the situation, we must assess our current drug development techniques and consider innovations that can be employed to bridge the gap between the discovery of new antibiotics and the rising need to tackle antimicrobial resistance. Therefore, it is crucial to rethink monotherapy and aggressively pursue combination therapies that will result in multi-target engagement and reduce spontaneous resistance development. Hence, a study of this type must be carried out to generate data that could give insights into the antibiotics combination therapy options that could be further explored *in vivo* to manage infections caused by multidrug-resistant Enterobacteriaceae encountered in the environment. The data generated will also provide helpful information which may be added to the national and global antibiotic resistance surveillance databases.



**1.3 Hypothesis**

The study was premised on a null hypothesis that multidrug-resistant Enterobacteriaceae isolates from environmental sources do not harbour integrons, and the combination of antibiotics is ineffective against them.

## 1.4 Aim and Objectives

### 1.4.1 Aim

The research aimed to characterize integrons and their associated gene cassettes in multidrug-resistant Enterobacteriaceae from environmental sources and explore antibiotic combination options against some multidrug-resistant strains.

### 1.4.2 Specific objectives

In achieving the main aim above, the following specific objectives were set:

1. Retrieve, resuscitate, and reconfirm Enterobacteriaceae isolates previously obtained from environmental sources in the AEMREG culture collection.
2. Characterize the Enterobacteriaceae isolates into Extended-spectrum and metallo β-lactamase producers and multidrug-resistance using phenotypic antimicrobial assays.
3. Detect the integrons' classes in the confirmed Enterobacteriaceae strains using polymerase chain reaction (PCR).
4. Detect and characterize the integrons' variable regions and their antibiotic resistance genes.
5. Determine the phylogenetic relatedness among some isolates of the same species from various sources using Enterobacterial repetitive intergenic consensus- PCR.
6. Determine the minimum inhibitory concentration and investigate the *in vitro* synergistic activity of selected combined antibiotics against some MDR isolates to evaluate the possible efficacy of the drug combinations.

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## CHAPTER TWO

## LITERATURE REVIEW

**Integrons as emerging contaminants facilitating the widespread of antimicrobial resistance in Enterobacteriaceae (Published in *Advances in Biomedical and Health Science*)**

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**Abstract**

Antibiotic resistance genes (ARGs) are classified as emerging environmental pollutants of global public health concern. These ARGs are disseminated through genetic elements such as integrons. Integrons can acquire, integrate, and express various rearrangeable gene cassettes (GCs), harbouring different ARGs that may be readily spread to other bacteria in widely varied niches. Different classes of integrons possessing diverse arrays of ARGs located within its GCs are commonly distributed in the Enterobacteriaceae family and are responsible for the high rate of multidrug resistance observed. The members of this family are natural commensals of the gastrointestinal tracts of humans and animals released into the different aquatic environments. Various water sources further disseminate the organisms and their diverse resistance gene repertoires. Thus, understanding the distribution and diversity of the significant integron classes in the clinically relevant Enterobacteriaceae members will be of utmost importance. It will provide a framework for health authorities to make decisions on the surveillance of these contaminants in the environment.



**Keywords**

Integrons, Gene cassettes, Enterobacteriaceae, Integrases, antibiotic resistance genes

## 2.1 Introduction

Members of the family Enterobacteriaceae are commonly associated with several community-acquired and hospital-acquired infections. The family's genera can be found in the gastrointestinal tracts of warm-blooded animals, humans, plants, soil, and water. Clinically significant genera include *Escherichia-*, *Citrobacter-*, *Klebsiella-*, *Enterobacter-*, *Salmonella-*, *Shigella-*, *Serratia-*, and *Yersinia*. Intestinal, genitourinary, and bloodstream infections have been linked to these microbes (Kaushik et al., 2018; Tärnberg, 2012). Antibiotics are used extensively in clinical and farming contexts to treat these infections. The fast rise of multidrug-resistant (MDR) microorganisms, particularly among these intestinal microbes, has been exacerbated by the use and overuse of antibiotics during the previous few decades. This necessitates an upsurge in the clinical failures observed with the currently available antibiotics, which is expected to rise if left unchecked.



The capacity of a bacteria to acquire and subsequently transfer antibiotic resistance genes (ARGs) to others in wide-ranging niches via horizontal gene transfer (HGT) mechanisms has been essential in developing MDR phenotypes. The three mechanisms of HGT include transformation, which entails the direct uptake of DNA from its surroundings; conjugation involves cell-to-cell contact, while transduction consists of a vector for inserting DNA into the recipient cell. Once the newly incorporated DNA is in the cell, site-specific recombination or transposition can further spread the ARGs to plasmids, chromosomes, transposons, and integrons in the recipient bacteria. These genetic elements then act as the primary vehicles facilitating the HGT. Integrons play a significant part in spreading antibiotic resistance, especially in Gram-negative pathogens (Chaturvedi et al., 2021; Gillings, 2014).

Integrons are genetic elements that act as natural gene capture and expression systems. Although integrons by themselves are immobile, however, they can link with other DNA elements. Transposons, conjugative plasmids, and insertion sequences (IS) elements are examples of such DNA elements which can act as an essential vehicle for ARG transmission (Deng et al., 2015; Jones-Dias et al., 2016; Kaushik et al., 2018; Mazel, 2006; Moura et al., 2012; Sandoval-Quintana et al., 2022). Generally, integrons are distinguished by their capacity to acquire small mobile elements known as gene cassettes (GCs) (Hall, 2012; Stokes & Hall, 1989). However, about one-third of them have been found in bacterial genomes without GCs and were called empty integrons. They are present in around 17% of bacterial chromosomes (Kaushik et al., 2018). These structures have been detected in various environments, including Antarctic soils, desert soils, forests, hot springs, plant surfaces, and river sediments, marine and deep-sea. In the late 1980s, integrons were identified and subsequently implicated in the distribution of ARGs through the presence of GCs, which they usually harbour. Integrons are now well-established and well-documented in disseminating resistance, especially within Gram-negative bacteria (Gillings, 2014; Hall, 2012; Hall & Collis, 1995). Hence, in this review, we carried out an overview of the distribution and diversity of the different integron classes in the clinically relevant Enterobacteriaceae members.



**2. 2 Integron structure**

Structurally, three essential properties of all integrons are found on a functional platform known as the 5′ conserved segment (CS). These are the gene integron integrase, *intI*, which encodes a site-specific tyrosine recombinase enzyme, with a primary function of catalyzing the integration and excision of GCs. Secondly, an integron-associated recombination site, *attI*, is recognized by the *intI* and functions as the site for acquiring new genetic material without disturbing the previously existing genes. Lastly, an effective constitutive promoter,

$P_c$, regulates the expression of captured GCs. It is located within the *intI* gene and oriented towards the integration point (Boucher et al., 2007; Hall & Collis, 1995). These basic features ensure that in a population of integron-containing cells, the newly created variants can instantly express genes that are likely to confer phenotypic advantages due to the presence of the promoter. The level of expression of the GCs is contingent on its proximity to the promoter, Pc, where the maximum level of expression occurs in the GC closest to the promoter (Hall, 2012; Hall & Collis, 1995; Kaushik et al., 2018). Therefore, integrons are essentially genetic elements capable of integrating and expressing various rearrangeable GCs harbouring different ARGs that may be readily mobilized to other neighbouring bacteria.

2.2.1 Gene cassettes

Gene Cassettes (GCs) are variable sequences that can exist as free, circular, nonreplicating DNA molecules and are usually linear when integrated into more prominent elements called integrons (Cambray et al., 2010). GCs do not carry the machinery for their movement and are referred to as the mobile parts of an integron (Bennett, 1999; Daly & Fanning, 2004). Their presence has been known to confer resistance against most classes of antibiotics. These include all known β-lactams, chloramphenicol, trimethoprim, erythromycin, aminoglycosides, quinolones, streptothricin, lincomycin, rifampicin, fosfomycin, and antiseptics of the quaternary ammonium compound family (Deng et al., 2015; Gillings, 2014; Kaushik et al., 2019; Mazel, 2006). The GCs have simple structures with two components which are an open reading frame (ORF) which encodes ARGs, and a cassette-associated recombination site (*attC*), also called the 59-basepair element (59-be) (Mazel, 2006; Stokes & Hall, 1989) and therefore used interchangeably in this text. These 59-be, which usually vary in length (generally between 57 and 141 bases), are imperfect inverted repeat sequences (IRs) that are found at the ORF's 3' end and are recognized by the integrase (*intI)* (Cambray et al., 2010; Gillings, 2014; Stokes & Hall, 1989).

The 59-be is an essential component for integrating GCs into integrons. The recombination of the 59-be in a closed-circular cassette molecule with the *attI* results in integrating GCs into the integron. Moreover, the excision of a GC occurs between two 59-be resulting in the formation of a free circular cassette (Collis et al., 1993; Hall et al., 1999; Hall & Collis, 1995; Stokes et al., 1997). There is a strong association between the *attI* and 59-be sites ensuring that new GCs are inserted next to the integron irrespective of already harbouring one or more GCs. The result of this system implies that the last GC integrated is the most proximal to the promoter (Collis et al., 1993; Collis et al., 2002a), as seen in Figure 2.1. As this association is conservative, the *attI* site is reconstituted; it can occur numerous times, thus making it possible for a particular integron to harbour a string of GCs. Individual *intI* is strictly associated with its *attI* site, but this does not apply to the *attC*. Therefore, even though there is a great diversity in the sequences of the *attC* sites, each different *intI* can recognize many other *attC* sites, thereby allowing GCs to be easily exchanged between different integron classes (Collis et al., 2002a; Collis et al., 2002b; Hall, 2012). Most GCs do not possess a promoter and therefore depend on an external promoter, Pc, located on the *intI* of an integron located upstream for their expression (Gillings, 2014; Hall & Collis, 1995; Stokes et al., 1997). Therefore, a promoter-less cassette must be closely situated to the Pc on an integron to express genes on its cassette. It thus implies that the downstream genes on integrons with a long array of cassettes may be unexpressed. The insertion event positions the gene in the cassette in the proper orientation, allowing expression from the integron's upstream promoter, Pc (Hall & Collis, 1995; White et al., 2000). The GC is flanked at both ends by the conserved sequences 'GTTRRRY'.

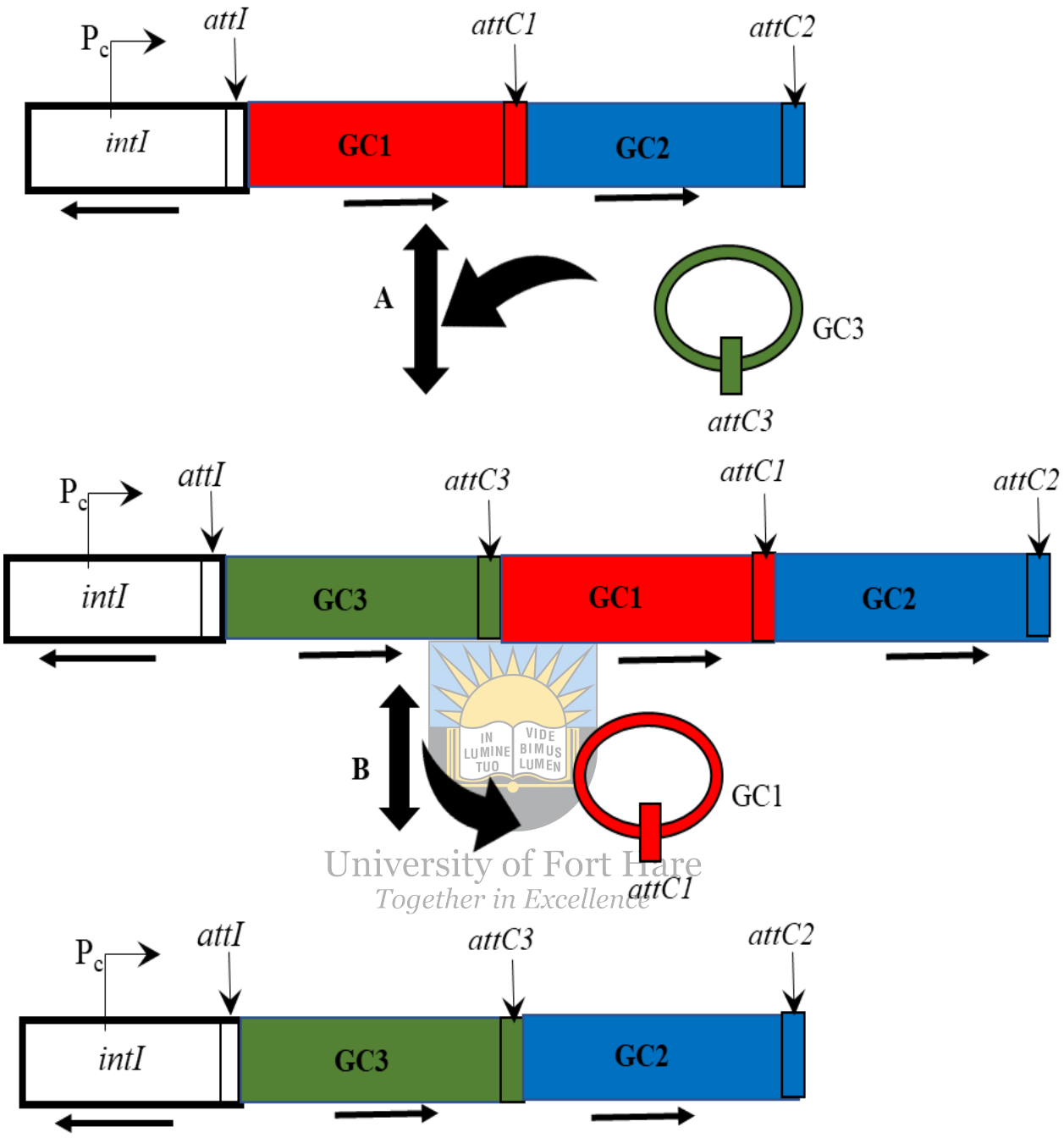




**Figure 2. 1: The organization of an integron and gene cassette (GC) recombination system.** The integron integrase, intI gene, catalyzes the insertion of the GC3 at A and the excision of GC1 at B. The integron recombination site, attI, is strongly associated with the GC's recombination site, attC (also known as 59-be). As shown at A, the circularized GC3 is inserted into the integron and linearized. It occurs through a particular recombination process between the attI site and the attC3 of the incoming GC3. Hence, the GC3 is placed closest to the promoter, Pc. The excision of the GC occurs preferentially between two attC sites. The GC1 is removed by recombination between attC1 and attC3 sites at B. The arrows indicate the direction of transcription.

### 2.3 Classes of integrons

Integrons have been categorized into various classes based on the amino acid sequence of the *intI* gene (Cambray et al., 2010). Integrons with *intI1* genes are classified as class 1, *intI2* genes are class 2, *intI*3 genes are class 3, and so on. The *intI1*, *intI*2, and *intI*3 were identified with mobile genetic elements, while *intI*4 and others were associated with chromosomal integrons (Deng et al., 2015). Most of the integrons that have been reported have been detected in Gram-negative bacteria, with very few detected among Gram-positive bacteria (Kaushik et al., 2018; Stalder et al., 2012). Class 1 and 2 integrons have been mostly reported among the Enterobacteriaceae family, while class 3 integrons seem far scarce and infrequently implicated in the spread of antibiotic resistance. Members within the same class have the same integrase but can be further differentiated based on their GCs. Integrons have been identified in several bacterial species from clinical and environmental samples (Boucher et al., 2007; Buongermino et al., 2020; Daly & Fanning, 2004; Hall, 2012; Jones-Dias et al., 2016; Koczura et al., 2012; Mokracka et al., 2012; Nield et al., 2001).



The other integrons classes, including classes 4 and 5, have not been extensively studied. They are yet to be reported within the Enterobacteriaceae family. In *Vibrio* spp., a class 4 integron was discovered. It was inserted in an integrative and conjugative element (ICE) and conferred resistance against trimethoprim/sulfamethoxazole (Hochhut et al., 2001). A class 5 integron was also found in a compound transposon on the *Vibrio salmonicida* pRSV1 plasmid (Sørum et al., 1992). The *attI* sites of each integron class differ, although they all have a common core site with the conserved sequences. So, there is little comparison between the sequences of *attI1* present in class 1 integrons and the corresponding sequences of *attI2* and *attI3* present in classes 2 and 3, respectively (Hall et al., 1999).

2.3.1 Class 1 integrons

In 1989, the first integron discovered was the Class 1 integron by Stokes and Hall (Stokes & Hall, 1989). The class now accounts for a substantial proportion of MDR nosocomial infections. It is the most frequently reported integron among the enterics and has been reported in pathogens such as *Salmonella*, *Escherichia coli*, *Enterobacter*, *Citrobacter*, *Shigella*, *Yersinia*, *Klebsiella*, *Serratia*, and *Proteus* (Chaturvedi et al., 2021; Goldstein et al., 2001; Guo et al., 2011; Kaushik et al., 2019; Pellegrini et al., 2011; Rosser & Young, 1999; Su et al., 2012). Class 1 integrons have the highest detection frequency among the other integrons. The *intI1* can recombine with different *attC* sites even when their nucleotide sequences are very dissimilar. The *attC* sites are usually associated with a particular ORF in the GCs, which are not necessarily integrated. Once integrated, they become part of the integron enabling this class to harbour the most known antibiotic resistance GCs. The class 1 integron-integrase (*intI1*) can recruit GCs from other classes (Deng et al., 2015).



A typical class 1 integron is associated with functional and non-functional transposons derived from Tn*402*. These structures are usually embedded in a large transposon such as Tn*21*/Tn*1696* with a 5′ region called the 5′-CS (Brown et al., 1996; Cambray et al., 2010; Kaushik et al., 2018). The 5′-CS consists of an integron's three essential features, *intI*, *attI*, and Pc. In addition, many of the class 1 integrons have a 3′ region, called 3′-CS. The length of the sequences of the 3′-CS is typically 2,384 bp and encodes four ORFs (Stokes & Hall, 1989). The first is the *qacEΔ1* gene, a truncated version of the *qacE* conferring resistance against quaternary ammonium compounds. A postulation was that the truncation arose due to the insertion of a *sul1* gene at the 3′ end. The gene encodes resistance against sulphonamides. The insertion led to the deletion of the 59-be of the *qacE* gene and some coding sequences. The *sul1* is the second ORF on the 3′ end to further lend credence to this theory. It encodes resistance against the antibiotic sulphonamide through the enzyme dihydropteroate synthetase

(Gillings, 2014; Kaushik et al., 2018). The third is the *orf5*, a gene of unknown function, similar to puromycin acetyltransferase. The last is the *orf6*, whose biological function is not yet ascertained (Daly & Fanning, 2004; Gaze et al., 2005; Kaushik et al., 2018; Stokes & Hall, 1989). The schematic representation of a typical class 1 integron is shown in Figure 2.2.

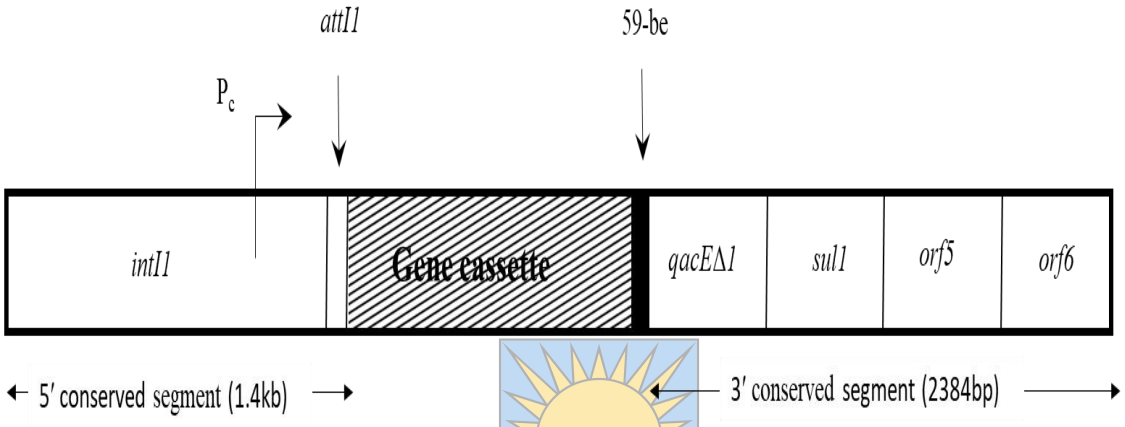


**Figure 2. 2: A schematic illustration of the structure of a typical class 1 integron.** The 5′-CS harbours the integrase gene (intI1), the promoter ($P_c$) and the attachment site (attI), while the 3′-CS contains four ORFs (qacEΔ1, sul1, orf5, and orf6, respectively). The hatched box shows a single gene cassette with its corresponding 59-be inserted between the 5′ and 3′ CS. The common promoter usually expresses the gene found proximal to the 5′ site of insertion.



It has been reported that the presence of the *qacE* gene protects bacterial cells from toxins found in their natural environment. The gene encodes multidrug exporters that confer resistance against bactericidal ammonium molecules (Gilbert & McBain, 2003; Paulsen et al., 1996; Shafaati et al., 2016). The *qacE* gene has been reported in over half of the GCs detected in class 1 integrons recovered from environmental isolates (Gillings et al., 2009). In the early 1930s, the era preceding the discovery of antibiotics, quaternary ammonium compounds served as hospital disinfectants (Russell, 2002). It explains the possession of the *qacE* gene in most class 1 integrons (Gaze et al., 2005). The first broadly introduced antibiotics in the mid to late 1930s were the sulphonamides (Sköld, 2000), commencing antibiotic resistance selection. Therefore, it was not unexpected that detecting the *sul1* gene on the class 1 integrons was the next event of evolution. The general abundance of class 1

integrons in other ecosystems apart from the clinical settings where they seemed to have originated makes them the natural environment's pollutants (Gillings, 2014).

Some enteric bacteria have been reported to have variations in the 3′-CS, which is inconsistent with most known class 1 integrons. An example is the Class 1 integron in Tn*402*, with a complete *qacE* gene cassette without the *sul1*, *orf5*, and *orf6* genes. Immediately after the *qacE* gene, it contains the genes *tniR*, *tniQ*, *tniB*, and *tniA*, which are referred to as the transposition gene module (*tni* module) on the 3′ end (Kholodii et al., 1995; Rådström et al., 1994; Sajjad et al., 2011). Tn*402* has typical inverted repeats of 25bp, flanking the integron, IRi, and the transposition modules, IRt (Figure 2.3). These IRi and IRt bordering the integron enable class 1 integrons within the Tn*402* to move horizontally through transposition. Many class 1 integrons do not have this complete *tni* module, as several additions and deletions have rendered it a defective transposon (Brown et al., 1996; Sajjad et al., 2011). For example, integron In2 has an insertion of IS*1326* and IS*1353* located at the 3′-CS giving rise to the deletion of parts of the *tni* module (Brown et al., 1996). Hence when the evolutionary history of class 1 integrons is considered, the Tn*402* is regarded as the ancestral lineage of class 1 integrons (Partridge et al., 2001). Various evidence show complex integrons in different bacterial species with numerous discrepancies in the functional structure and ORFs harbouring different GCs (Gillings, 2014; Jiang et al., 2017; Kaushik et al., 2018).

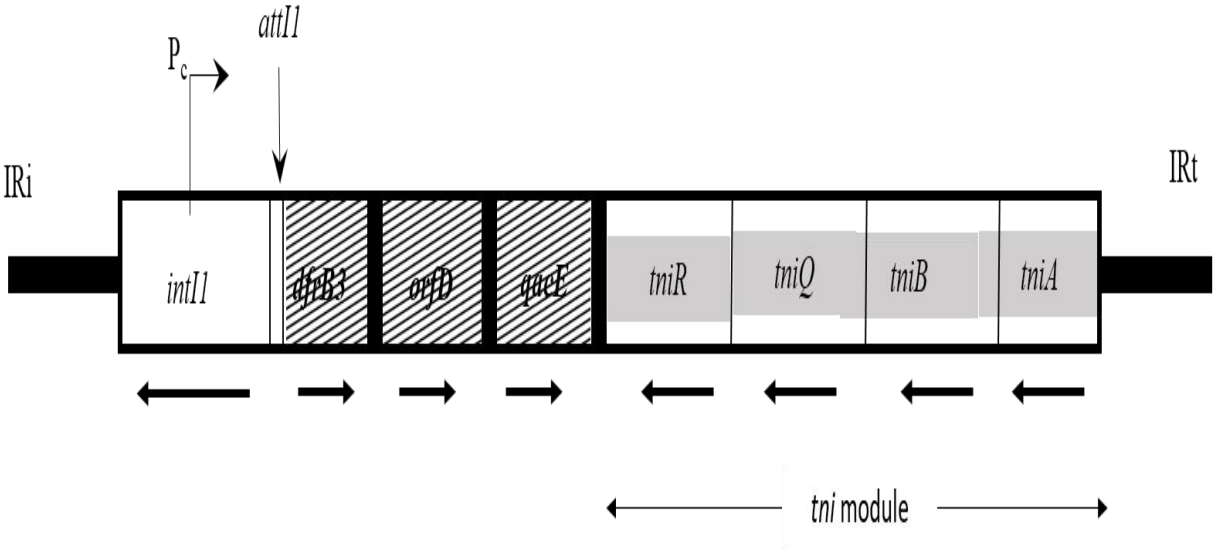


**Figure 2. 3: The representation of a class 1 integron found in Tn402 named In16.** Three gene cassettes harboured on this integron are dfrB3, orfD, and qacE with their individual 59 -be represented by filled rectangles. The complete transposition gene modules are located downstream of the cassettes. The structure is bounded by 25bp inverted repeats, IRi and IRt, as indicated by the thick line. The arrows show the direction of transcription.

2.3.2 Class 2 integrons

Class 2 integrons are also found within transposons called Tn*7* and their related transposon derivatives, such as Tn*825*, Tn*1826*, and Tn*4132*, which facilitate their dissemination (Stalder et al., 2012). Their 3' -CS harbours five *tns* genes (*tnsA*, *tnsB*, *tnsC*, *tnsD*, and *tnsE*) which are also involved in their movement (Collis et al., 1993). They contain integrase genes (*intI2*) whose sequences share a 46% similarity with class 1 integrase (*intI1*) (Gillings, 2014; Hall, 2012; Hansson et al., 2002). Within the Enterobacteriaceae family, *intI2* occur less frequently when compared with class 1 integrons (Gillings, 2014). However, the essential distinctive feature is that the *intI2* gene is interrupted by an early stop codon with a shortened, non-functional 178 amino acid protein product (Hansson et al., 2002). The *intI2* gene's mutation is liable for the low variety of GCs compared to class 1 integrons (Gillings, 2014; Hansson et al., 2002), giving rise to a stable GC array harbouring *dfrA1*, *sat2*, *aadA1*, and *orfX* genes. The *dfrA1* encodes resistance against trimethoprim, while *sat2* confers resistance against streptothricin. The *aadA1* genes confer resistance against streptomycin and spectinomycin,

while *orfX* encodes a protein of unknown biological activity (Hansson et al., 2002), as seen in Figure 2.4. The GC *orfX* is not followed by the usual 59-be with a typical palindromic and core structure, but its activity as a recombination site has been established (Hansson et al., 1997, 2002).

There are slight differences reported in the GCs of some class 2 integrons. It has been inferred that these might be due to GCs movements even when the cognate integrase is inactive. It is proposed that these differences might have either been due to the recombination activity supplied by another active integrase from class 1 or the occasional stop codon mutation leading to the restoration of recombination activity (Mazel, 2006). Therefore, a typical wild-type class 2 integrase cannot move GCs as its site-specific recombination activity is defective. Two class 2 integrons have been described with functional *intI2*. One had 9 GCs whose biological activities were unknown. The other harboured the *dfrA14* GC; a novel GC predicated as a lipoprotein signal peptidase gene (Barlow & Gobius, 2006; Márquez et al., 2008). Only five class 2 integrons were characterized, each associated with antibiotic resistance before the reports of active *intI2* integrases.



Further inference from the report of Márquez and colleagues suggests that active class 2 integrase, as described above, exists but is not widespread (Márquez et al., 2008). For instance, a hybrid of class 2 integron in *Acinetobacter baumannii* was reported by Ploy and colleagues. The integron contained an *intI2* gene, with two GCs usually found on class 2 integrons, d*frA1* and *sat*, in addition to the 3′-CS of class 1 integrons (Ploy et al., 2000). This description of a hybrid integron shows that recombination between two different integron classes is possible.

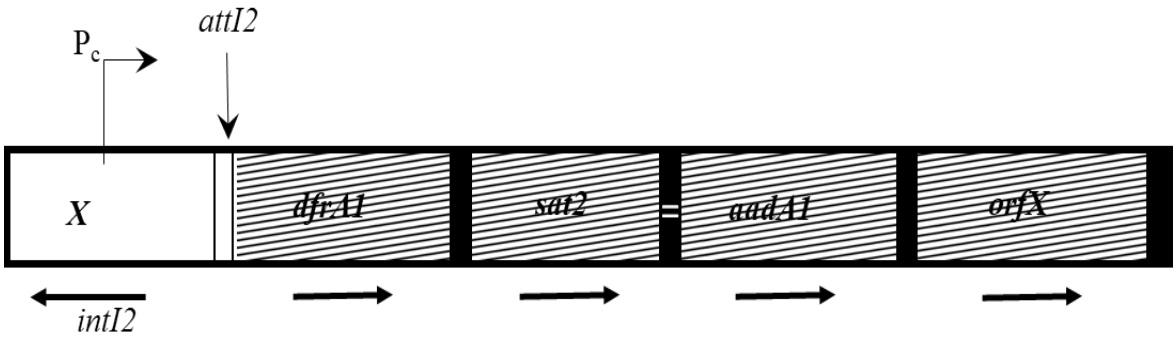


**Figure 2. 4: The schematic illustration of a class 2 integron from Tn7.** The intI2 gene characterizes the class 2 integron and is shown in the unshaded rectangle box with 'X' indicating the premature stop codon resulting in the transcription and translation of an inactive integrase. The attI2 site is the class 2 recombination site. The Tn7 harbours three gene cassettes, dfrA1, sat2, and aadA1, with their respective 59 -be indicated with the filled rectangles. The last GC is the orfX with an unknown biological activity which contains an unusual 59 -be structure. Arrows indicate the direction of transcription.

2.3.3 Class 3 integrons

Class 3 integron integrase genes (*intI3*) share 60% of their sequence identity with class 1 integrons. They are also embedded in Tn*402* but have a reverse orientation compared with class 1 integrons (Collis et al., 2002a). Although rapidly evolving, they are the least prevalent integrons class, colonizing new species and acquiring novel GCs. Their GCs mainly confer resistance against aminoglycosides and β-lactams (Gillings, 2014). The class 3 integron was initially reported in a *Serratia marcescens* strain Tn*9106* recovered from a patient in Japan diagnosed with a urinary tract infection. The integron harboured two GCs. One was the $bla_{IMP}$ which encodes resistance against carbapenems and broad-spectrum metallo-β-lactamases. The second was the *aacA4* which encodes resistance against aminoglycosides. It was the first report of the $bla_{IMP}$ gene on an integron, although the *aacA4* gene had been earlier identified in class 1 integrons (Hall et al., 1991). The class 3 integron is flanked by IRi and a resolvase-encoding *tniR* gene at the 3′ -CS end (Arakawa et al., 1995; Collis et al., 2002a). Such class 3 integrons are quite common in Japan (Correia et al., 2003) and were not usually detected in other parts of the world (Kor et al., 2013; Laroche et al., 2009; van Essen-Zandbergen et al., 2007). However, they have been reported in Canada (Xu et al., 2007) and,

more recently, Iran (Goudarzi & Azimi, 2017; Kargar et al., 2014) and Portugal (Jones-Dias et al., 2016). They also do not harbour a wide array of GCs, probably due to the capacity of the *intI3* gene being less active than the *intI1* and *intI2* integrases (Collis et al., 2002b). Class 3 integrons, although not as widely disseminated as class 1 integrons, have the potential to play an essential function in widespread ARGs. Unlike the typical class 2 integrase, the class 3 integrase is active. It catalyzes the site-specific recombination between GCs, just as in class 1 integrons (Collis et al., 2002a).

### 2.4 Integrons and their role as an emerging contaminant

Antibiotic pressure may have critically impacted the selection and spread of integrons in bacteria. Over 130 GCs impart resistance against diverse antibiotic classes, while over 60 GCs with unknown functions have been identified (Partridge et al., 2009). GCs have conferred antibiotic resistance in practically every antibiotic family. Furthermore, the GCs harbouring the *qacE*, which encodes resistance against the quaternary ammonium compounds, are also found in integrons (Deng et al., 2015; Mazel, 2006). Integrons were detected in bacterial populations under direct or indirect antibiotic pressure in clinical, agricultural, and environmental contexts, according to studies (Chaturvedi et al., 2021; Gillings, 2014; Haberecht et al., 2019; Soltan Dallal et al., 2018). Many bacteria containing integrons and resistance genes from wastewater make their way into the environment due to the high rate of integrons in commensals in humans and animals.



### 2.5 Integrons in different water matrices

It has been estimated that only 1% of the wide range of bacteria found within the environment is culturable using the various available cultivation techniques (Amann et al., 1995; Kotlarska et al., 2015). To overcome this limitation, more researchers have developed methods based on metagenomes. The metagenomic approach and cultivation techniques used to study bacterial communities in the environment have highlighted integrons' roles in

disseminating antibiotic resistance. So, there is mounting evidence that the environment plays a critical part in disseminating antibiotic-resistant bacteria impacting human and animal health (Aminov, 2009; Fadare et al., 2020; Kotlarska et al., 2015).

Integrons were found in estuarine, stream water sediments and biofilms, creek, and lake sediments (Hardwick et al., 2008; Koczura et al., 2014; Roe et al., 2003) using metagenomic and culture-dependent approaches. In some of these reports, the integrons harboured one to three GCs with many unknown functions. Water has gotten a great deal of attention since it is the primary vector of contaminants in the environment. Antibiotic resistance genes have been reported in freshwater bodies, including rivers (Cho et al., 2019; Fadare et al., 2020; Haberecht et al., 2019; Piccirilli et al., 2019; Ye et al., 2017), hospital effluents (Fadare & Okoh, 2021a; King et al., 2020; Korzeniewska & Harnisz, 2013), and sewage effluents (Aristizábal-Hoyos et al., 2019; Blaak et al., 2015; Fadare & Okoh, 2021b; Kotlarska et al., 2015; Tesfaye et al., 2019). Table 2.1 shows the distribution of various GCs in the integron classes among some members of the Enterobacteriaceae family in different environmental matrices. Most GCs encode the *dfrA* gene, which confers resistance against trimethoprim. Others include the *aadA* and *aac(6)-Ib*, which confers resistance against the aminoglycosides. The *sat2* and *sat1* encode resistance against streptothricin, while the $bla_{IMP}$ and $bla_{OXA}$ encode resistance against β-lactamases. Most gene cassettes were detected in class 1 integrons, while class 3 integrons were the least. Class 3 integrons were detected in hospital effluents and mostly harboured the β-lactamases.

**Table 2. 1: The distribution of gene cassettes in the integron classes among some members of the Enterobacteriaceae family in different environmental matrices.**

| Organism isolated | Sample type | Class 1 integron gene cassette array | Class 2 integron gene cassette array | Class 3 integron gene cassette array | Reference |
|---|---|---|---|---|---|
| *E. coli* | WWTP Final effluent | *dfrA17-aadA5* | - | - | (Mokracka et al., 2012) |
| | River | *aadA2-InuF* | *dfrA1-sat2-aadA1* | - | (Su et al., 2012) |
| | Surface water | *dfrA12-orfF-aadA2* | *dfrA1-sat2-aadA1* | - | (Kaushik et al., 2019) |
| | Irrigation water | *aadA1-dfrA12* | *sat1-aadA1* | - | (Roe et al., 2003) |
| | Lakes | *dfrA12-orfF-aadA2, dfrA17-aadA5, dfrA1-aadA1* | *dfrA1-sat2-aadA1* | - | (Koczura et al., 2015) |
| *Enterobacter* spp. | WWTP Final effluent | *aadA2* | - | - | (Mokracka et al., 2011) |
| | River | *dfrA12-orf40A-aadA2* | - | - | (Chakraborty et al., 2013) |
| | River | *dfrA5* | - | - | (Mukherjee & Chakraborty, 2006) |
| | Hospital effluent | - | - | $bla_{OXA-256}$-*aac(6)-Ib* | (Barraud et al., 2013) |
| *Klebsiella* spp. | WWTP Final effluent | $bla_{GES}$-*aacA4-dfrA7* | - | - | (Mokracka et al., 2012) |
| | River | *dfrA1-aadA1* | - | - | (Chakraborty et al., 2013) |
| *Citrobacter* spp. | WWTP Final effluent | *dfrA1-aacA7-aadA6* | *sat2-aadA1* | - | (Mokracka et al., 2012) |
| | River | $bla_{IMP-9}$-*aacA4* $bla_{oxa-10}$-*aadA2* | - | - | (Chakraborty et al., 2013) |
| | Hospital effluent | - | - | $bla_{OXA-2}$-$bla_{GES-1}$ | (Simo Tchuinte et al., 2016) |
| | River | *dfrA12* | - | - | (Mukherjee & Chakraborty, 2006) |
| *Salmonella* spp. | River | *dfrA7* | - | - | (Mukherjee & Chakraborty, 2006) |
| | River | *dfrA1-orf38* | - | - | (Chakraborty et al., 2013) |

WWTP: Wastewater treatment plant **a**nd -: Not detected.

### 2.5.1 Integrons in wastewater treatment plants (WWTPs)

The wastewater treatment plants (WWTPs) link human waste and the aquatic and soil ecosystems. WWTPs gather effluents from several sources, including private residences, hospitals, factories, and animal husbandries. As a result, the influents of the WWTP include microbiological, chemical, and organic wastes. Due to the high bacterial density in these WWTPs, they are hotspots for HGT, with numerous recombination events coexisting since wastewater releases traces of antimicrobial agents and resistance determinants (Fadare & Okoh, 2021b; Tesfaye et al., 2019). At various stages of the WWTP process, Class 1 integrons have been identified, with varying prevalence or relative abundance (Koczura et al., 2012; Mokracka et al., 2012). Unfortunately, the presence of these genetic elements in WWTP's final effluents demonstrates the inadequacy of the procedure for removing bacteria that harbour them. These may, in turn, contribute to the increased frequency of integron-positive isolates and their elevated resistance level.

## 2.6 Integrons in some clinically relevant Enterobacteriaceae



The relationship between integrons and antibiotic resistance has been mostly considered within the family of Enterobacteriaceae. The members of this family are usually of clinical and veterinary interest. The continuous use of antibiotics within the medical and agricultural settings has accelerated the dissemination of integrons as they harbour various ARGs. The class 1 integrons are the most often detected. They have been reportedly found in multiple niches, including their detection in commensal bacteria of farm animals, healthy humans and even infants not yet exposed to antibiotics (Dawes et al., 2010; Sunde et al., 2015; Vo et al., 2010). These commensal bacteria harbour integrons with varying structures and play a critical role in transferring resistance genetic markers from commensal bacteria to environmental and pathogenic bacteria.

2.6.1 *Escherichia coli*

The bacterium *E. coli* is a dominant free-living organism that has been extensively studied. It is the most ubiquitous facultative anaerobe in the gut microbiome of humans. It is commonly found in animals' gastrointestinal tracts, fostering its presence as an indicator of faecal contamination. It has been reported as an important human pathogen causing various intestinal and extraintestinal illnesses (Croxen & Finlay, 2010; Lamprecht et al., 2014). Antibiotics are commonly used to treat infections arising from this organism, resulting in selected antibiotic-resistant strains. A substantial relationship between an enhanced rate of resistance and integrons in various environmental matrices from different geographical locations has been reported. It indicates that the functions of integrons in disseminating ARGs and the emergence of *E. coli* with multidrug resistance traits have been well established in the past few decades (Kaushik et al., 2018; Laroche et al., 2009; Leverstein-van Hall et al., 2003).



Before their detection in other parts, *E. coli* harbouring class 1 integrons were initially reported from European countries (Martinez-Freijo et al., 1998). The integron 1-positive isolates harboured various GCs in diverse combinations. The most frequently observed occurred either singly or in combination. They conferred resistance against trimethoprim (*dfrA1*, *dfrA5*, *dfrA12*, *dfrA17*, and *dfrB2*), erythromycin (*ere2*), and streptomycin, aminoglycosides, and spectinomycin (*aadA1*, *aadA2*, and *aadA5*) (White et al., 2000, 2001). The most predominant GC among the integron-mediated antibiotic resistance in *E. coli* in the 1980s was *aadA1*. It was followed by the combination of GCs *dfrA17-aadA5*, which evolved in the 1990s. The presence of the *aadA* gene in integron classes 1 and 2 indicates a selection mechanism for the propagation of this GC in enteric bacteria (Roe et al., 2003). Su and colleagues in China, from 1998 to 2004, reported a higher occurrence of multigene cassettes in the clinical *E. coli* isolates recovered in the final year than when the study commenced (Su

et al., 2006). Over time, the increased prevalence of combination GCs indicates that class 1 integrons are implicated in acquiring resistance against broad-spectrum antibiotics. An example of integron-positive *E. coli* harbouring a variety of GCs is a pathogenic *E. coli* strain isolated from a hospitalized patient in France. The isolate harboured a class 1 integron named In53 which contained nine functional GCs coding for resistance against various compounds. These GCs include *aacA1b/orfG* (amikacin, tobramycin, and netilmicin), *aadA1* (spectinomycin and streptomycin), *aadB* (gentamicin), *arr-2* (rifampicin), $bla_{\text{OXA-10}}$ (penicillin), $bla_{\text{VEB-1}}$ (extended-spectrum β-lactamase, (ESBL)), *cmlA5* (chloramphenicol), *qacI* (Naas et al., 2001). The common GCs harboured in this class 1 integron result in the resistance against aminoglycosides, trimethoprim, erythromycin, and the β-lactams.

Although many class 2 integrons have also been detected in numerous commensal, environmental, and pathogenic *E. coli*, their occurrence has been much lower than their class 1 integron counterpart. The lower rate is attributable to the defective class 2 integrase. There is a predominance of the GC combination *dfrA1-sat1-aadA1* in *E. coli* globally (Goldstein et al., 2001; Su et al., 2006; Sunde, 2005; Sunde et al., 2015). In comparison, another GC array with a slight modification in the *sat* allele has also been reported (*dfrA1-sat2-aadA1*) (Su et al., 2012). A novel class 2 integron with a functional *intI2* gene in a pathogenic *E. coli* has been reported (Márquez et al., 2008). The integron possessed a GC *dfrA14* typically found in class 1 integrons and a novel ORF. A functioning *intI2* gene suggests that these integrons no longer rely on other mobilizing elements and have developed the ability to capture and distribute these genes to other bacteria. Class 3 integrons have not been often discovered in prior investigations, and when they are, they co-occur with classes 1 and 2 integrons in MDR isolates (Kargar et al., 2014), indicating that class 3 integrons may play a vital role in the spread of antibiotic resistance soon.

2.6.2 *Klebsiella* spp.

*Klebsiella* species are omnipresent. They are mostly found in two major habitats. First is environmental sources, including soil, surface water, plants, and sewage. The other is in the mucosal surfaces of warm-blooded vertebrates such as horses, swine, or humans (Broberg et al., 2014; Podschun & Ullmann, 1998). They also constitute members of the microbiome of the human gut. There are three major clinically important species of this genus. The most important is *Klebsiella pneumoniae* (responsible for 75% to 86% of *Klebsiella* infections), followed by *K. oxytoca* (accountable for 13% to 25% of *Klebsiella* infections) and less commonly *K. granulomatis* (Broberg et al., 2014). The two most common pathogens have been implicated in human infections. Examples are meningitis, urinary tract infections (UTI), wound infections, cholecystitis, pneumonia, and septicaemia (Tumbarello et al., 2015). These species have acquired resistance against many antibiotics, including ESBLs, cephalosporins, and aminoglycosides. The first carbapenem-hydrolyzing enzyme was isolated from a *K. pneumoniae* strain in North Carolina in 1996 and named *Klebsiella pneumoniae* carbapenemase (KPC) (Yigit et al., 2001). Shortly after, this enzyme spread rapidly among other members of Enterobacteriaceae and, within two decades, became a global public concern. Due to an aggravated incidence of MDR *K. pneumoniae* and the associated resistance challenges they pose, they have been included in the ESKAPE pathogens list (Boucher et al., 2009). The inclusion of resistance genes within mobile elements, particularly integrons, has been ascribed to the MDR trait's great potential to propagate rapidly in *Klebsiella* spp. (Chowdhury et al., 2011). The class 1 integrons in this genus have been extensively investigated and reported to harbour a variety of GCs. The most associated GCs are *aadA* and *dfrA,* although multiple resistance traits have reported the *dfrA-orfC* and *dfrA12-orfF-aadA2* (Gu et al., 2008, 2017; Xu et al., 2017). Some novel GCs reported include *aacA4-catB8-aadA1* and *aadB-catB-like-bla*$_{\text{OXA-10}}$*/aadA1* (Gu et al., 2008). Some

class 2 integrons have typical GCs *dfrA*, *sat1*/*sat2*, and *aadA1* (Wu et al., 2016), while class 3 harbouring *bla*$_{GES-1}$ has also been previously reported (Correia et al., 2003).

*K. pneumoniae* are generally known to produce the enzymes *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$ ESBLs. The genes that produce these enzymes may be found as independent entities or in complex integrons enhancing their rapid spread to other pathogens (Cantón et al., 2008). Several reports have reported higher integrons in ESBL producers than non-ESBL producers (Machado et al., 2013; Rao et al., 2006). Thereby further revealing the pivotal role integrons play in transferring extended-spectrum resistance.

2.6.3 *Salmonella* spp.

*Salmonella* is the causative agent of salmonellosis, a global zoonosis and a foodborne disease posing a significant public health risk. They have been frequently reported in water and are commonly isolated foodborne pathogens of dairy products, poultry and eggs, fruits, and vegetables. With a repertoire of over 2600 serovars, those implicated in enteric fever include Typhi, Sendai, and Paratyphi A, B, and C. The non-typhoidal *Salmonella* includes serovars such as Enteritidis and Typhimurium (Gal-Mor et al., 2014). Since the development of MDR *Salmonella* in 1990, they have posed a significant public health concern and are associated with different integrons classes with the usual presence of one to three antibiotic resistance GCs (Boyd et al., 2002). Various reports establish that their MDR traits have been harboured by different classes, with the most prevalent being class 1 integrons. Most of these integrons are present on conjugative plasmids and may be readily transferred to other strains, giving rise to similar GCs found in environmental, food, and human strains. The class 1 integrons have been more often involved in the widespread ARG in *Salmonella* spp. Class 1 has several variants of GCs encoding resistance against trimethoprim (*dfrA1*, *dfrA7*, *dfrA12*, *dfrA17*), aminoglycosides (*aadA*, *aadA*1, *aadA2*, and *aadA5*), gentamicin and kanamycin (*aadB*) either alone or in combination. Ab initio, only single GCs were reported among the different

*Salmonella* serovars (Peirano et al., 2006; Vo et al., 2010). However, just as seen in other members of Enterobacteriaceae, the occurrence of integrons harbouring multiple GCs has increased over time. Several reports of novel and atypical class 1 integron-mediated GCs in *Salmonella* spp. emerged (Lopes et al., 2016). For example, the unusual GC array *aac(6')-IIc*, *ereA2* and *aadA2* in *Salmonella enterica* serovar *Keurmassar* was reported in Senegal (Gassama-Sow et al., 2004), while the array *dfrA21-bla*$_{\text{OXA-129}}$*-aadA1* was reported in *S. enterica* serovar Bredeney in Brazil (Michael et al., 2008). The significant variety of class 1 integrons in terms of GCs, location, and distribution in different serovars is vital in disseminating resistance genes among *Salmonella* spp.

Class 2 integrons have also been detected in this bacterium, albeit at a relatively lower occurrence percentage than class 1. No class 3 integrons in *Salmonella* using molecular confirmation methods have been reported. The serovars of *Salmonella* harbouring class 2 integrons have a seemingly consistent GC array of *dfrA1-sat1-aadA1* (Antunes et al., 2006; Rodríguez et al., 2006). However, an array replacing *sat1* with *sat2* was observed in *S. enterica* serovar *Paratyphi B* dT$^{+}$ strains (Doublet et al., 2014; Miko et al., 2003). Novel GC with arrays of *sat1- ere*(A)*-aadA1* and *sat-sat1-aadA1* were also identified in class 2 integrons among non-typhoid *S. enterica* serovars in Japan (Ahmed et al., 2005).



2.6.4 *Shigella* spp.

In terms of prevalence and distribution, *Shigella* spp. is found worldwide. It is the causative agent of shigellosis and continues to be an important cause of diarrheal diseases. Shigellosis is a human colon infection that causes various symptoms, from short-term watery diarrhoea to severe inflammatory bowel illness with tenesmus, fever, and neurologic symptoms. It is linked to a high rate of disease, especially in children under the age of five, who account for the majority of cases (70%) and deaths (60%) (Barrantes & Achí, 2016; Kotloff et al., 1999).

The prompt treatment of shigellosis using effective antibiotics has been reported to reduce the period of clinical symptoms and decrease their transmission from person to person.

However, excessive use of these antibiotics has led to a progressive increase in antibiotic resistance. With resistance against ampicillin, trimethoprim/sulphonamides, tetracycline, and streptomycin. The rapid dissemination of the genes mediating this antimicrobial resistance phenomenon has been found on GCs harboured by integrons, especially class 1 and class 2 (Ahmed et al., 2006; Gu et al., 2017). Within the Enterobacteriaceae family, there seems to be a high occurrence of class 2 integron being reported in *Shigella* spp. than the class 1 (Gu et al., 2017; Peirano et al., 2005; Soltan Dallal et al., 2018).

2.6.5 *Enterobacter* spp.

This genus was first described in 1960 by Hormaeche and Edwards and has undergone several taxonomic modifications within the last six decades. The members of this genus are facultative anaerobic bacilli which are widely found in nature. As commensals, they are part of the gut human microbiome, while as saprophytes, they thrive in soil and sewage (Hormaeche & Edwards, 1960; Mezzatesta et al., 2012; Tindall et al., 2017). *Enterobacter* spp. has become clinically significant by emerging as nosocomial pathogens from intensive care patients accounting for 5 to 7% of all nosocomial infections in the United States in the past few decades. They are also important pathogens in plants and insects since they are easily found in terrestrial and aquatic environments (Sanders & Sanders, 1997; Mezzatesta et al., 2012). Several *Enterobacter* spp. have been reported harbouring class 1 integrons with GCs, including *aadA2* (Corkill et al., 2005) and *dfrA12-orfF-aadA2* (Mokracka et al., 2011). Ramrez and colleagues (Ramírez et al., 2010) found that class 2 integrons containing a typical *intI2* GC with extra genes (*dfrA1–sat2–aadA1–orfX–ybfA–ybfB–ybgA*) were a prominent GC array in *Enterobacter*. Class 3 integrons with GCs $bla_{\text{OXA-256}}$ and *aac(6')-Ib*

have also been found in *E. cloacae*, conferring resistance against oxacillin and gentamicin, respectively (Barraud et al., 2013).

2.6.6 *Citrobacter* spp.

The members of this genus are widely distributed and are a significant part of the normal intestinal flora of animals and humans. Species of this genus are also among the several members of the Enterobacteriaceae family, which are the leading cause of neonatal sepsis and meningitis (Liu et al., 2021). A large GC array containing a typical *intI2* GC with extra genes (*dfrA1*‑*sat2*‑*aadA1*‑*orfX*‑*ybfA*‑*ybfB*‑*ybgA*) like the *Enterobacter* array was reported to be a major GC array in *Citrobacter* (Ramírez et al., 2010). Recently, a class 3 integron has also been identified in *Citrobacter* spp. with resistance against oxacillin (Simo Tchuinte et al., 2016).

**2.7 Conclusion**



The Enterobacteriaceae family comprise bacteria in the gastrointestinal tract of animals and humans. Because several species of this family coexist in the gut, where they are constantly exposed to different classes of antibiotics, there is a higher probability that these bacteria will act as possible carriers of resistance gene determinants which should not be underestimated. These resistance genes are often found on gene cassettes which can be easily integrated into integrons and then subsequently expressed, thereby conferring the resistant phenotype on the organism. Integrons also harbour resistance determinants for other antimicrobials and pollutants. The physical relationship between integrons and such resistance determinants may result in invariable selection, which adds to the complexity and substantially influences public health.

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# CHAPTER THREE

## LITERATURE REVIEW

## Combination therapy: an alternate approach to combating multidrug-resistant bacterial infections

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**Abstract**

Multidrug-resistant gram-negative bacteria's ability to produce enzymes such as carbapenemases, β-lactamases, and aminoglycoside-modifying enzymes has made pathogens resistant against most of the present therapeutic choices, thus increasing the difficulty in managing life-threatening illnesses. The number of drugs currently available is insufficient to combat this global danger, hence the need to explore an alternative approach to solving the problem through the combination of existing antibiotics. Therefore, we highlighted some current antibiotic treatment options for multidrug-resistant gram-negative infections and some combinations of antimicrobials that have been explored *in vitro* to detect synergistic interactions.

**Keywords:** Multidrug resistance, checkerboard assay, time-kill assay



## 3.1 Introduction

The rising prevalence of illnesses mediated by antibiotic-resistant bacteria (ARB) is a worldwide health issue made worse by the lack of novel antibiotic classes available to clinics in the last decades (Worthington & Melander, 2013). According to reports, over 700,000 individuals die each year from ARB infection, which is anticipated to increase to about ten million by 2050 (Morris & Cerceo, 2020; Na et al., 2018). The spread of ARB has thus challenged the tremendous gains of the existing antibiotics and their efficacy in decreasing morbidity and mortality (Magiorakos et al., 2012). Microorganisms that exhibit resistance against one or more antibiotics from a minimum of three different antibiotic classes have been classified as multidrug-resistant (MDR) bacteria. Recently, the infections caused by MDR bacteria such as Enterobacteriaceae have increased. The World Health Organization (WHO) identified Enterobacteriaceae as critical pathogens requiring new antibiotics because of the increased multidrug resistance (World Health Organization, 2017).

Effective antibiotics are crucial in modern medicine, but the rapid growth of antibiotic resistance and the slow discovery rate of new antibiotics jeopardize future antibiotic treatment choices (O'Neill, 2016). Infections caused by the Enterobacteriaceae family are treated with different classes of antibiotics. However, the clinical, environmental, and agricultural settings have exerted selective pressures due to enormous antibiotic use and misuse over the decades and contributed to the emergence, re-emergence and spread of multidrug resistance globally (Fadare & Okoh, 2021; Jacoby, 2009).

In modern medicine, only half of the problem is simply having MDR bacteria and the danger of nosocomial or community-acquired infections. The rising occurrence of MDR organisms and the ease of transfer of resistance genes in environmental settings complicates the situation (Berendonk et al., 2015; Skandalis et al., 2021). The amount of resistance seen among *Escherichia coli*, *Enterobacter* species, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella* species, and *Pseudomonas aeruginosa*, for example, is especially concerning because these bacteria constitute the most common etiologies of hospital and community-acquired illnesses (Guo et al., 2020; Lynch et al., 2013). The acquisition of novel resistance components, mostly extended-spectrum β-lactamases (ESBLs) and carbapenemase-producing Enterobacteriaceae (CPEs) has resulted in a substantial rise in antimicrobial resistance among Enterobacteriaceae (Lynch et al., 2013). Bacterial species adopt a variety of defence mechanisms to counter the adverse effects of antibiotics designed against them. To overcome this, some novel therapeutic alternatives for gram-positive and gram-negative pathogens have recently received clinical approval in the United States and Europe (Terreni et al., 2021). Nonetheless, given the significance of this scientific milestone, further concerted efforts must still be put in place to create more drugs with broad-spectrum activity to reduce the spontaneous development of antibiotic resistance.

The resistance phenomenon complicated by the slow development of new antibiotics has rendered most current antibiotics ineffective, prompting the search for novel, effective treatments (Brennan-Krohn & Kirby, 2019a; Spellberg, 2014), such as exploring synergistic interactions in currently available drugs. Even if the bacteria are resistant against one or both antibiotics separately, two drugs together can have a more significant impact, allowing current antibiotics to be salvaged in treating MDR bacteria. Therefore, we highlighted some current antibiotic treatment options for multidrug-resistant gram-negative infections and some combinations of antimicrobials that have been explored *in vitro* to detect synergistic interactions.

### 3.2 Current antibiotics widely used in treating MDR Enterobacteriaceae infections

3.2.1 The β-lactams

In clinical practice, β-lactam antibiotics rank among the essential therapeutic alternatives for infections caused by Enterobacteriaceae family members (Li et al., 2019). Over time, microbes have evolved with the capacity to break down the chemical backbone in β-lactams via the development of enzymes known as β-lactamases (Cantón et al., 2012; Fadare et al., 2020; Rawat & Nair, 2010). The ESBLs have been identified as a significant concern in clinical practice. Penicillins, monobactams, penicillin inhibitors, and third and fourth-generation cephalosporins inhibited by clavulanic acid can all be hydrolyzed by class A β-lactamases. These ESBLs with great dissemination potential have evolved significantly, particularly in clinical settings (Bush & Jacoby, 2010; Jacoby, 2009; Tacconelli et al., 2018). The TEM genotype was the first reported ESBL (Bush & Jacoby, 2010; Datta & Kontomichalou, 1965), followed by the SHV genotype (Brunton et al., 1986; Bush & Jacoby, 2010). Several additional β-lactamases of clinical significance have been discovered. These include the PER, GES, and VEB, called minor ESBLs. Carbapenems also have the β-lactams structure, and resistance against them is mediated by organisms producing the carbapenem

hydrolyzing β-lactamases called carbapenemases. Clinically relevant carbapenemases include the metallo β-lactamases (MBL) Imipenem-type carbapenamases (IMP), *Klebsiella pneumoniae* carbapenemases (KPC), Verona integron-encoded metallo β-lactamases (VIM), and oxacillinase group of β-lactamases (OXA)-type such as OXA-1, and OXA-48. Furthermore, pAmpCs are class C β-lactamases, divided into six families: DHA, ACC, EBC, MOX, FOX, and CIT (Dallenne et al., 2010; Paterson & Bonomo, 2005). The evolution of these resistance genotypes complicates using this class of antibiotics as effective therapeutics.

### 3.2.2 The aminoglycosides

Another important class of antibiotics used to treat MDR bacteria is aminoglycosides (AMGs). They are broad-spectrum drugs that comprise aminocyclic alcohols and amino sugars because these sugar units typically contain numerous hydroxyl and amino groups, with high water solubility and a broad antibacterial activity (Johnston et al., 2002). By inhibiting the protein synthesis pathway, they have been used for many years to treat infections facilitated by non-fastidious gram-negative bacteria (Castanheira et al., 2018). However, as these antibiotics have become more widely used, adverse effects such as nephrotoxicity, ototoxicity, and neuromuscular inhibition have been discovered, with bacterial resistance increasing. As a result, third-generation AMGs were created, including netilmicin (1976), arbekacin (1973), and amikacin (1972) (Kudo & Eguchi, 2016).



However, it was later seen that these AMGs showed poor antibacterial activity against bacteria that produce aminoglycoside modifying enzymes (AMEs). One of the most common bacterial resistance mechanisms against AMGs is the enzymatic modification of aminoglycoside antibiotics (Wachino et al., 2020). The enzymes belong to families such as aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs) and aminoglycoside adenyl transferases (ANTs) (Sparo et al., 2018; N. Wang et al., 2022). The creation and use of broad-spectrum antibiotics such as β-lactam antibiotics and

fluoroquinolones with fewer adverse effects further made AMGs unattractive, with consequent low sales and further apathy in the hunt for newer, more effective AMGs (Castanheira et al., 2018). Although, recently, a new type of parenteral antibiotic, Plazomicin (previously ACHN-490) targets MDR Enterobacteriaceae, such as ESBL, carbapenemase and AME producers, was approved for treating pyelonephritis and complicated urinary tract infections (Saravolatz & Stein, 2020).

3.2.3 The quinolones

Quinolones are a class of synthetic antimicrobials with great clinical use. The first member, nalidixic acid, was effective against Gram-negative bacterial infections. Other member derivatives were, however, used as broad-spectrum antibiotics (Hooper & Jacoby, 2015). It is a class of antibiotics that targets the enzymes bacterial topoisomerases DNA gyrase and topoisomerase IV (Khodursky & Cozzarelli, 1998), which are enzymes involved in DNA transcription, repair, recombination, and replication. When the drugs bind to these enzymes, they prevent the progression of the DNA replication fork, thereby leading to bacterial cell death.



As synthetic drugs, it was predicted that resistance development could only arise owing to alterations in genes encoding target enzymes or transporter as there are no quinolone-producing microorganisms in the environment. Hence, topoisomerase mutations continue as the most predominant method for bacterial populations to develop significant levels of resistance against these antibiotics. Mutation in the gyrase *gryA* and *gryB* subunits is the primary cause of quinolone resistance in gram-negative bacteria. In contrast, mutations in genes *parC* and *parE* encoding topoisomerase IV are secondary because the gyrase subunits are the primary target for quinolones. Mutations in the *par* genes have only been found in bacteria that also show changes in *gryA*. Another means of resistance against quinolones is the presence of efflux pumps on the bacterial species. When the possession of the efflux

pump and mutational processes co-occur, the level of resistance against quinolones is at its maximum (Hooper & Jacoby, 2015).

Also, there have been reports of the plasmid-encoded gene, *qnr*, which causes resistant quinolone phenotypes. The *qnr* gene is a member of the pentapeptide repeat protein (PRP), which protects the bacterial topoisomerases from the activity of quinolones (Blanco et al., 2016; Martínez-Martínez et al., 1998; Martinez et al., 2009). The *qnr* families *qnrA*, *qnrC*, *qnrB*, *qnrD*, and *qnrS* have been described in plasmids from bacterial pathogens of environmental origins (Cavaco et al., 2009; Jacoby et al., 2006; Ruiz, 2019). The genetic environments surrounding *qnrA* or *qnrB* genes show that they are usually integrated into complex *sul1*-type integrons and associated with IS*CR1* (Nordmann & Poirel, 2005). In addition to *qnr*, these integrons usually harbour other resistance genes, such as aminoglycoside inactivating enzymes or β-lactamases (Strahilevitz et al., 2007; Wang et al., 2003). Although the *qnrS* genes are not harboured by integrons, they are frequently associated with Tn*3* transposon, which contains a TEM-1 β-lactamase gene (Strahilevitz et al., 2009).



Finally, the plasmids may hold additional resistance-determining genes. These genes are especially pertinent given the correlation between the *qnr* genes and the extended-spectrum β-lactamase and AmpC β-lactamase genes. Since the extra resistance genes will allow for co-selection of *qnr*, these interactions with other resistance determinants have probably favoured the spread and maintenance of *qnr* genes even in settings where quinolones are not used for therapy (Strahilevitz et al., 2009).

### 3.3 Combination strategies to overcome resistance

Since no antibiotic drug is effective against all infections, one of the principal reasons for combining drugs was the possibility of greater efficacy than single antibiotics. Several

objectives are accomplished by combining antibiotics. The first is the ability to widen the antibacterial spectrum while administering empirical therapy when it is still difficult to ascertain the organism's identity. The second objective is to produce synergistic results that boost treatment effectiveness. Other objectives include averting the development of resistance and lowering host toxicity (Moellering, 1983; Silver, 2007, 2016). A deeper understanding of the underlying mechanisms of synergies obtained in combination studies would help interpret the results and forecast the effects of other combinations. For instance, colistin is more likely to overcome impermeability than alterations in the target molecule, whereas ertapenem can only function as a competitive carbapenemase inhibitor in the periplasmic space if the antibiotic can pass through the bacterial's outer membrane.

Streptomycin and penicillin were the first medications to be combined in 1950 (Jawetz et al., 1952), while trimethoprim and sulphonamides were mixed in 1968 (Bushby & Hitchings, 1968). These combinations improved the potency and antibacterial spectrum of the medicines. Antibiotic resistance can be reduced using fewer and more effective antibacterial drugs. Since the duration of antibiotic exposure is linked to resistance development (Andersson et al., 2020), it is critical to use medications with broad-spectrum activity and pharmacokinetic qualities that allow them to reach the target site quickly (Krause et al., 2016). However, because most existing medicines lack all these features, combining therapies can result in a synergistic and more successful outcome (Lehár et al., 2009; Silver, 2016), which is an alternate option. It has been demonstrated that combining medications produces a far more potent effect than using each drug alone (Hussein et al., 2020; Sick et al., 2014; Tamma et al., 2012). Colistin, which is now only used as a last resort, worked well with protein synthesis inhibitors like linezolid, fusidic acid, and clindamycin, which individually have a negligible effect on gram-negative bacteria (Brennan-Krohn et al., 2018). Such combinations are still used on the front lines today because they are supported by laborious

clinical and epidemiological data (Laxminarayan et al., 2013; Silver, 2016; Tyers & Wright, 2019).

The clinical application of aminoglycosides has been hindered due to bacterial resistance and their toxic side effects, such as nephrotoxicity and ototoxicity. So, investigations have been carried out to combine aminoglycosides with synergistic effects leading to a reduction in the dosing concentration of AMGs, which will abate the intensity of their side effects (Sick et al., 2014; Tamma et al., 2012). When AMGs were combined with β-lactams, it was observed that they accelerated the clearance of bacteria, thereby expanding the scope of clinical treatment and reducing the occurrence of the emergence of resistance (Le et al., 2011). The β-lactams assist in non-destructive damage of the bacterial cell wall, consequently allowing the AMGs to enter the bacterial cell, thereby increasing their killing activity (Castanheira et al., 2018; Davis, 1982; Sick et al., 2014). Polymyxins have also been combined with AMGs with synergistic antibacterial interactions against gram-negative *Pseudomonas aeruginosa* (Hussein et al., 2020). Combining non-inhibitory drugs can have more significant effects than their individual activities due to complementing actions or different targets of action in microbial cells. These combinations are among the most efficient strategies to treat diseases caused by pathogens.



### 3.4 Effective *in vitro* combination techniques

Various techniques are frequently employed in testing for synergy in drug combinations which do not always produce the same outcomes. Selecting an appropriate technique is intricate as no established gold standard synergy reference method exists. Although many studies have not compared *in vitro* synergy testing findings with clinical outcomes, an ideal procedure should reliably predict treatment effects. Also, different techniques might be most effective for various organisms or medications (Brennan-Krohn & Kirby, 2019b; Okoliegbe et al., 2021). The checkerboard approach or time-kill assays (TKAs) are typically used to

investigate the effectiveness of antibiotic combinations utilizing static antibiotic concentrations *in vitro*.

### 3.4.1 The checkerboard

In the checkerboard method, standard broth-based minimal inhibitory concentration testing is used with at least a two-dimensional array of serial concentrations of the test drugs. The array is typically created in a 96-well microtiter plate, with each well containing the standardized bacterial inoculum and proper concentrations of drugs incubated at appropriate conditions (Brennan-Krohn & Kirby, 2019b). The results of the microtiter plates are then used for computing the fractional inhibitory concentration (FIC) index in the well with growth inhibition. The FIC of each drug is calculated by dividing the drug's concentration in a well by the drug's MIC. The FIC index is obtained by summating the FIC values of the drugs in a well. A typical layout of the checkerboard array is indicated in Figure 3.1. Here, synergy is defined when there is an enhanced combination's antibacterial impact compared to the effects of each antibiotic after 24 hours with the FIC index $\leq 0.50$, where values greater than 4 are considered antagonistic with intermediate values classified as indifferent (Fadare et al., 2022; Odds, 2003). It is pertinent to note that for the combination to be classified as synergistic, the concentration of each drug in the inhibited well must be less than one-half of its MIC value due to the accepted ±1 two-fold dilution difference of a drug during MIC testing (CLSI, 2020).

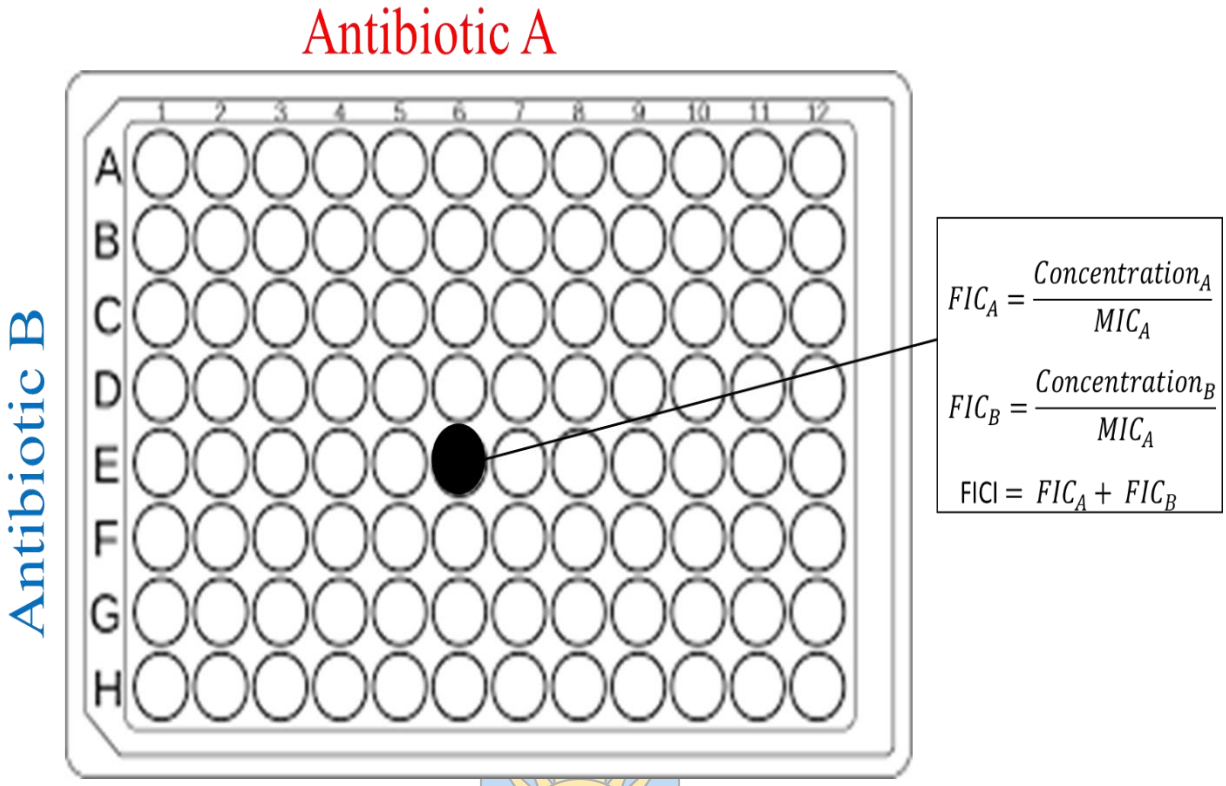


**Figure 3. 1: A typical checkerboard array and the Fractional Inhibitory Concentration (FIC) Index calculation.** $FIC_A$: FIC of antibiotic A; $FIC_B$: FIC of antibiotic B; FICI: FIC index.



In the evaluation of Berçot and colleagues, the checkerboard assay was used for synergy combination testing using colistin, fosfomycin and tigecycline against eight clinical Enterobacteriaceae isolates. Although most of the interactions were indifferent, synergistic activity was reported for the colistin-fosfomycin combinations, while colistin-tigecycline combinations were rare (Berçot et al., 2011). Another *in vitro* study combined pentamidine, an antiprotozoal drug, with amikacin, tobramycin, tigecycline, and doripenem against clinical strains of carbapenemase-producing colistin-resistant Enterobacteriaceae. The results indicated synergistic and bactericidal interactions (Cebrero-Cangueiro et al., 2018). The *in vitro* success in combining colistin with rifampicin against clinical isolates of carbapenemases-producing *Klebsiella pneumoniae* was further confirmed *in vivo* using a murine peritoneal sepsis model. *In vitro*, synergistic interactions were observed against all strains, while *in vivo*, the combination reduced bacterial tissue concentrations against half of

the clonally unrelated strains when compared with the controls, albeit improving their survival. The combination revealed efficacy in less severe pneumonia models and prevented the development of resistant mutants (Pachón-Ibáñez et al., 2018).

3.4.1 The time-kill assasy (TKA)

One of the drawbacks of the checkerboard assay is that it assesses the inhibition of bacterial growth at the end of the assay and, as such, is not used to predict the bacterial killing rate. The time-kill assay, however, addresses this concern. The time-kill assay reveals both time-dependent and concentration-dependent drug interactions making it a potent tool for learning about the dynamic interaction between the test agents and the organisms. Although it is more laborious and expensive than the checkerboard assay, it is the best method for determining the bactericidal activity of the drugs (Balouiri et al., 2016). Although, it also has its drawback in that its results are delayed by a day as they depend on quantifying bacterial cultures. In a time-kill experiment, the bacterial inoculum is inoculated in liquid culture tubes containing each drug alone at given concentrations, the two drugs combined at their given concentrations, and antibiotic-free growth control. Aliquots are removed from each tube for colony counts at the beginning and at specific time intervals throughout the 24-hour assay. When viable bacteria are reduced by at least 2 log10 CFU/mL compared to the most potent single antibiotic, the combination's effectiveness is synergistic. When there was a reduction of 3 log10 CFU/mL or more at 24 hours compared to the starting inoculum, the effectiveness of the combination therapy was also categorized as bactericidal (Brennan-Krohn et al., 2018; CLSI, 2020).



**3.5 Limitations of *in vitro* studies**

Contradictory findings from published *in vitro* research may have resulted from methodological variations, bacterial inoculum size, drug concentrations, and strain-dependent variables. However, many of these investigations found that antibiotic combinations had

bactericidal or synergistic effects when used against bacteria that had developed resistance against the individual antibiotics. For instance, synergistic interactions have been observed in double and triple antibiotics combinations against carbapenemase-producing Enterobacteriaceae (Berçot et al., 2011; Pachón-Ibáñez et al., 2018; Tängdén et al., 2014; Urban et al., 2010).

The fact that *in vitro* experiments do not accurately mimic the conditions of cells in an organism, as is the case with isolated and cultured primary cells, which typically differ significantly from the corresponding cell type in an organism, limits the usefulness of *in vitro* data in predicting *in vivo* behaviour. Since it is impossible to correctly predict *in vivo* outcomes from *in vitro* evaluations of antimicrobial synergy, adequate safety measures must be followed before using such combination therapy in clinical settings. First, a pharmacokinetic/pharmacodynamic simulation is required since the concentrations measured can be higher than the tolerated limit for the actual serum levels. Another critical concern is that the inoculum size utilized for these *in vitro* assays may differ dramatically *in vivo* vis-a-vis host defence mechanisms, and the results from the checkerboard and rate of kill assays may not reflect the actual outcome when utilized in clinical settings. Therefore, future research in which the synergistic and bactericidal connections observed in *in vitro* combinations need to be investigated in animal models, pharmacokinetic/pharmacodynamic investigations, and human subjects will be vital in establishing the possible clinical effects applications of our findings.



### 3.6 Conclusion

Combination therapy may frequently be required for positive patient outcomes, but human clinical trials are still lacking and frequently constrained by retrospective and noncomparative study designs. The utilization of different combination regimens for MDR Enterobacteriaceae has been investigated in numerous studies. However, these studies frequently lack variable

dosage exposures or *in vivo* validation, and the best antimicrobial agent/class combinations for treating infections must be optimized in the shortest possible time.

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**CHAPTER FOUR**

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**Abundance, molecular characterization of integrons and its associated gene cassettes in *Klebsiella pneumoniae* and *Klebsiella oxytoca* recovered from diverse environmental matrices (Under review in *Scientific Reports*)**

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**Abstract**

The high prevalence of infections arising from *Klebsiella* species is related to their ability to acquire and disseminate exogenous genes associated with mobile genetic elements such as integrons. We assessed the prevalence, diversity, and associated gene cassettes (GCs) of integrons in *Klebsiella* species. The isolates recovered from wastewater and hospital effluents, rivers, and animal droppings were identified using conventional molecular PCR with primers targeting the *gryA*, *pehX*, and *16S-23S* genes. The antimicrobial resistance profile and the production of extended-spectrum β-lactamase and metallo β-lactamase were detected using disk diffusion, double disk synergy, and ethylenediaminetetraacetic acid tests, respectively. PCR, DNA sequencing analyses, and restriction fragment length polymorphism were used to characterize the integrons and their associated GCs. Furthermore, the genotypic relationships between the different isolated *K. pneumoniae* were determined using enterobacterial repetitive intergenic consensus (ERIC)-PCR. About 98% (51/52) of the confirmed isolates harboured an integrase gene, with 80% *intI1*, while the remaining 20% co-currently harboured *intI1* and *intI2* genes, with no *intI3* observed. About 78% (40/51) of the bacterial strains were positive for a specific promoter, the *P2R2* gene, investigated, while 80% (41/51) harboured at least one of the *qacEΔ1* and *sul1* genes. Three different GCs arrangements identified were *aac(6')-Ib*, *aadA1-dfrA1*, and *dfrA1-sat2*. At a similarity index of 60%, the ERIC-PCR fingerprints generated were categorized into nine clusters. Our study is the first to reveal the features of integrons in *Klebsiella* spp. recovered from environmental sources in the Eastern Cape Province, South Africa. We conclude that the organisms' sources are repositories of integrons harbouring various antibiotic resistance gene cassettes, which can be readily mobilized to other microorganisms in similar or varied niches.



**Keywords:** Integrons, *Klebsiella* spp., gene cassettes, antimicrobial resistance, ERIC-PCR

## 4.1 Introduction

The Klebsiella genus is becoming more often implicated as opportunistic pathogens linked to severe hospital-acquired illnesses, including urinary tract infections, septicaemia, and pneumoniae. The most common species in this genus are *Klebsiella oxytoca* and *Klebsiella pneumoniae*, accounting for approximately 8% of all hospital-acquired infections in Europe and the United States (Magill et al., 2014). Antibiotics are generally given to treat the illnesses these organisms cause. Unfortunately, over the decades, the increased use of antibiotics in veterinary, agricultural, and clinical sceneries has aggravated the emergence and widespread of multidrug-resistant (MDR) pathogens even in environmental sources (Jones-Dias et al., 2016). This scenario has reduced treatment options with current antibiotics, especially when MDR bacteria harbour genes exhibiting resistance against extended-spectrum β-lactamases (ESBLs) and carbapenemases. These bacteria can readily acquire the genes that produce hydrolyzing enzymes, which can be harboured on various mobile genetic elements, further accelerating their dissemination process (Firoozeh et al., 2019).



Integrons are mobile genetic elements found on pathogenicity islands, transposons, and plasmids, easing their distribution among various bacteria. They are considered efficient gene expression systems that naturally capture, integrate gene cassettes (GCs) and immediately express the captured antimicrobial resistance genes (ARGs) due to the inherent promoters located on their structures (Ghaly et al., 2021; Hall & Collis, 1995; White et al., 2001). All integrons have a functional platform on the 5′-Conserved Segment (CS) that harbours the integron-integrase, *intI* gene, the integron-associated recombination site, *attI*, and a highly constitutive promoter, $P_c$. Integrons are categorized into classes according to the sequences of the *intI* gene (Boucher et al., 2007; Cambray et al., 2010; Gillings, 2014; Hall & Collis, 1995). The classes often detected within the Enterobacteriaceae family include the class 1, class 2, and class 3 integrons (Deng et al., 2015). The most prevalent integrons are class 1,

frequently reported in *K. pneumoniae* (Firoozeh et al., 2019; Lima et al., 2014). Typical class 1 integrons have a second CS known as the 3′ CS. The first open reading frame (ORF) harbours the *qacE* gene that encodes resistance against quaternary ammonium compounds—followed by the ORF harbouring *sul1* gene conferring resistance against sulphonamides. Others include the *orf5*, a gene whose exact function is unknown but with some similarity to puromycin acetyltransferase. In addition, the *orf6* with a yet-to-be-discovered biological activity (Daly & Fanning, 2004; Kaushik et al., 2018; Okoh & Fadare, 2022; Stokes & Hall, 1989). In *Klebsiella* spp., class 2 integrons have sometimes been identified, whereas class 3 integrons have been the least frequently discovered (Firoozeh et al., 2019; Lima et al., 2014).

Gene cassettes are variable sequences that can occur as free, circular, nonreplicating DNA molecules and are usually linear when integrated into integrons (Cambray et al., 2010). Their presence has been known to confer resistance against most antibiotic classes. These include all known aminoglycosides, erythromycin, antiseptics of the quaternary ammonium compounds, β-lactams, chloramphenicol, fosfomycin, trimethoprim, lincomycin, quinolones, rifampicin, and streptothricin (Deng et al., 2015; Gillings, 2014; Kaushik et al., 2019; Mazel, 2006). Integrons harbouring several cassette arrays have been described in North and South America, Europe, and Asia (Chowdhury et al., 2011; Deng et al., 2015; Firoozeh et al., 2019). Although the main focus of detecting integrons and their GCs was within the clinical environment, they have also been identified in several bacterial species from various environmental sources (Boucher et al., 2007; Kaushik et al., 2019; Su et al., 2012). However, restricted studies exist on integron characterization in *Klebsiella* spp. from environmental sources in the Eastern Cape Province (ECP) in South Africa. This study reports the prevalence and characteristics of integrons and their associated GCs in *Klebsiella* species from various environmental matrices.

## 4.2 Materials and methods

### 4.2.1 Bacterial strains

Fifty-two *Klebsiella* species isolates recovered from various environmental matrices in Amathole and Chris Hani District Municipalities in the ECP and reported in our previous papers (Fadare et al., 2020; Fadare & Okoh, 2021a, 2021b) were included in this study. Of these, 33 *Klebsiella pneumoniae* were recovered from freshwater sources (n=7), hospital wastewater (HWW) (n=9), and wastewater treatment plant effluents (WWTP) (n= 7), while the remaining were isolated from animal droppings (FD). In addition, 19 *K. oxytoca* bacterial strains were recovered from HWW (n=11), WWTP (n=2), and FD (n=6). The bacterial isolates were recovered between October and November 2017. The bacterial isolates were stored at -80 ℃ in 20% glycerol stock as part of the Applied and Environmental Research Group (AEMREG) culture collection. Ethical clearance was obtained with project number OKO011SFAD01 to access these bacterial cultures.



### 4.2.1 Resuscitation and DNA extraction of bacterial cultures

The retrieved glycerol stocks were resuscitated in Brain Heart Infusion (BHI) broth (Merck, South Africa) and incubated at 37 ℃ for 18 hours. A loopful was streaked on nutrient agar (Oxoid, UK) and further purified twice. For subsequent analysis, single pure colonies were transferred to 2 ml BHI broth and incubated overnight at 37 ℃. The boiling method (Maugeri et al., 2004) extracts the genomic DNA with slight modifications, as previously reported elsewhere (Fadare et al., 2020). The DNA-containing supernatant was then transferred to sterile microcentrifuge tubes (Eppendorf, Germany) and stored at -20 ℃ for future assays.

### 4.2.2 PCR-based confirmation of *Klebsiella* spp.

The molecular confirmation of the *Klebsiella* genus was by the conventional Polymerase Chain Reaction (PCR) assay using primer sets that target the *gryA* gene. All the genus-confirmed *Klebsiella* isolates were speciated into *K. pneumoniae and K. oxytoca* using

primers targeting the 16S-23S ITS and *pehX* genes, with primers and conditions described in Table 4.1. *K. pneumoniae* ATCC 35657$^{TM}$ and *K. oxytoca* NCTC 11686 were included as positive controls (Microbiologics, Medimark, France), while *E. coli* ATCC 8739 was used as the negative control.

Each PCR mixture of a total of 25 µl contained PCR master mix (12.5 µl) (Thermo scientific (EU), Lithuania), forward and reverse primers (1 µl) (Inqaba Biotechnical Industries, South Africa). Furthermore, 5.5 µl nuclease-free water and DNA template (5 µl) were added. The DNA amplification was executed in a BioRad thermal cycler (LASEC, South Africa). A 1.5% (w/v) agarose gel stained with ethidium bromide (5 µl) was used to resolve 5 µl of the amplicons. The gel was run using Mupid-One (Eurogentec, Belgium) electrophoresis set-up at 100 volts for 1 hour in 0.5X TBE buffer with a 100-base-pair molecular marker (Biolabs, New England). The results were viewed using UV transillumination (ALLIANCE 4.7, UVtec, London, UK).

**Table 4. 1: The list of primers and thermocycling conditions used for PCR amplification.**

| Primer | Primer Sequence (5′ →3′) | Amplicon size (bp) | Thermocycling conditions | Reference |
|---|---|---|---|---|
| *gryA*-F<br>*gryA*-R | CGCGTACTATACGCCATGAAGT A<br>ACCGTTGATCACTTCGGTCAGG | 441 | 94 ℃ for 5 mins; 35 [94 ℃, 30 s; 55 ℃, 45 s; 72 ℃, 45s]; 72 ℃ for 7 min. | (Brisse & Verhoef, 2001) |
| *16S-23S ITS*-F<br>*16S-23S ITS* -R | ATT TGA AGA GGT TGC AAA CGA T<br>TTC ACT CTG AAG TTT TCT TGT GTT C | 130 | 94 ℃ for 5 mins; 30 [94 ℃, 30 s; 55 ℃, 30 s; 72 ℃, 40 s]; 72 ℃ for 10 min. | (Liu et al., 2008) |
| *pehX*-F<br>*pehX*-R | GATACGGAGTATGCCTTTACGG TG<br>TAGCCTTTATCAAGCGGATACT GG | 343 | 94 ℃ for 5 mins; 30[94 ℃, 30 s; 55 ℃, 30 s; 72 ℃, 40 s]; 72 ℃ for 10 min. | (Kovtunovych et al., 2003) |
| *INTI1*-F<br>*INTI1*-R | CAG TGG ACA TAA GCC TGT TC<br>CCC GAG GCA TAG ACT GTA | 164 | 94 °C for 5 mins; 35[94 °C, 60 s; 55 °C, 60 s, 72 °C, 30 s] 72 °C, 10 min | (Koeleman et al., 2001) |
| *INTI2*-F<br>*INTI2*-R | TTATTGCTGGGATTAGGC<br>ACGGCTACCCTCTGTTATC | 232 | 94 °C for 5 mins; 32[94 °C, 60 s; 59 °C, 60 s; 72 °C, 2 mins]; 72 °C for10 mins. | (Goldstein et al., 2001) |
| *INTI3*-F<br>*INTI3*-R | AGTGGGTGGCGAATGAGTG<br>TGTTCTTGTATCGGCAGGTG | 600 | 94 °C for 5 mins; 32[94 °C, 60 s; 48 °C, 60 s; 72 °C, 2 mins]; 72 °C for 10 mins. | (Goldstein et al., 2001) |
| INTF<br>P2R2 | AGTGGGTGGCGAATGAGTG<br>TGTTCTTGTATCGGCAGGTG | 540 | 94 °C for 5 mins; 35[94 °C, 60 s; 55 °C, 60 s; 72 °C, 30 s]; 72 °C for 10 mins. | (Wei et al., 2011) |
| *qacEΔ1*-F<br>qacEΔ1-R | ATC GCA ATA GTT GGC GAA GT<br>CAA GCT TTT GCC CAT GAA GC | 225 | 94 for 9mins; 30[94 ℃, 30 s; 55 ℃, 30s; 72 ℃, 60s]; 72 ℃ for 10 mins. | (Stokes & Hall, 1989) |
| *Sul1*-F<br>*Sul1*-R | ATGGTGACGGTGTTCGGCATCT GA<br>CTAGGCATGATCTAACCCTCGG TCT | 840 | 94 for 9mins; 30 [94 ℃, 30 s; 55 ℃, 30 s; 72 ℃, 1 min]; 72 for 10 mins. | (Grape et al., 2005) |
| 5′CS<br>3′CS | GGCATCCAAGCAGCAAG<br>AAGCAGACTTGACCTGA | Variable | 94 °C, 5 min; 35[94 °C, 1 min; 45 °C, 1 min; 72 °C, 2 min]; 72 °C for 10 mins. | (Lévesque et al., 1995) |
| hep 74<br>hep51 | CGGGATCCCGGACGGCATGCA CGATTTGTA<br>GATGCCATCGCAAGTACGAG | Variable | 94 °C, 5 min; 33[94 °C, 1 min; 59 °C, 45 s; 72 °C, 5 min]; 72 °C, 5 min | (White et al., 2001) |

4.2.3 Phenotypic identification of antibiotic susceptibility, metallo and extended-spectrum β-lactamase production

The susceptibility profile was determined using the diffusion technique (DDT), approved by the Clinical and Laboratory Standard Institutes (CLSI). Isolates were assessed for extended-spectrum β-lactamase (ESBL) production using the double-disk synergy test (DDST). It was done using ceftazidime-10 µg, cefoxitin-30 µg, and cefotaxime-30 µg in combination with amoxicillin/clavulanic acid -20 µg/10 µg. The isolates were also exposed to a panel of the following nine antibiotics trimethoprim-sulfamethoxazole-25 µg, ampicillin-10 µg, colistin sulphate-10 µg, imipenem-10 µg, chloramphenicol-30 µg, gentamicin-10 µg, ciprofloxacin-5 µg, nalidixic acid-30 µg, and tetracycline-30 µg. The antibiotics were purchased from Mast Diagnostics, South Africa.

The inoculum of *Klebsiella* spp. was suspended in sterile saline solution with the turbidity adjusted to the 0.5 McFarland standard and evenly spread on Mueller-Hinton agar plates (Oxoid, UK). A disc dispenser (Mast Diagnostics, South Africa) was used to place the antibiotics. The plates were incubated at 37 °C for 18 hours. After that, the inhibition zones' width was measured to the nearest millimetre and compared to the CLSI established breakpoints to categorize the isolates as resistant, intermediate, or susceptible. Isolates resistant against more than two antimicrobial classes were considered MDR (CLSI, 2020).



The ethylenediaminetetraacetic acid (EDTA) test was used to determine the generation of metallo β-lactamase (MBL) according to CLSI standards. Briefly, 0.5 McFarland adjusted test isolate was exposed to two 10 µg imipenem discs. Then, 10 µl of 0.5 M EDTA was added to only one disc to obtain a concentration of 750 µg, and an increase in inhibitory zone width of 5mm in the disc potentiated with EDTA after 18 hours of incubation at 37 °C was recorded as positive for MBL generation (CLSI, 2020).

4.2.4 Molecular detection and characterization of integrons

The confirmed bacterial strains were screened with conventional PCR assays as described above. The presence of *intI1*, *intI2*, and *intI3* genes located on the 5′-CS was assayed for the classification of the integrons. In addition, a *P2R2* gene on the 5′-CS of a typical integron was also screened to detect the promoter. Furthermore, the *intI1*-positive strains were assessed for genes (*qacEΔ1* and *sulI*) on the ORFs at the 3′-CS of a typical class 1 integron. The positive control for a typical class 1 integron used was *Acinetobacter baumannii* ATCC 19606. All primers and thermal cycling conditions are listed in Table 4.1.

4.2.5 Mapping of integrons

In separate PCR assays, isolates positive for the *intI1* and *intI2* genes were assessed to detect their internal variable regions (IVRs). Specific primer set 5′-CS and 3′-CS, which joins the *attI1* site of the 5'-CS and the 3'-CS of *intI1*, was used for *intI1* positive isolates while the primer set hep74 and hep 51, which binds to the *attI2* and the *orfX* sites located downstream the GC regions, were for *intI2* positive isolates, used as indicated in Table 4.1. The PCR assays were carried out in triplicates to ensure reproducibility.



4.2.6 Restriction analysis and DNA sequencing of amplicons

Amplicons of variable regions that appeared similar in size were exposed to the *AluI* restriction enzyme (Biolabs, England) for the restriction fragment length polymorphism (RFLP) to assess if the products were in the same sequence. For the identification of similar GC arrays in the integrons, *AluI* was chosen due to its recognition sequence being only four bases, thereby increasing the likelihood of its activity over the other enzymes that target the six-base sequence. Briefly, 30 µl of the amplicon was exposed to 1.0 µl of 10U/ml *AluI*. The reaction mixture was then incubated for 4 hours at 37 ℃. The products were then run on a 2% agarose gel and visualized. These were characterized according to their distinct restriction profiles, and two randomly selected representative amplicons from each different RFLP class

were selected for sequencing. This step was taken to help reduce the risk of sequencing multiple identical variable regions needlessly.

4.2.7 Variable sequence analysis, cassette identification

The content and arrangement of the inserted GCs within the amplified IVRs were analyzed through sequencing. The amplicons were sequenced in both directions on an ABI 3500XL sequencer using the Nimagen, BrilliantDye™ Terminator Cycle sequencing kit V3.1 (Inqaba Biotechnical Industries, South Africa). The sequences were then modified with Chromas 2.7 and pairwise aligned using BioEdit sequence alignment editor software. BLAST nucleotide search analysis (https://blast.ncbi.nlm.nih.gov/Blast) was performed on the generated consensus sequences to identify the contents of the inserted gene cassette. The position of each gene in each cassette was determined using ABRicate 0.8.4 (https://github.com/tseemann/abricate) (ResFinder, ARG-ANNOT, CARD, and NCBI databases).



4.2.8 Molecular typing of isolates

Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) was done to evaluate the genotypic similarities of the integron-harbouring *K. pneumoniae* despite being isolated from different environmental matrices. PCR was performed using the primer sets ERIC1: (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC 2: (5′ -AAGTAAGTGACTGGGG TGAGCG-3′) under conditions as described (Versalovic et al., 1991). The amplicons were resolved in 3% agarose gel in a 0.5X TBE buffer and allowed to run at 100 volts for 240 mins.

4.2.9 Statistical analysis

The descriptive statistical software in Microsoft Excel 2016 and the Statistical Package for the Social Sciences (SPSS) version 25 were used to examine the data (SPSS Inc., Chicago,

IL). The data was validated, and relationships were calculated with 2 X 2 cross-tabulation tables utilizing exploratory data analysis. Pearson's Chi-square test was used to examine the statistical significance of susceptibility and the number of integron-positive isolates. A *P* value of less than 0.05 was considered significant.

Using Gelj v.2.0 software, computer-assisted pattern analysis was used to examine the DNA fingerprints generated from the ERIC-PCR. Pearson's correlation coefficient was used to compute the percentage similarity of digitized bands. The relatedness of the isolates was estimated using the unweighted pair group method with arithmetic mean (UPGMA) and complete linkage methods, which were shown as dendrograms.

**4.3 Results**

4.3.1 PCR confirmation of bacterial strains

From the 52 isolates that were positive for the *gryA* gene to confirm *Klebsiella* spp., 63% (33/52) were positive for *16S-23S ITS* to confirm *K. pneumoniae*. About 37% (19/52) of the remaining isolates were positive for the polygalacturonase *pehX* gene and identified as *K. oxytoca*.



4.3.2 Prevalence of antibiotic resistance, ESBL and MBL production

Figure 4.1 shows the antibiotic susceptibility fingerprints of each isolate against the 13 tested antibiotics. All the *K. pneumoniae* were resistant against nalidixic acid and colistin sulphate. It was followed keenly by their resistance against ampicillin, cefotaxime, and ceftazidime (98%). The percentage frequencies of resistance observed against the remaining antibiotics assayed are as follows: tetracycline (94%), amoxicillin/clavulanic acid (85%), ciprofloxacin (73%), trimethoprim-sulfamethoxazole (73%), cefoxitin and chloramphenicol (45%), and gentamicin (39%). No resistance was observed against imipenem, a carbapenem, one of the drugs of last resort. All the *K. oxytoca* investigated exhibited resistance against nalidixic acid, ampicillin, and tetracycline. The least resistance (11%) was also observed against the

imipenem. The remaining resistance frequencies were as follows: cefoxitin (47%), chloramphenicol (68%), trimethoprim-sulfamethoxazole and gentamicin (84%), ciprofloxacin amoxicillin/clavulanic acid (89%), and colistin, cefotaxime, ceftazidime (95%). Here intermediate resistance observed was also classified as resistance. The percentage resistance frequencies of each species are shown in Figure 4.2.

Screening using the DDST revealed that 50% (26/52) of the isolates were ESBL producers (Figure 4.1). Of the 26 ESBL-producing isolates, 73% (19/26) were *K. pneumoniae*, while 27% (7/26) were *K. oxytoca*. For the MBL production, only 11.5% (6/52) of the isolates were positive, with two *K. pneumoniae* strains and four *K. oxytoca* strains. One *K. pneumoniae* strain isolated from hospital wastewater (Isolate No. 17) co-produced ESBL and MBL.

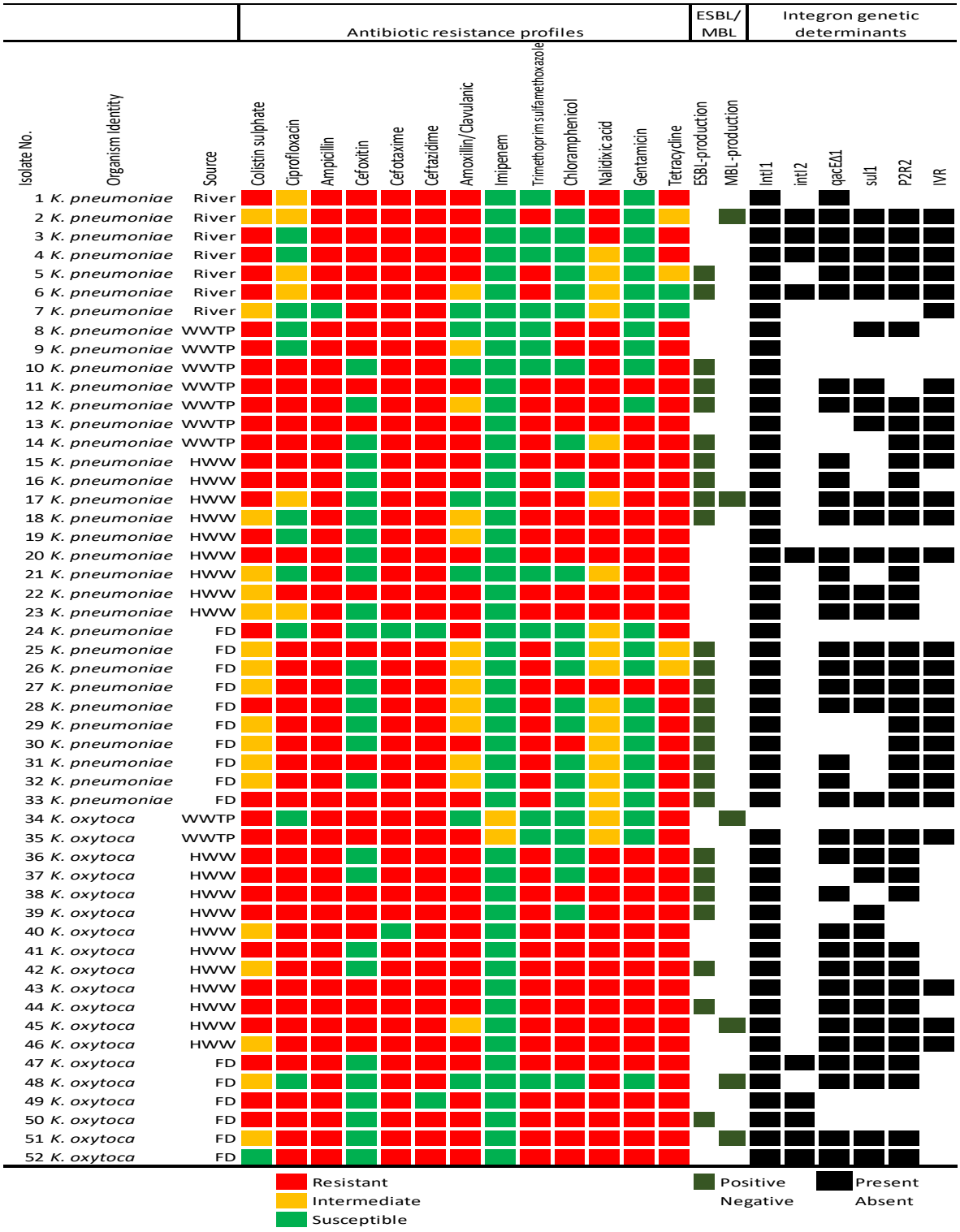


**Figure 4. 2: The phenotypic antibiotic susceptibility profiles, the production of ESBL and MBL, and the detection of integron genetic determinants of isolates from environmental matrices.** Each isolate's susceptibility profiles against certain antibiotics are colour coded to indicate whether it is resistant, intermediate-resistant, or susceptible. The ESBL and MBL production are colour coded as negative or positive, while absent or present represents the occurrence of the integron genetic elements. WWTP: Wastewater treatment plants effluent, HWW: Hospital wastewater, FD: Animal faecal droppings.

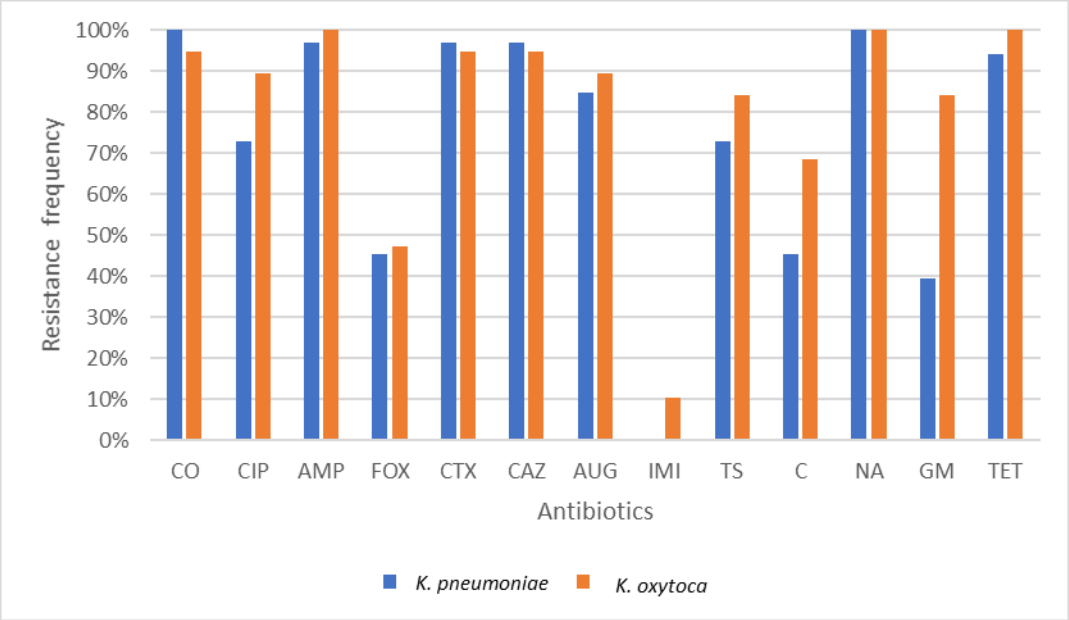


**Figure 4. 3: Total resistance frequencies of K. pneumoniae (n=33) and K. oxytoca (n=19) against 13 antibiotics.** Antibiotics code: CTX-cefotaxime, CO-colistin sulphate, CIP-ciprofloxacin, TS-trimethoprim/sulfamethoxazole, FOX-cefoxitin, NA-nalidixic acid, AMP-ampicillin, AUG-amoxicillin/clavulanic acid, IMI-imipenem, C-chloramphenicol, GM-gentamicin, CAZ-ceftazidime, and TET-tetracycline.

4.3.3 Characterization of 5′ conserved segment (Integrase + Promoters)

Among the 52 confirmed *Klebsiella* spp., 98% (51/52) were integron-positive as they harboured the integron integrase (*intI1*) gene. All the *K. pneumoniae* harboured the *intI1* gene. The *intI1* gene was absent in only one *K. oxytoca* isolate, while 20% (10/51) of these *intI1* positive isolates concurrently harboured the *intI2* gene and were thus classified as class 1+2 integrons. Those carrying only the *intI1* gene classified as class 1 integrons were 80% (41/51). Notably, none of the isolates harboured the *intI2* gene only, as when present, they concurrently were found with *intI1*. No *intI3* gene for class 3 integrons was detected (Figure 4.1). As shown in Table 4.2, detecting the integrase gene in each isolate was associated with resistance against three antibiotics assayed at significant levels. These were ciprofloxacin, amoxicillin-clavulanic acid, and imipenem ($P<0.05$).



Out of the 51 integrase-positive isolates examined, 78% (40/51) were positive for the *P2R2* gene investigated for a typical promoter gene on an integron located on the 5′ CS. Of these,

65% (26/40) were detected in *K. pneumoniae* isolates, while 35% (14/40) were harboured in *K. oxytoca* integrons. We could not amplify promoters targeting the *P2R2* gene on the remaining 11 integron-positive isolates.

**Table 4. 2: The association between resistance against antibiotics and the presence of integrons in *Klebsiella* spp.**

| Antibiotics | No. of resistant isolates | | No. of susceptible isolates | | |
|---|---|---|---|---|---|
| | Total (%) | Integron-positive (% of resistant isolates) | Total (%) | Integron-positive (% of susceptible isolates) | *P*-value ($\chi^2$ test) |
| Colistin sulphate | 51 (98.1) | 50 (98) | 1 (2) | 1 (2) | 0.888 |
| Ciprofloxacin | 41 (78.8) | 41 (80.4) | 11 (21) | 10 (20) | **0.051*** |
| Ampicillin | 51 (98.1) | 50 (98) | 1 (2) | 1 (2) | 0.888 |
| Cefoxitin | 24 (46.2) | 23 (45.1) | 28 (54) | 28 (55) | 0.275 |
| Cefotaxime | 50 (96.2) | 49 (96.1) | 2 (4) | 2 (4) | 0.840 |
| Ceftazidime | 50 (96.2) | 49 (96.1) | 2 (4) | 2 (4) | 0.840 |
| Amoxicillin/ clavulanic acid | 45 (86.5) | 45 (88.2) | 7 (13) | 6 (12) | **0.010*** |
| Imipenem | 2 (3.8) | 1 (2) | 50 (96) | 50 (98) | **0.000*** |
| Trimethoprim-sulfamethoxazole | 40 (76.9) | 40 (78.4) | 12 (23) | 11 (22) | 0.065 |
| Chloramphenicol | 28 (53.8) | 28 (54.9) | 24 (46) | 23 (45) | 0.275 |
| Nalidixic acid | 52 (100) | 51 (100) | 0 (0) | 0 (0) | [a] |
| Gentamicin | 29 (55.8) | 29 (56.9) | 23 (44) | 22 (43) | 0.257 |
| Tetracycline | 50 (96.2) | 49 (96.1) | 2 (4) | 2 (4) | 0.840 |

*****Significant values are represented in bold.
[a]No statistics are computed because Nalidixic acid exhibited a constant resistance attribute.



4.3.4 Characterization of the 3′ conserved segment (*qacEΔ1 + sul1*)

For the ORFs located on 3′-CS, 80% (41/51) of the integron-positive isolates assayed harboured at least one of the *qacEΔ1* and *sul1* genes. Approximately 73% (37/51) were positive for the *qacEΔ1* gene, while 67% (34/51) had the *sulI* gene. All the *qacEΔ1* and *sul1*-

positive isolates concurrently harboured both genes except four (Isolate No. 8, 13, 37 and 39). From the *intI1*-positive isolates, 20% (10/51) lacked any of the 3′-CS genes, while 14% (7/51) harboured the *qacEΔ1* without *sul1,* and 8% (4/51) were positive for *sul1* without *qacEΔ1*. About 65% (33/51) of the isolates were classified as typical class 1 integrons as they contained both genes on the 3′ CS.

4.3.5 Characterization of internal variable regions (IVRs)

The internal variable regions that harbour GCs and are between the CS of the integron harbouring isolates were 53% (27/51). The mapping of *intI1* and *intI2* positive isolates revealed that 59% (22/37) and 50% (5/10) of the integrons were positive for IVRs, respectively. The *K. oxytoca* isolates with class 1+2 integrons did not harbour any IVRs. The amplified IVRs ranged from approximately 160 bp to 1400 bp, with the mode being the least base pair. The analysis of the IVRs yielded eight amplicons of distinctly different sizes as follows: ≈160bp (n=18), ≈190bp (n=2), ≈280 (n=2), ≈350 (n=1), ≈550 (n=1), ≈ 700bp (n=1), ≈1200 (n=1), and ≈1400 (n=1).



The analysis of the sequences yielded three distinct cassette arrays. These detected cassette arrays were *aac(6')-Ib, aadA1-dfrA1,* and *dfrA1-sat2* (Table 4.3). They harboured genes that encode resistance against aminoglycosides, trimethoprim, and streptothricin. The most detected cassette was *aac(6')-Ib* representing 82% (18/22) of all identified cassettes. The gene *dfrA1* was detected in 9% of the cassettes, while *sat2* and *aadA1* genes occurred singly. The sequences of the remaining IVR amplicons yielded empty or undetermined cassette arrays. The schematic representation of some of the integrons and their associated gene cassette arrays are shown in Figure 4.3.

**Table 4. 3: The content and arrangement of genes within the integron's internal variable regions (n=27).**

| Class of integron | No. of isolates | Cassette array | Approximate size (bp) | *qacEΔ1+sul1* |
|---|---|---|---|---|
| Class 1 | 16 | *aac(6')-Ib* | 160 | +1[a] |
| | 2 | _[b] | 190 | +1 |
| | 1 | *aadA1-dfrA1* | 1200 | +1 |
| | 1 | _[b] | 350 | +1 |
| | 2 | _[b] | 280 | +1[c] |
| Class 1+2 | 1 | *dfrA1-sat2* | 1400 | +1 |
| | 2 | aac(6')-Ib | 160 | +1 |
| | 1 | _[b] | 550 | +1 |
| | 1 | _[b] | 700 | +1 |

[a]Except for three isolates that were positive for *qacEΔ1* only, three did not harbour both *qacEΔ1+sul1*, and one was positive for *sul1* only.
[b]The amplicon yielded an undetermined/empty cassette array.
[c]One isolate lacked both *qacEΔ1+sul1* genes.

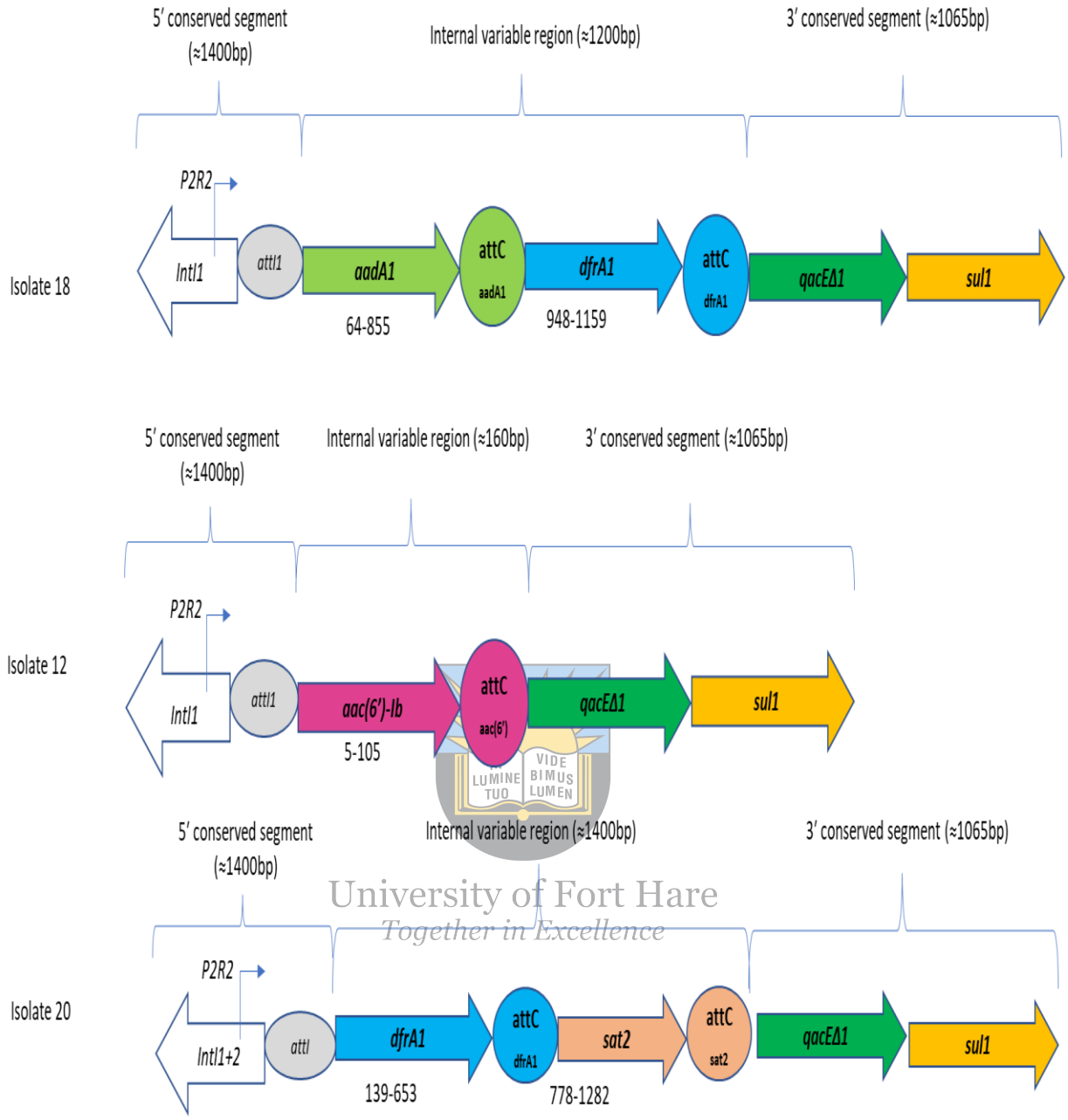




**Figure 4. 4: Schematic representation of some integron-positive K. pneumoniae with their associated gene cassette arrays.** These isolates were positive for *intI1* and *intI1+2* for class 1 and class 1+2 integrons, respectively. The *P2R2* gene for the promoter on the 5′ conserved segment was shown. Also detected were *sul1* and *qacEΔ1* genes found on the 3′ conserved segment conferring resistance against sulphonamides and quaternary ammonium compounds, respectively. The cassette arrays include the *aadA1* and *aac(6')-Ib* gene, which encodes resistance against aminoglycosides. The *dfrA1* and *sat2* genes confer resistance against trimethoprim and streptothricin, respectively. The start and stop nucleotide sequences were also depicted, while the arrows indicate the transcription direction.

4.3.6 Genetic relatedness of *K. pneumoniae* from various sources

The genotypic relatedness of all the *K. pneumoniae* investigated is indicated in the dendrogram generated from the ERIC-PCR fingerprinting of isolates. The fingerprints generated nine different clusters (Figure 4.4, A to I) at a similarity cut-off value of 60%. Cluster I was the highest ERIC-genotype cluster. It comprises seven isolates with representatives from various water sources, with similarities ranging from 67% to 78%. Cluster C was grouped with five isolates from HWW and WWTP, while the least ERIC-genotype cluster was observed at F and G (one isolate from HWW and river, respectively). The eight FD isolates were spread into three different clusters, where three were clustered at A with 68%, while four clustered at E with 65% similarity indices. The remaining FD isolates clustered with an isolate from the river at D with 63% similarity. Cluster B contained two isolates from WWTP, while cluster H contained an isolate each from WWTP, HWW, and river. It should be noted that 5 of the *K. pneumoniae* isolates did not generate any visible bands on the ERIC-PCR gel and were excluded from the analysis.

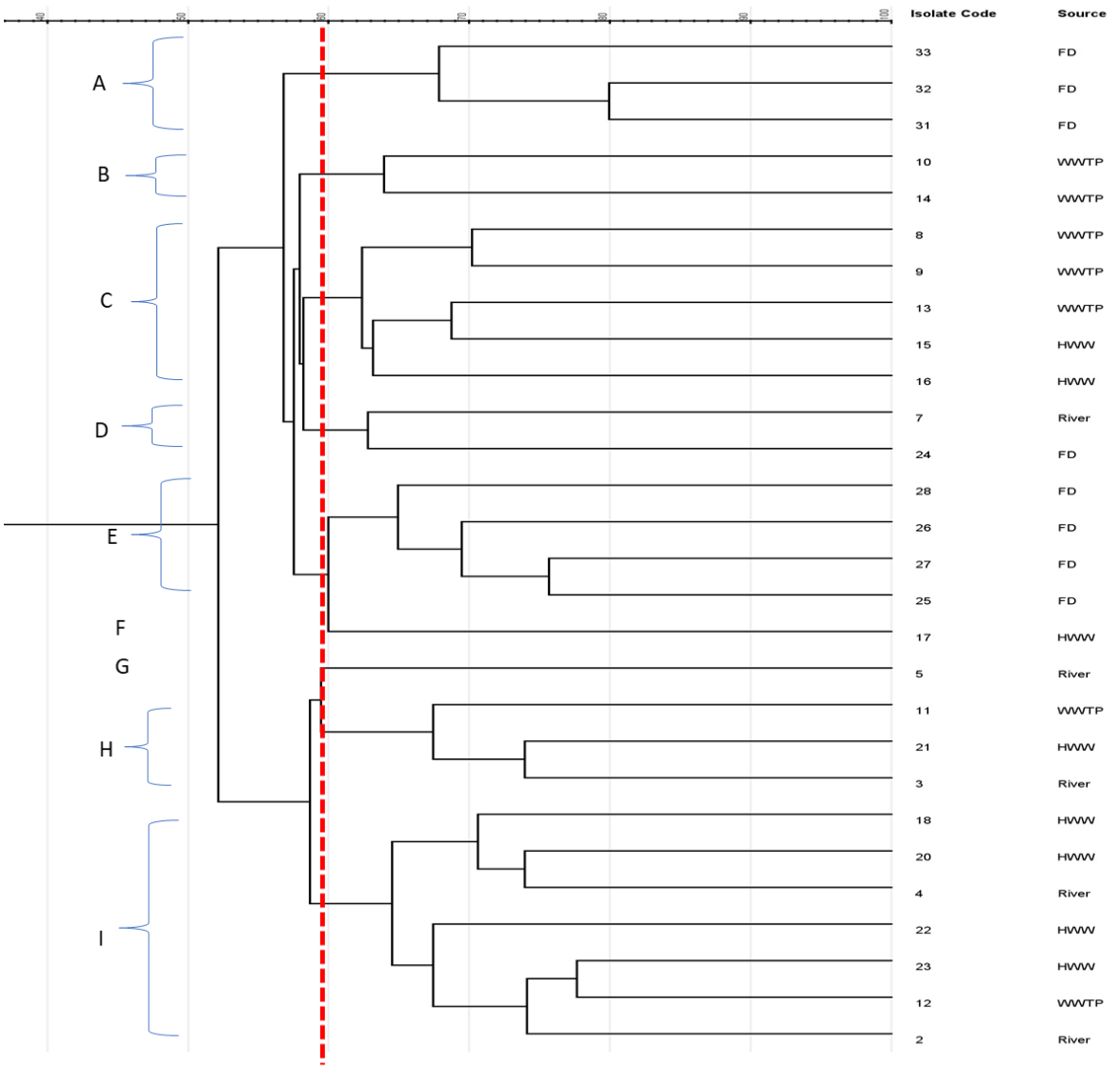


**Figure 4. 5: UPGMA dendrogram image obtained from clustering analysis indicating the genetic relatedness of K. pneumoniae (n=28) recovered from various environmental sources using the ERIC-PCR technique.** HWW: Hospital wastewater, WWTP: wastewater treatment plant, FD: animal droppings.

### 4.4 Discussion

On speciation of the *Klebsiella* investigated in this study, *K. pneumoniae* (63%) was more frequently detected than *K. oxytoca* (37%). It agrees with the reports that *K. pneumoniae* is the most prevalent species of the genus as it is the causative agent of most nosocomial *Klebsiella* infections, thereby referred to as the most medically significant species of the genus (Magill et al., 2014; Podschun & Ullmann, 1998). *K. pneumoniae* is becoming more recognized as an invasive and aggressive pathogen that carries some ARGs (Martin & Bachman, 2018). It has been reported to cause fatalities in various provinces within South Africa (Jacobson et al., 2015; Pedersen et al., 2018).

Antibiotic resistance was shown to be prevalent among the isolates in this investigation. They were all categorized as MDR because they were resistant against more than two distinct classes of antibiotics (Figure 4.1). Many antibiotics are not entirely metabolized after consumption and end up in wastewater systems (Fadare & Okoh, 2021b). Antibiotics in wastewater have been demonstrated to impose selection pressure on antimicrobial-resistant bacteria, allowing them to spread to new environments (Li et al., 2019). This widespread usage of antimicrobials has resulted in an increased rate of antimicrobial resistance detection in *Klebsiella* spp. Almost all the isolates exhibited resistance against the β-lactams and the third-generation cephalosporins. The least resistance was observed against imipenem, which is not usually a front-line prescription antibiotic. This low detection rate in carbapenems is somewhat expected and similar to reports of other studies where the resistance frequencies ranged from 7% to 15% (Conte et al., 2017; King et al., 2020). MDR *K. pneumoniae* has emerged as a critical problem in treating nosocomial infections worldwide (Firoozeh et al., 2019); therefore, its environmental detection is quite concerning. The production of β-lactamase by *Klebsiella* spp. is one of their resistance mechanisms against β-lactam antibiotics (Rawat & Nair, 2010). The study revealed a prevalence phenotype of 50% and

12% of ESBL and MBL, respectively. The presence of these β-lactamases is also associated with co-resistance against other classes of antibiotics by bacterial species harbouring them, further limiting possible treatment options. These β-lactamases are usually carried on mobile genetic elements such as integrons, which aid in the horizontal spread of ARGs among diverse bacterial species (Cantón et al., 2012; Martin & Bachman, 2018; Rawat & Nair, 2010).

In this study, integrons were detected in all but one isolate, with class 1 integrons being the most predominant. The studies of (Firoozeh et al., 2019) and (Lima et al., 2014) have stated that all MDR *K. pneumoniae* harboured the *intI* gene, although these reports were from clinical samples. Other studies from environmental origins have also reported the detection of the *intI1* gene as the most prevalent in similar studies (Kaushik et al., 2018; Su et al., 2012; Zhang et al., 2019). There is a strong association between the occurrence of *intI1* integrons and the prevalence of MDR in Gram-negative bacteria. Although our results indicate a significant association between *intI1* and three out of thirteen antibiotics investigated (Table 4.2), in research by Li and colleagues (Li et al., 2013), integron harbouring isolates demonstrated resistance against a substantially greater number of antibiotics than negative isolates. Other investigations have found a high prevalence of integron-positive MDR *Klebsiella* spp. (Firoozeh et al., 2019; Wu et al., 2012). Integrons give a selective advantage to bacteria in settings where antibiotic use causes selective pressures, which may explain the high occurrence of integrons among MDR strains.



Typical class 1 integrons usually harbour quaternary ammonium compounds and sulphonamide resistance genes (Paulsen et al., 1996; Stokes & Hall, 1989). In this study, 59% (30/51) of the integrase-positive isolates harboured both *sul1* and *qacEΔ1* genes, although 27% (8/30) of these concurrently have the *intI2* gene (Figure 4.1). Invariably, this shows that some typical class 1 integrons can still harbour the class 2 integrase gene, a scenario that has

been previously reported (Zhang et al., 2019). Furthermore, we report a prevalence of 78% of a promoter type, the *P2R2* gene, located on the integron. It further confirms the role of an integron as an expression vector as this promoter can immediately express the genes in the cassette arrays acquired from the environment once incorporated into the integrons. The inability to detect the *P2R2* gene on the remaining isolates via PCR could have been due to mutation in the promoter gene located within the integrase, or those integrons could have a promoter type different from the assayed gene.

Three unique GC arrays detected in the integrons are shown in Table 4.3. These results prove that environmental sources also serve as a potential repertoire of mobile genetic elements harbouring different antibiotic resistance GCs. The GCs contained genes encoding resistance against trimethoprim (*dfrA1*), streptothricin (*sat2*), and aminoglycosides (*aadA1* and *aac(6)-Ib*). The most prevalent GC was the *aac(6)-Ib*, which occurred singly. Our results indicate that 53% (27/51) of the isolates harbouring the *intI1* gene contained the internal variable regions (Figure 4.1). The *aac(6)-Ib* gene encodes an enzyme 6′-N-acetyltransferase and belongs to the family of aminoglycoside acetyltransferases, while the *aadA1* encodes the aminoglycoside adenyl transferase. They confer resistance against aminoglycosides such as amikacin, gentamicin, tobramycin, neomycin, and streptomycin. The *Sat2* encodes streptothricin-N-acetyltransferase, which confers resistance against streptothricin. The detection of the *dfrA* genes encodes dihydrofolate reductase, which confers resistance against trimethoprim, has been associated with selection pressure and high use of trimethoprim, especially in clinical sources (Firoozeh et al., 2019). However, this study infers that such a selective burden may exist in contaminated aquatic environments.



According to the sequencing results, 13.7% of these isolates did not contain any GCs (Table 4.3), and these could be a result of defects or mutations at the 3′ CS or the GCs belonging to an unusual or complex class 1 integrons (Lee et al., 2011). The detection of integrons with

empty GCs has been reported, and it shows the readiness of such isolates to capture GCs readily and subsequently express the ARGs harboured on them. The ERIC-PCR dendrogram image, as seen in Figure 4.4, shows the clonal relatedness of the integron-positive *K. pneumoniae* from the various sources clustered in similar groups, indicating high genetic relatedness among the integron-bearing MDR isolates. The result further reiterates the ability of bacterial species to exchange genetic elements from various niches, which can confer specific adaptability properties.

### 4.5 Conclusions

MDR bacterial strains have been widely disseminated due to the distribution of antibiotic-resistant strains, particularly the ESBL and carbapenemase producers. The occurrence of integrons in these MDR bacterial strains has further accelerated the spread of ARGs into diverse environmental sources. The data obtained from this study show that integrons with their associated gene cassettes are widely distributed in *Klebsiella* spp. from environmental matrices that may further constitute a problem when treating bacterial infections. The baseline properties of integrons in *Klebsiella* species isolated from several environmental matrices in the Eastern Cape Province, South Africa, have never been previously reported.

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**CHAPTER FIVE**

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**Integron-harbouring *Citrobacter* spp. isolated from rivers, animal dropping, wastewater and hospital effluents: A public health risk (Submitted to *Environmental Sciences*)**

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**Abstract**

Globally, the environmental resistome is a hotspot of antimicrobial resistance genes. We investigated the antibiotic resistance phenotypes, the prevalence of integrons, and the diversity of gene cassettes (GCs) in *Citrobacter* species from various environmental matrices following standardized methods. All the bacterial strains (n=16) were resistant against two antibiotic classes, and 93.8% of the strains were resistant against more than three antibiotic classes indicating that they were multidrug-resistant (MDR). Interestingly, all the isolates had multiple antibiotic resistance indices greater than 0.2, implying that these environmental sources were heavily contaminated with antibiotics. All the isolates harboured the *intI1* gene, and three were concurrently positive for the *intI2* genes. About 62.5% (10/16) of the isolates were typical class 1 integrons due to *qacEΔ1* and *sulI* genes. The analysis of the sequences of the integrons variable regions revealed various GCs. These include four aminoglycoside adenyltransferases (*aadA1* and *aadA5*), six aminoglycoside acetyltransferases (*aac3-Ib* and *aac(6')-Ib*) and five trimethoprim resistance genes (*dfrA1*, *dfrA*5, *dfrA17*, and *dfrA21*). About 50% of the isolates were positive for the *P2R2* promoter genes coupled with the high prevalence of integron-harbouring MDR among the environmental *Citrobacter* spp. implies that ARGs harboured on the GCs could be easily expressed and subsequently involved in ARG transmission. The study reports integron-harbouring *Citrobacter* spp. for the first time in the Eastern Cape Province, South Africa, suggesting that these environmental niches are important reservoirs of antimicrobial resistance determinants.



**Keywords:** *Citrobacter* spp., Integrons, gene cassette arrays, MARI, integrons variable region

## 5.1 Introduction

The Enterobacteriaceae family includes the *Citrobacter* genus. They are present in the soil, sewage, and water and are a part of humans' and animals' intestines (Canario et al., 2004; Ekwanzala et al., 2020). There are 5 out of the 11 recognized species generally considered human commensals. These include *C. youngae*, *C. freundii*, *C. amalonaticus*, *C. koseri*, and *C. braakii*. However, the latter two are the major opportunistic pathogens that cause central nervous system and meningitis illness in newborns (Ekwanzala et al., 2020; Rodrigues et al., 2014). Once pathogenic, other kinds of infections they cause are infective endocarditis (Dzeing-Ella et al., 2009), Harrington rod infection (Canario et al., 2004), and empyema and pneumonia (Ariza-Prota et al., 2015). Antibiotics such as cephalosporins, quinolones, aminoglycosides, chloramphenicol and carbapenems are often used to treat *Citrobacter* spp. infections (Deveci & Coban, 2014). However, the global expansion in the ability of bacterial strains to render antibiotics ineffective, especially in Enterobacteriaceae, is a significant concern.



The phenotypic antibiotic resistance observed in bacteria is mediated by antibiotic resistance genes (ARGs) found on mobile genetic elements (MGEs) like integrons. Integrons are genetic structures with a primary platform that enables them to acquire gene cassettes (GCs) and consequently express the antibiotic resistance determinants harboured on the GCs. The primary platform comprises the 5′ - conserved segment (CS), which harbours the integron integrase, *intI*, a constitutive promoter, $P_{c,}$ and a primary recombination site, *attI*. The *intI* encodes a site-specific tyrosine recombinase enzyme, with a primary function of catalyzing the integration and excision of GCs. The *attI* is recognized by the *intI* and functions as the site for acquiring new genetic material without disturbing the previously existing genes, while the $P_c$, located inside the *intI* gene and faces the integration point, controls the

expression of inserted GCs (Boucher et al., 2007; Hall & Collis, 1995). The GCs have simple structures with two components: an open reading frame (ORF) that encodes ARGs and a cassette-associated recombination site (*attC*). Their presence has been known to confer resistance against most classes of antibiotics. These include chloramphenicol, antiseptics of the quaternary ammonium compound family, β-lactams, erythromycin, fosfomycin, trimethoprim, lincomycin, aminoglycosides, rifampicin, and quinolones (Deng et al., 2015; Gillings, 2014; Kaushik et al., 2019; Mazel, 2006). Hence, bacterial strains harbouring integrons are usually resistant against more than two antibiotics from different classes and have been implicated in conferring multidrug resistance (MDR) on the organisms (Koczura et al., 2014; Leverstein-van Hall et al., 2003; Mokracka et al., 2012; Zhang et al., 2020).

Integrons are categorized into several classes according to the sequences of the *intI* gene, with class 1 being the most predominant (Cambray et al., 2010; Kaushik et al., 2019; Stokes & Hall, 1989). Integrons harbouring several GC arrays have been described in North and South America, Asia, and Europe (Chowdhury et al., 2011; Deng et al., 2015; Firoozeh et al., 2019). Although the main focus of detecting integrons and their GCs has been within the clinical environment, they have also been identified in several bacterial species from various environmental sources (Boucher et al., 2007; Kaushik et al., 2019; Su et al., 2012). However, restricted studies exist on the characterization of integrons from environmental sources in the Eastern Cape Province (ECP) in South Africa. This paper evaluated integrons' prevalence, diversity, and characterization and their associated GCs in *Citrobacter* spp. recovered from various environmental matrices.

## 5.2 Materials and Methods

### 5.2.1 *Citrobacter* isolates

Sixteen *Citrobacter* isolates, including 12 *C. freundii*, 3 *C. koseri* and 1 *C. braakii*, were obtained from rivers (4), effluents of hospital wastewater (3), and wastewater treatment plants (6) and animal faecal dropping (2). The isolates were recovered from Amathole and Chris Hani District Municipalities in the ECP between October and November 2017. The identity of each isolate was determined using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) (Bruker Daltonics, Germany) at the time of isolation and stored as glycerol stocks at -80 ℃.

### 5.2.2 DNA extraction and PCR identification of isolates



The retrieved glycerol stocks were resuscitated in Brain Heart Infusion (BHI) broth (Merck, South Africa) and incubated for 18 hours at 37 ℃. A loopful was streaked on nutrient agar (Oxoid, UK) and further purified twice. For subsequent analysis, single pure colonies were transferred to 2 ml BHI broth and incubated overnight at 37 ℃. The boiling method (Maugeri et al., 2004) was used with slight modifications, as previously reported elsewhere (Fadare et al., 2020). The DNA-containing supernatant was then transferred to sterile microcentrifuge tubes (Eppendorf, Germany). It was used as the DNA template in subsequent PCR reactions. The genus was identified with primers targeting the *Citrobacter* urease gene primers described in Table 5.1.

The PCR mixture (25 µl) contained PCR master mix (12.5 µl) (Thermo scientific (EU), Lithuania), forward and reverse primers (1 µl) (Inqaba Biotechnical Industries, South Africa). Furthermore, a DNA template (5 µl) and nuclease-free water (5.5 µl) were added. The DNA amplification was done in a BioRad thermal cycler (Lasec, South Africa). A 1.5% (w/v) agarose gel was used to resolve 5 µl of the amplicons. The gel was run using Mupid-One

(Eurogentec, Belgium) electrophoresis set-up at 100 volts for 1 hour in 0.5X TBE buffer with a 100-base-pair molecular marker (Biolabs, New England). The results were viewed using UV transillumination (ALLIANCE 4.7, UVtec, London, UK).

**Table 5. 1: PCR primers and their expected band sizes.**

| Target gene | Amplicon size (bp) | Primer | Primer Sequence (5′ →3′) | Reference |
|---|---|---|---|---|
| *Citrobacter* urease | 454 | Urease-F | TGAAGCTGAACTACCCGGAATC | (Lü et al., 2011) |
| | | Urease -R | TGTCCAGGCTCAAAACGTAC | |
| *intI1* | 164 | INTI1-F | CAG TGG ACA TAA GCC TGT TC | (Koeleman et al., 2001) |
| | | INTI1-R | CCC GAG GCA TAG ACT GTA | |
| *intI2* | 232 | INTI2-F | TTATTGCTGGGATTAGGC | (Goldstein et al., 2001) |
| | | INTI2-R | ACGGCTACCCTCTGTTATC | |
| *intI3* | 600 | INTI3-F | AGTGGGTGGCGAATGAGTG | (Goldstein et al., 2001) |
| | | INTI3-R | TGTTCTTGTATCGGCAGGTG | |
| *P2R2* Promoter | 540 | INTF | AGTGGGTGGCGAATGAGTG | (Wei et al., 2011) |
| | | P2R2 | TGTTCTTGTATCGGCAGGTG | |
| *qacEΔ1* | 225 | qac-F | ATCGCAATAGTTGGCGAAGT | (Stokes & Hall, 1989) |
| | | qac-R | CAAGCTTTTGCCCATGAAGC | |
| *sul1* | 840 | Sul1-F | ATGGTGACGGTGTTCGGCATCTGA | (Grape et al., 2005) |
| | | Sul1-R | CTAGGCATGATCTAACCCTCGGTC T | |
| Class 1 gene cassette | Variable | 5′ CS | GGCATCCAAGCAGCAAG | (Lévesque et al., 1995) |
| | | 3′ CS | AAGCAGACTTGACCTGA | |
| Class 2 gene cassette | Variable | hep 74 | CGGGATCCCGGACGGCATGCACGA TTTGTA | (White et al., 2001) |
| | | hep51 | GATGCCATCGCAAGTACGAG | |

5.2.3 Molecular detection and characterization of integrons

The confirmed bacterial strains were screened with conventional PCR assays as described above. Different integrase genes on the 5′-CS were targeted with specific primers to classify the isolates into various integron classes. For the integrons' characterization, a *P2R2* promoter gene on the 5′-CS of a typical integron was also assayed using primer pairs listed in Table 5.1. For the detection of typical class 1 integrons, isolates were further screened for the detection of *qacEΔ1* and *sul1* genes on the 3′-CS. The positive control, *Acinetobacter baumannii* ATCC 19606 for a typical class 1 integron, was used.

5.2.4 Detection of integron variable regions (IVRs)

The isolates were assessed to detect the IVRs in separate PCR assays. Specific primer sets 3′ CS and 5′ CS that joins the *attI1* site of the 5′ -CS and the 3′-CS of *intI1* were used for *intI1* positive isolates. The primer set hep74 and hep 51, which bind to the *attI2* and the *orfX* sites downstream of the GC regions, were used for *intI2* positive isolates (Table 5.1). The PCR assays were carried out in triplicates to ensure reproducibility.



5.2.5 Restriction analysis and DNA sequencing of amplicons

Amplicons of IVRs that appeared similar in size were exposed to the *AluI* restriction enzyme (Biolabs, England) for restriction fragment length polymorphism (RFLP) procedure to assess if the products had the same sequence. For the identification of similar GCs in the integrons, *AluI* was chosen due to its recognition sequence being only four bases, thereby increasing the likelihood of its activity over the other enzymes that target the six-base sequence. Briefly, 30 µl of the amplicon was exposed to 1.0 µl of 10U/ml *AluI*. The reaction mixture was then incubated at 37 ℃ for 4 hours. The products were then run on a 2% agarose gel and visualized. These were characterized according to their distinct restriction profiles, and two

randomly selected representative amplicons from each different RFLP class were selected for sequencing. This step was taken to help reduce the risk of needlessly sequencing multiple identical IVRs.

5.2.6 Variable sequence analysis, cassette identification

The content and arrangement of the inserted GCs within the amplified IVRs were analyzed through sequencing. The amplicons were bi-directionally sequenced on sequencer ABI 3500XL using the Nimagen, BrilliantDye™ Terminator Cycle sequencing kit V3.1 (Inqaba Biotechnical Industries, South Africa). The sequences were then modified with Chromas 2.7 and pairwise aligned using BioEdit sequence alignment editor software. BLAST nucleotide search analysis (https://blast.ncbi.nlm.nih.gov/Blast) was performed on the generated consensus sequences to identify the contents of the inserted GC. The position of each gene in each cassette was determined using ABRicate 0.8.4 (https://github.com/tseemann/abricate) (ResFinder, ARG-ANNOT, CARD, and NCBI databases).



5.2.7 Antibiotic susceptibility assay

Antibiotic susceptibility assay was carried out following the disk diffusion technique (DDT) described in our previous report (Fadare & Okoh, 2021a). Isolates were exposed to a panel of thirteen antibiotics which includes the following: imipenem (IMI) (10 µg), ciprofloxacin (CIP) (5 µg), amoxicillin/clavulanic acid (AUG) (20 µg/10 µg), ceftazidime (CAZ) (10 µg), ampicillin (AMP) (10 µg), cefotaxime (CTX) (30 µg), trimethoprim-sulfamethoxazole (TS) (25 µg), colistin (CO) (10 µg), nalidixic acid (NA) (30 µg), chloramphenicol (C) (30 µg), cefoxitin (FOX) (30 µg), gentamicin (GM) (10 µg), tetracycline (TET) (30 µg). The antibiotics were purchased from Mast Diagnostics, South Africa. The results of the diameter of the inhibition zones were read according to the Clinical and Laboratory Standard Institutes (CLSI) guidelines (CLSI, 2020).

5.2.8 The determination of multiple antibiotic resistance (MAR) indices and phenotypes

When an isolate exhibited resistance against more than two antibiotic classes, it was classified as MDR (Magiorakos et al., 2012). The MAR indices of the *Citrobacter* spp. were determined using the mathematical computation below, and the various MAR phenotypes were also evaluated (Ateba & Bezuidenhout, 2008).

$$MAR\ index = a/b$$

"a" denotes the total number of antibiotics against which the bacterial species showed phenotypic resistance, whereas "b" denotes the total antibiotics tested. A value greater than 0.2 shows the extensive use of antibiotics in that location, implying a significant risk of antimicrobial resistance spreading (Krumperman, 1983; Osundiya et al., 2013).

5.2.9 Production of extended-spectrum and metallo β-lactamase

The production of extended-spectrum β-lactamase (ESBL) was assessed using the double-disk synergy test (DDST). It was done using ceftazidime-10 µg, cefoxitin-30 µg, and cefotaxime-30 µg in combination with amoxicillin/clavulanic acid -20 µg/10 µg. The ethylenediaminetetraacetic acid (EDTA) test was used to determine the generation of the metallo β-lactamase (MBL) according to Clinical and Laboratory Standard Institute (CLSI) guidelines. Briefly, 0.5 McFarland adjusted test isolate was exposed to two 10 µg imipenem discs. Then, 10 µl of 0.5 M EDTA was added to only one disc to obtain a concentration of 750 µg. An increase in inhibitory zone width of 5mm in the disc potentiated with EDTA after 18 hours of incubation at 37 °C was regarded as positive for MBL generation (CLSI, 2020).

## 5.3 Results and discussion

### 5.3.1 Integrons in *Citrobacter* spp.

All the isolates harboured the *Citrobacter* urease gene for the PCR confirmation of *Citrobacter* spp. Interestingly, all the sixteen confirmed isolates were integron-harbouring even though they were recovered from different environmental niches suggesting the possible ubiquity of integrons. Although the reports of the detection of *Citrobacter* spp. have not been commonplace in environmental sources, this has limited the detection of integrons in this genus. However, high incidences of integrons in Enterobacteriaceae have been severally reported in effluents of urban wastewater treatment plants (WWTPs) and hospitals (Kotlarska et al., 2015; Pellegrini et al., 2011), rivers (Chaturvedi et al., 2021; Singh et al., 2021; Wright et al., 2008), as well as from animals (Kadlec & Schwarz, 2008; Lopes et al., 2016). Also, some studies with clinical isolates in Egypt and Iran have stated over 50% occurrence of *intI1* gene in *Citrobacter* spp. (Lorestani et al., 2018; Malek et al., 2015). Initially, integrons were only reported in clinical sources but are now widely detected in environmental isolates (Boucher et al., 2007; Buongermino Pereira et al., 2020; Daly & Fanning, 2004; Gillings, 2014; Hall, 2012; Jones-Dias et al., 2016; Koczura et al., 2012; Mokracka et al., 2012; Nield et al., 2001).



All the isolates in this study harboured the *intI1* gene. Although three isolates concurrently harboured the *intI2* gene (Table 5.2), these were classified as class 1+2 integrons. The detection of isolates with *intI1* and *intI2* genes has been described in other members of Enterobacteriaceae. For instance, class 1+2 integrons were detected in rivers (Chaturvedi et al., 2021; Kaushik et al., 2019) and waterfowl birds (Zhang et al., 2019). The high rate of detection of the *intI1* gene corroborates previous studies that have shown an abundance of class 1 integron due to the high incidence of anthropogenic pressure (Cambray et al., 2010; Chaturvedi et al., 2021), while the *intI2* genes, when compared with *intI1* genes, have been

less common (Chaturvedi et al., 2021; Koczura et al., 2012; Kotlarska et al., 2015; Mokracka et al., 2012). We did not detect any *intI3* gene. Class 1 and 2 integrons have been more often detected among the Enterobacteriaceae family, while class 3 integrons seem to be scarce and infrequently involved in disseminating ARGs. Although with a 60% sequence identity with *intI1*, *intI3* are the least prevalent in colonizing new species (Collis et al., 2002). They are embedded in a reverse orientation within the Tn*402* hence being less active. However, only a few studies (Barraud et al., 2013; Simo Tchuinte et al., 2016) have found the *intI3* gene in hospital effluents with exceptionally low prevalence.

**Table 5. 2: Characterization of integrons with their associated resistance gene cassette arrays in *Citrobacter* spp. recovered from various sources.**

| Isolate code | Source | Integrase gene | *P2R2* gene[a] | Integron's variable region length (≈bp) | Gene Cassette array | *qacEΔ1+sul1* gene |
|---|---|---|---|---|---|---|
| 176 | WWTP | *intI1+intI2* | + | 800 | *dfrA5-aac3-Ib* | + |
| 177 | WWTP | *intI1* | – | ND | N/A | – |
| 178 | River | *intI1* | – | ND | N/A | +[c] |
| 179 | River | *intI1* | – | 160 | *aac(6')-Ib* | + |
| 180 | HWW | *intI1* | + | 160 | *aac(6')-Ib* | + |
| 181 | HWW | *intI1* | – | 400 | –[b] | + |
| 182 | WWTP | *intI1* | + | 160 | *aac(6')-Ib* | + |
| 183 | FD | *intI1* | – | ND | N/A | – |
| 184 | River | *intI1* | – | ND | N/A | – |
| 185 | River | *intI1+intI2* | – | 160 | *aadA1* | – |
| 186 | WWTP | *intI1* | – | ND | N/A | – |
| 187 | WWTP | *intI1+intI2* | + | 1500 | *aadA1-dfrA1-aadA1* | + |
| 188 | FD | *intI1* | + | 1500 | *aadA1-dfrA1* | + |
| 189 | HWW | *intI1* | + | 1600 | *aadA5-dfrA17* | + |
| 190 | WWTP | *intI1* | + | 160 | *aac(6')-Ib* | + |
| 191 | River | *intI1* | + | 800 | *dfrA21-aac3-Ib* | + |

[a]*P2R2* gene assayed to target a typical class 1 promoter type.
[c]Only *Sul1* detected.
[b]Empty/undetermined gene cassette.
HWW: hospital wastewater; WWTP: wastewater treatment plant.; ND: Not detected; N/A: Not applicable since no amplicon was detected; +: present; –: absent.

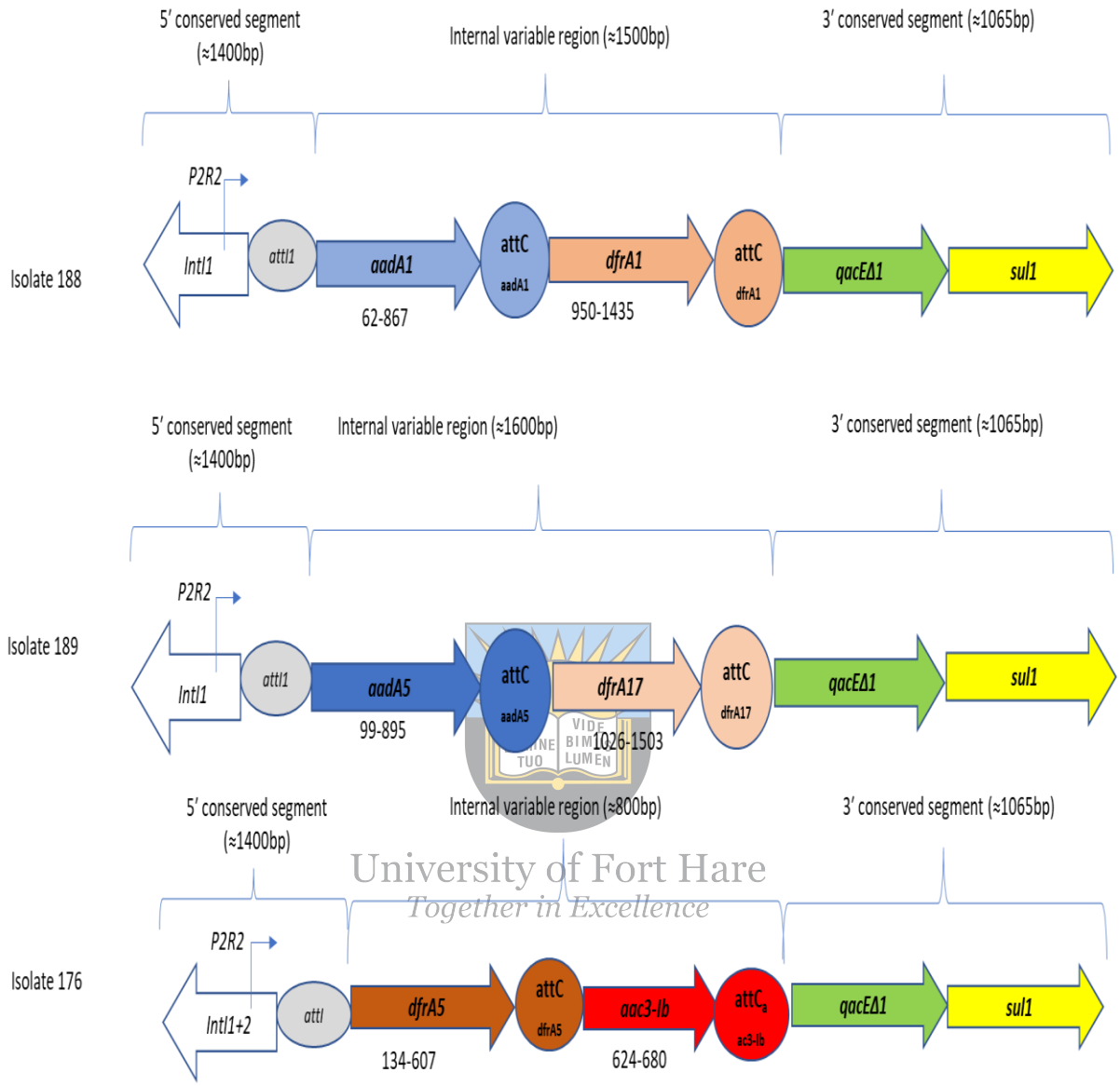




**Figure 5. 1. Schematic representation of some integron-harbouring *Citrobacter* spp. with their associated gene cassette arrays.** The represented isolates were positive for *intI1* and *intI1+2* for class 1 and class 1+2 integrons, respectively. They all harboured the *P2R2* promoter situated on the 5′ conserved segment. Also detected were the *qacEΔ1* and *sul1* genes found on the 3′ conserved segment and confer resistance against quaternary ammonium compounds and sulphonamides, respectively. The cassette arrays shown are the *aadA1*, *aadA5*, and *dfrA* variants (*dfrA1/5/17*). These encode resistance against spectinomycin, streptomycin, and trimethoprim, respectively. The start and stop nucleotide sequences were also depicted. The direction of the arrows indicates the direction of transcription.

5.3.2 Characterization of the 3′ conserved segment (*qacEΔ1* + *sul1*)

Our analysis of the 3′ CS revealed that 62.5% (10/16) of the isolates were typical class 1 integrons because they harboured both the *qacEΔ1* and *sul1* genes on the 3′ CS. One isolate harboured only the *sul1* gene, while the remaining 31.2% (5/16) lacked *qacEΔ1* and *sul1* genes. Generally, when the evolutionary history of class 1 integrons is being considered, the truncated *qacE* gene, *qacEΔ1*, which confers resistance against quaternary ammonium compounds, precedes the insertion of a *sul1* gene at the 3′ end, which encodes resistance against the antibiotic sulphonamide (Gillings, 2014). Our detection of the *sul1* gene in an isolate without the *qacEΔ1* was unusual; however, this result is similar to a previous report in China wherein a class 1 integron harboured the *sul1* gene but lacked the *qacEΔ1* gene (Zhang et al., 2019).

5.3.3 Analysis of integron variable regions



The integron variable regions that harbour GCs and are located between the CS of the integron-positive isolates were 69% (11/16). All the isolates with the typical class 1 structure were positive for IVRs. In addition, only one isolate (Isolate code 165), which lacked the *qacEΔ1* and *sul1* genes, was positive for the IVR (Table 5.2). Notably, a class 1 integron positive for only the *sul1* gene (Isolate code 178) lacked the IVR. Furthermore, we could not amplify the IVR of four class 1 integrons. These isolates lacked both the *sul1* and *qacEΔ1* genes. The lack of these 3′ CS genes could result from a mutation in these segments and could be attributed to the failure to amplify the IVRs of these isolates (Koczura et al., 2014; Laroche et al., 2009).

The amplified IVRs detected ranged from approximately 160 bp to 1600 bp, with the least base pair size most frequently detected. The analysis of the IVRs yielded eleven amplicons of distinctly different sizes as follows: ≈160bp (n=5), ≈400bp (n=1), ≈800 (n=2), ≈1500 (n=2),

and ≈1600 (n=1). The analysis of the IVR yielded seven different GC arrays. Four class 1 integrons with 160bp size yielded GC *aac(6')-Ib*, while the class 1+2 integron with IVR of 160 bp harboured *aadA1*. Sequence analysis revealed the different variants of *dfrA* (*dfrA17*, *dfrA1*, *dfrA21*, *dfrA5*) and *aadA* (*aadA5* and *aadA1*) GCs. The *aadA* gene is an aminoglycoside adenyl transferase that gives resistance against aminoglycosides. The *aadA1* confers resistance against streptomycin and spectinomycin, while the *aadA5* gene gives resistance against spectinomycin but not streptomycin (White et al., 2000). The *dfrA* variants, trimethoprim resistance encoding genes, are the most frequently detected GCs. Most of the GC arrays occurred uniquely except when they occurred singly, as seen with the GC *aac(6')-Ib*. Our results indicate the occurrence of the combination of an *aadA* GC with a *dfrA* GC. Other studies have reported the association of the *aadA* and *dfrA* genes (Kaushik et al., 2019; Lorestani et al., 2018). It thus indicates co-transmission and subsequent integration of *dfrA* and *aadA* GCs (Chang et al., 2007). In other studies *Citrobacter* spp. recovered from a WWTP effluent (Mokracka et al., 2012) and clinical samples (Lorestani et al., 2018), the *dfrA1-aadA1* GCs were the most prevalent. Similar GC arrays in *E. coli* were reported (Koczura et al., 2014; Rehman et al., 2017). The detection of ARGs in this study indicates the high prevalence of integrons in *Citrobacter* spp., which we recovered from various environmental sources. This study recorded only one typical class 1 integron with approximately 400 bp IVR with an empty cassette. It could be that the region sequences have not been previously reported, and therefore, further research can be carried out to determine its content. However, here, we categorized it as undetermined. Figure 5.1 shows a schematic representation of some integron-harbouring isolates and their associated GC arrays.



It has been reported that most GCs are promoter-less and rely on an external promoter located on the 5′ CS for their expression (Gillings, 2014). In this study, about half of the isolates harboured the assayed *P2R2* promoter gene on an integron, as seen in Table 5.2. The

promoter gene on the 5′ CS ensures the expression of the genes on the GCs. Their detection in this study indicates that these GC arrays can immediately confer phenotypic advantages on the isolates harbouring them. Although this research did not investigate the expression of the GCs, some other studies have reported that the maximum expression level usually occurs in the GC closest to the promoter (Hall, 2012; Kaushik et al., 2018).

### 5.3.4 Antibiotic resistance phenotypes of integron-harbouring strains

The antibiotic resistance frequencies of the isolates are presented in Figure 5.2. All the bacterial strains showed resistance against cefotaxime, a third-generation cephalosporin. It was followed keenly by their resistance against another third-generation cephalosporin, ceftazidime (94%). The percentage frequencies of resistance observed against the remaining antibiotics assayed are as follows: colistin (86%), ampicillin and tetracycline (81%), amoxicillin/clavulanic acid (75%), cefoxitin (69%), trimethoprim-sulfamethoxazole (56%), chloramphenicol (44%), nalidixic acid (31%), ciprofloxacin and gentamicin (25%). No resistance was observed against imipenem, a carbapenem, one of the drugs of last resort. The low prevalence of resistance detected against this antimicrobial is expected, given that they are seldom administered as a first-line antimicrobial.



MAR was shown to be prevalent among the isolates in this investigation. The various MAR phenotypes and their corresponding indices values are shown in Table 5.3. All the bacterial species tested were MDR except one. Although the isolate was resistant against three antibiotics out of the thirteen tested, two belonged to the same class, cephalosporins. The multiple antibiotic resistance index (MARI) ranged from 0.23 to 0.85. Most of the multiple antibiotic resistance phenotypes (MARPs) occurred uniquely except for three. A MARI value of more than 0.2 was found in all the isolates, indicating that antibiotic pressure in the various sources is high. Many antibiotics are not entirely metabolized after consumption and end up

in wastewater systems (Fadare & Okoh, 2021b). Antibiotics in wastewater have been demonstrated to impose selection pressure on antimicrobial-resistant bacteria, allowing them to spread to new environments (Li et al., 2019). MGEs are linked to multidrug resistance due to their potential to transmit several antimicrobial resistance genes simultaneously.

One of the three major classes of carbapenemases is the Class B metallo-β-lactamases (MBLs) which includes the Verona integrin-encoded MBL (VIM), imipenemase (IMP), and New Delhi MBL (NDM). The EDTA test for the phenotypic detection of MBL revealed that 25% (4/16) of the isolates produced MBLs while only 12.5% (2/16) produced ESBL using the DDST protocol. None of the isolates co-produced the MBL and ESBL. The ESBL producers include isolates with code numbers 189 and 180. They displayed resistance against 10 and 11 antibiotics with MARI values of 0.77 and 0.85, respectively. As indicated in Table 5.2, they harboured IVRs with GC arrays *aadA5-dfrA17* and *aac(6')-Ib*, respectively. The presence of these β-lactamases is also associated with co-resistance against other classes of antibiotics by bacterial species harbouring them, further limiting possible treatment options. These β-lactamases are usually carried on MGEs like integrons, which aid in the horizontal spread of ARGs among diverse bacterial species (Cantón et al., 2012; Martin & Bachman, 2018; Rawat & Nair, 2010).

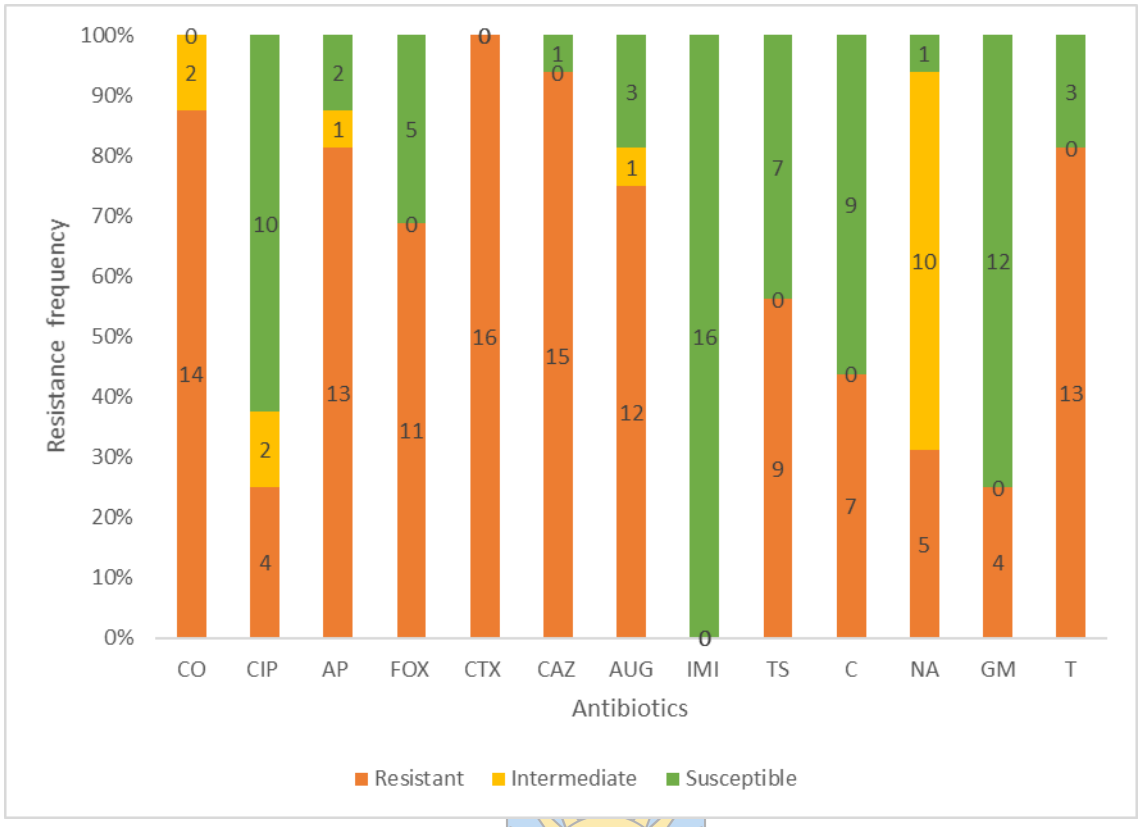


**Figure 5. 2: The phenotypic antibiotic resistance profile of Citrobacter spp. (n=16).** Antibiotics code: CTX-cefotaxime, TS-trimethoprim/sulfamethoxazole, CO-colistin sulphate, CIP-ciprofloxacin, FOX-cefoxitin, AMP-ampicillin, IMI-imipenem, C-chloramphenicol, NA-nalidixic acid, GM-gentamicin, AUG-amoxicillin/clavulanic acid, CAZ-ceftazidime, and TET-tetracycline.

**Table 5. 3: The MARI and MARP of *Citrobacter* spp. from different environmental matrices.**

| S/N | No of the antibiotics | No of the isolates | MARI | MARP |
|---|---|---|---|---|
| 1 | 11 | 1 | 0.85 | CO-CIP-AP-FOX-CTX-CAZ-AUG-TS-C-NA-T |
| 2 | 11 | 2 | 0.85 | CO-CIP-AP-FOX-CTX-CAZ-AUG-TS-NA-GM-T |
| 3 | 11 | 1 | 0.85 | CO-CIP-AP-FOX-CTX-CAZ-AUG-TS-C-NA-GM |
| 4 | 10 | 1 | 0.77 | CO-CIP-AP-CTX-CAZ-AUG-TS-NA-GM-T |
| 5 | 9 | 2 | 0.69 | CO-AP-FOX-CTX-CAZ-TS-C-NA-T |
| 6 | 9 | 2 | 0.69 | CO-AP-FOX-CTX-CAZ-AUG-C-NA-T |
| 7 | 9 | 1 | 0.69 | CO-AP-CTX-CAZ-AUG-TS-C-NA-T |
| 8 | 8 | 1 | 0.62 | CO-AP-FOX-CTX-CAZ-AUG-NA-T |
| 9 | 8 | 1 | 0.62 | CO-AP-FOX-CTX-AUG-C-NA-T |
| 10 | 8 | 1 | 0.62 | CO-AP-CTX-CAZ-AUG-TS-NA-T |
| 11 | 7 | 1 | 0.54 | CO-CIP-CTX-CAZ-AUG-NA-T |
| 12 | 7 | 1 | 0.54 | CO-AP-FOX-CTX-CAZ-AUG-NA |
| 13 | 3 | 1 | 0.23 | CO-CTX-CAZ |

Antibiotics code: CTX-cefotaxime, TS-trimethoprim/sulfamethoxazole, CO-colistin sulphate, CIP-ciprofloxacin, FOX-cefoxitin, AMP-ampicillin, IMI-imipenem, C-chloramphenicol, NA-nalidixic acid, GM-gentamicin, AUG-amoxicillin/clavulanic acid, CAZ-ceftazidime, and TET-tetracycline.



### 5.4 Conclusion

Our findings have revealed a high prevalence of multidrug-resistant *Citrobacter* spp. from rivers, animal droppings, hospitals and wastewater treatment plants effluents. These harboured different classes of integrons and a repertoire of various gene cassette arrays they possess. The integrons can rapidly acquire and express ARGs harboured on GCs, which further confers phenotypic resistant advantages on the isolates. Since integrons are mobile, the GCs can be easily exchanged among various bacterial species in different environmental

matrices. MDR phenotypes, ESBL and MBL-producers, and class 1 and 2 integron genes in *Citrobacter* spp. poses a severe public health issue because these strains can act as strong carriers for the extensive propagation of ARGs to other bacterial pathogens. The study reports integron-harbouring *Citrobacter* spp. for the first time in the Eastern Cape Province, South Africa, suggesting that the various environmental niches from which the bacteria were isolated are essential reservoirs of antimicrobial resistance determinants in the environment.

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**CHAPTER SIX**

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**Classes 1 and 2 integrons in antibiotic-resistant *Escherichia coli* recovered from aquatic and animal origins in the Eastern Cape Province, South Africa (Submitted to *Journal of Exposure Science & Environmental Epidemiology*)**

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**Abstract**

Integrons are genetic elements that can identify and capture gene cassettes (GCs) with the ability to express the antimicrobial resistance genes harboured on the GCs. This paper evaluated the prevalence of integrons and the characterization of their associated GCs in *Escherichia coli* recovered from aquatic and animal origins. The isolates were recovered from food animals, including cattle, goats, and sheep, and aquatic sources, including rivers and effluents of wastewater treatment plants. Integrase genes and their associated GCs were assessed in 71 *E. coli* isolates using PCR, RFLP, and DNA sequencing. The phylogenetic relationships of the isolates were investigated using ERIC-PCR. A total of 88.7% (63) integron integrase genes were amplified from the 71 isolates. These were classified into class 1, 2 and 1+2 integrons with 76.2% (48), 1.6% (1), and 22.2% (14), respectively. The *intI1* internal variable regions (IVRs) were detected in 19 isolates with various amplicon sizes ranging from 200 to 1700bp. These harboured GCs such as *dfrA1*, *dfrA21*, *dfrA5*, *dfrA17*, *aadA5*, *aadA1*, *aadA5*, and *aac-3-Ib*, while the remaining IVRs contained an empty or undetermined GCs. The *intI2* IVRs were detected in 4 isolates at 1400bp and harboured a constant GC array of *dfrA1-sat2*. The outcome of the ERIC-PCR revealed that the isolates were characterized into fourteen clusters at a similarity cut-off of 55%, indicating that the isolates had unique DNA fingerprints and were unrelated. Due to the high rate of occurrence of integrons with similar GCs in the unrepetitive *E. coli* from various sources, precautionary measures must be employed to prevent the spread of integrons. To our knowledge, this is the first comprehensive analysis in South Africa that shows the baseline features of *E. coli* integrons isolated from farm animals and water sources.



**Keywords:** *Escherichia coli*, integrons, multidrug resistance, gene cassettes, DNA fingerprinting

## 6.1 Introduction

Globally, there has been an upsurge of antimicrobial resistance (AMR) in *Escherichia coli* due to the continued usage of antibiotics as therapeutic and prophylactic agents in animals and humans, resulting in the widespread distribution of antimicrobial-resistant *E. coli* with often complicated treatment options. These complications usually arise due to the acquisition of various antimicrobial resistance genes (ARGs) harboured by transposons, plasmids, and integrons (Chaturvedi et al., 2021; Dessie et al., 2013). Integrons are genetic elements that can identify and capture gene cassettes (GCs) (Hall, 2012; Stokes & Hall, 1989). Integrons have a platform which includes the integron integrase gene, *intI*, the constitutive promoter, $P_c$, and the integron-associated integration site, *attI*, located on the 5′ conserved segment (CS). The GCs exist as sequences with an approximate range of 100-1000bp and encode a single gene with the addition of an extra short sequence referred to as the 59-base element (59-be) or the cassette-associated recombination site (*attC*). The genes on the GCs encode resistance against various classes of antibiotics. The GCs, usually promoter-less exist in circularised form when free-living, become linearised once incorporated into an integron and depend on the integron's $P_c$ to express their genes. Hence, the closer a GC is to the $P_c$, the stronger the expression of the genes they harbour. The structure of integrons allows them to act as natural expression vectors for any GCs captured on them (Gillings, 2014; Hall, 2012; Kaushik et al., 2018; McMillan et al., 2019). The characteristic of integrons as an expression vector has attracted much attention. Several studies have investigated the roles of integrons in the spread of ARGs (Chaturvedi et al., 2021; Chowdhury et al., 2011; Firoozeh et al., 2019; Kaushik et al., 2019; McMillan et al., 2019; Soltan Dallal et al., 2018; Wei et al., 2011; Zhang et al., 2020).



Integrons have been grouped into various classes depending on the amino acid sequences of the *intI* gene (Cambray et al., 2010; Gillings, 2014). Integrons with *intI1* genes are classified

as class 1, *intI2* genes are class 2, and *intI*3 genes are class 3. Integrons with *intI1* and *intI2* genes are often described among the Enterobacteriaceae family, while class 3 integrons seem to be far scarce and, consequently, infrequently associated with the spread of AMR. Members within the same class have the same integrase but can be further differentiated based on their GCs. The most frequently detected integron is class 1 among Enterobacteriaceae, including *E. coli*. Typical class 1 integrons have a 3′ region called 3′ CS, including the *qacEΔ1* and *sulI*, which confers resistance against antiseptics and sulphonamides. The remaining two ORFs, *orf5* and *orf6*, contain genes with unknown functions (Gillings, 2014; Kaushik et al., 2018; Okoh & Fadare, 2022; Stokes & Hall, 1989).

Integrons have been identified in several bacterial species from clinical samples and samples recovered from the environment (Boucher et al., 2007; Buongermino Pereira et al., 2020; Daly & Fanning, 2004; Hall, 2012; Jones-Dias et al., 2016; Koczura et al., 2012; Mokracka et al., 2012; Nield et al., 2001). Therefore, the detection of integrons is not restricted to pathogenic organisms but has also been reported in bacteria recovered from healthy animal and plant hosts and environmental sources (Chaturvedi et al., 2021; Dawes et al., 2010; Dessie et al., 2013; Rosser & Young, 1999). Hence, this study investigated the prevalence of integrons in *E. coli* strains isolated from aquatic and animal origins. In addition, we characterized the inserted GCs and assessed the possible associations between the presence of the integrase genes and antibiotic resistance in unique bacterial phylogenetic groups.

## 6.2 Materials and methods

### 6.2.1 Bacterial strains

The *E. coli* (n=71) selected for this study were obtained from Chris Hani and Amathole District Municipalities. The aquatic sources where the isolates were recovered include rivers, and wastewater treatment plant effluents (WWTP), while those recovered from animal origin include swabs from cattle, goats, and sheep. The *E. coli* strains recovered from October to November 2017 were preserved in 20% glycerol stock at -80 ℃ as part of the Applied and Environmental Research Group (AEMREG) culture collection. Ethical clearance was obtained with project number OKO011SFAD01 to access these bacterial cultures.

### 6.2.2 The extraction of genomic DNA

The retrieved glycerol stocks were resuscitated in Brain Heart Infusion (BHI) broth (Merck, Modderfontein) and incubated for 18 hours at 37 ℃. A loopful was streaked on nutrient agar (Oxoid, UK) and further purified twice. For subsequent analysis, single pure colonies were transferred to 2 ml BHI broth and incubated overnight at 37 ℃. The isolates' genomic DNA was extracted using the boiling process (Maugeri et al., 2004) with minor modifications, as described elsewhere (Fadare & Okoh, 2021). The DNA-containing supernatant was then decanted to sterile microcentrifuge tubes (Eppendorf, Germany) and stored at -20 ℃ for future assays.



### 6.2.3 PCR confirmation of the identities of the bacterial cultures

The identities of the recovered bacterial cultures were revalidated using the conventional polymerase chain reaction (PCR) before further analysis. Primer pair targeting the *uidA* gene was used to identify the bacterial species (Bej et al., 1991). The positive control *E. coli* ATCC 8739 (Microbiologics, Medimark, France) was used to validate the assays. All the primers and thermal cycling conditions used for all the PCR assays are described in Table 6.1.

The PCR reactions (25µl) were done with the BioRad Thermal Cycler PCR system (LASEC, South Africa). Each reaction comprised 12.5 µl double strength master mix and 5.5 µl PCR grade water supplied by Thermo Scientific (USA), and 1 µl of each primer set synthesized by Inqaba Biotechnical Industries (RSA). Previously extracted DNA (5 µl) was used as the DNA template for all reactions. For negative controls, the DNA template was replaced by PCR-grade water. Each amplicon (5 µl) was resolved in 1.5% (w/v) Ethidium bromide-stained agarose gel in 0.5X TBE buffer at 100 volts for 45 mins. The gel image was photographed using UV transillumination (Alliance 4.7, London, UK).

### 6.2.4 Molecular characterization of integrons

All confirmed *E. coli* isolates were assayed for integrase genes (*intI1*, *intI2*, and *intI3*) on the 5′ CS to classify the integrons using PCR. The *intI1*-positive strains were further assessed for the *qacEΔ1* and *sul1* genes on the 3′ CS of typical class 1 integrons. *Acinetobacter baumannii* ATCC 19606 (Microbiologics, Medimark, France) was used as a positive control for the typical class 1 integrons.



### 6.2.5 Characterization of integrons variable regions

Separate PCR assays were carried out to characterize the internal variable regions (IVRs) located between the 5′ CS and 3′ CS in all *intI1* and *intI2* genes harbouring *E. coli*. The primer set hep74 and hep 51, which bind to the *attI2* and the *orfX* sites downstream of the IVRs, were used for *intI2* positive isolates. Meanwhile, the primer pair 5′ CS and 3′ CS was used for the *intI1* positive isolates. These PCR reactions were done in triplicates to ensure repeatability.

**Table 6. 1. List of primers and thermocycling conditions for this study.**

| Primer | Primer Sequence (5′ →3′) | Amplicon size (bp) | Thermocycling conditions | Reference |
|---|---|---|---|---|
| uidA-F<br>uidA-R | AAAACGGCAAGAAAAAGCAG<br>ACGCGTGGTTACAGTCTTGCG | 147 | 94 ℃ for 5 mins; 30[94 ℃, 30 s; 58 ℃, 1 min; 72 ℃, 1 min]; 72 ℃ for 8 min. | (Bej et al., 1991) |
| INTI1-F<br>INTI1-R | CAG TGG ACA TAA GCC TGT TC<br>CCC GAG GCA TAG ACT GTA | 164 | 94 °C for 5 mins; 35[94 °C, 60 s; 55 °C, 60 s, 72 °C, 30 s] 72 °C, 10 min | (Koeleman et al., 2001) |
| INTI2-F<br>INTI2-R | TTATTGCTGGGATTAGGC<br>ACGGCTACCCTCTGTTATC | 232 | 94 °C for 5 mins; 32[94 °C, 60 s; 59 °C, 60 s; 72 °C, 2 mins]; 72 °C for10 mins. | (Goldstein et al., 2001) |
| INTI3-F<br>INTI3-R | AGTGGGTGGCGAATGAGTG<br>TGTTCTTGTATCGGCAGGTG | 600 | 94 °C for 5 mins; 32[94 °C, 60 s; 48 °C, 60 s; 72 °C, 2 mins]; 72 °C for 10 mins. | (Goldstein et al., 2001) |
| qac-F<br>qac-R | ATC GCA ATA GTT GGC GAAGT<br>CAA GCT TTT GCC CAT GAAGC | 225 | 94 for 9mins; 30[94 ℃, 30 s; 55 ℃, 30s; 72 ℃, 60s]; 72 ℃ for 10 mins. | (Stokes & Hall, 1989) |
| Sul1-F<br>Sul1-R | ATGGTGACGGTGTTCGGCATCTGA<br>CTAGGCATGATCTAACCCTCGGTCT | 840 | 94 for 9mins; 30 [94 ℃, 30 s; 55 ℃, 30 s; 72 ℃, 1 min]; 72 for 10 mins. | (Grape et al., 2005) |
| 5′CS<br>3′CS | GGCATCCAAGCAGCAAG<br>AAGCAGACTTGACCTGA | Variable | 94 °C, 5 min; 35[94 °C, 1 min; 45 °C, 1 min; 72 °C, 2 min]; 72 °C for 10 mins. | (Lévesque et al., 1995) |
| hep 74<br>hep51 | CGGGATCCCGGACGGCATGCACGATTTGTA<br>GATGCCATCGCAAGTACGAG | Variable | 94 °C, 5 min; 33[94 °C, 1 min; 59 °C, 45 s; 72 °C, 5 min]; 72 °C, 5 min | (White et al., 2001) |
| ERIC1<br>ERIC2 | ATGTAAGCTCCTGGGGATTCAC<br>AAGTAAGTGACTGGGGTGAGCG | Variable | 94 °C, 5 min; 39[94 °C, 1 min; 35 °C, 1 min; 72 °C, 2 min]; 72 °C, 10 min | (Versalovic et al., 1991) |

6.2.6 Restriction analysis and DNA sequencing of amplicons

Restriction fragment length polymorphism (RFLP) was carried out using the restriction enzyme *AluI* (Biolabs, England) to identify similar GC arrays in the integrons. Amplicons of variable regions that appeared similar in size were subjected to RFLP to determine if the products were of the same sequence. The *AluI* enzyme was used because its recognition sequence is only four bases, thereby increasing the likelihood of its activity over the other enzymes that target the six-base sequence. Briefly, 30 µl of the amplicon was exposed to 1.0 µl of 10U/ml *AluI*. The reaction mixture was incubated for 4 hours at 37 ℃. The products were resolved on a 2% agarose gel and visualized. These were characterized based on their distinct restriction profiles, and two randomly selected representative amplicons from each different RFLP class were selected for sequencing. This step was taken to help reduce the risk of sequencing multiple identical variable regions needlessly.

6.2.7 Variable sequence analysis, cassette identification

The PCR products of each representative RFLP class were sequenced with the Nimagen, BrilliantDye™ Terminator Cycle sequencing kit V3.1 cycle sequencing on an ABI 3500XL sequencer in both directions (Inqaba Biotec, Johannesburg). The resulting sequences were edited with Chromas 2.7 and aligned using BioEdit sequence alignment editor software. The generated consensus sequences were subjected to BLAST nucleotide search analysis (https://blast.ncbi.nlm.nih.gov/Blast) to identify the contents of the GCs. The position of each gene in each cassette was determined using ABRicate 0.8.4 (https://github.com/tseemann/abricate) (ResFinder, ARG-ANNOT, CARD, and NCBI databases).



6.2.8 Antibiotic sensitivity test

The antibiotic sensitivity test was carried out on all integron-harbouring *E. coli* following the disk diffusion technique (DDT) described in our previous report (Fadare & Okoh, 2021).

Isolates were exposed to a panel of thirteen antibiotics selected based on the antibiotics usually recommended by the Clinical and Laboratory Standard Institutes (CLSI) for treating *E. coli* infections. The antibiotics, their codes and concentration include the following: nalidixic acid (NA/30 µg), imipenem (IMI/10 µg), ciprofloxacin (CIP/5 µg), amoxicillin/clavulanic acid (AUG/20 µg/10 µg), cefotaxime (CTX/30 µg), ceftazidime (CAZ/10 µg), trimethoprim-sulfamethoxazole (TS/25 µg), colistin (CO/10 µg), cefoxitin (FOX/30 µg), gentamicin (GM/10 µg), tetracycline (T/30 µg), ampicillin (AMP/10 µg), chloramphenicol (C/30 µg). The antibiotics were purchased from Mast Diagnostics, South Africa. The CLSI recommendations were used to interpret the inhibition zones' findings (CLSI, 2020).

6.2.9 Phenotypic screening of ESBL and MBL-producers

The integron-harbouring *E. coli* isolates were subjected to the double-disk synergy test (DDST) for the phenotypic production of extended-spectrum β-lactamase (ESBL) according to established CLSI guidelines. The antibiotics used were cefoxitin-30 µg, cefotaxime-30 µg, and ceftazidime-10 µg, in combination with amoxicillin/clavulanic acid -20 µg/10 µg. The ethylenediaminetetraacetic acid (EDTA) test was used to determine the metallo β-lactamase (MBL) production according to CLSI standards. Briefly, the 0.5 McFarland adjusted test isolate was exposed to 10 µg imipenem discs A and B. Then, 10 µl of 0.5 M EDTA was added to disc A to obtain a concentration of 750 µg. An increase in inhibitory zone width of 5mm in disc A compared to disc B after 18 hours of incubation at 37 °C was regarded as positive for MBL generation (CLSI, 2020).



6.2.10 Phylogenetic typing of *E. coli*

Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR was done to appraise the genotypic diversities of the bacterial species despite being recovered from diverse environmental matrices. Sixty-four isolates were chosen randomly from various sources and

subjected to PCR with the primer sets ERIC1 and ERIC 2 under conditions described by (Versalovic et al., 1991) (Table 6.1). The amplicons were resolved in 3% agarose gel in a 0.5X TBE buffer and allowed to run at 100 volts for 240 mins.

6.2.11 Statistical analysis

The data were examined using the descriptive statistical tools in Microsoft Excel 2016 and the Statistical Package for the Social Sciences (SPSS) version 25 (SPSS Inc., Chicago, IL). The relationship between susceptibility and the presence of integrase genes was investigated using Pearson's correlation test. Significant was defined as a *P* value of less than 0.05. Qualitative data were expressed as percentage and frequency.

Using Gelj v.2.0 software, computer-assisted pattern analysis was used to examine the DNA fingerprints generated from the ERIC-PCR. Pearson's correlation coefficient was used to compute the percentage similarity of digitized bands. The relatedness of the isolates was estimated using the unweighted pair group method with arithmetic mean (UPGMA) and complete linkage methods, which were shown as dendrograms.



**6.3 Results and discussion**

6.3.1 PCR Confirmation of isolates

All the assayed isolates (n= 71) were positive for the *uidA* gene for the molecular confirmation of *E. coli*. The breakdown of the confirmed isolates and their sources include rivers (n=9), and WWTP (n=5), while those recovered from animal origin include swabs from cattle (n=9), goats (n=21), and sheep (n=27).

6.3.2 Prevalence and characterization of integrons

Approximately 88.7% (63) were integron-harbouring out of the 71 *E. coli* isolates. The integron-positive isolates were all classified according to the *intI* gene harboured. The *intI1* were 76.2% (48/63), *intI2* were detected in 1.6% (1/63), while the remaining 22.2% (14/63)

harboured both *intI1* and *intI2* genes. No *intI3*-positive isolates were found in this study. The high detection of integrons from the various sources of the isolates signifies a widespread distribution of integrons even in non-clinical sources. The most frequently detected integrase was the *intI1*, similar to the high occurrence rate in similar studies. According to other investigations, a high rate of detection of integrons was found in other *E. coli* collections. These include 82.3% in isolates from the river (Su et al., 2012), 78.3% in isolates from stool specimens (Kargar et al., 2014), 64% in isolates of clinical origin (Chang et al., 2007), 65% in isolates from poultry meat (Soufi et al., 2009), and 100% of isolates from human, animal and food origins (Sáenz et al., 2004). Other studies, however, have found a low incidence of integrons in *E. coli*, such as 6.7% in *E. coli* recovered from free-ranging livestock (Rehman et al., 2017).

The presence of class 2 integron was low in this investigation, with a 1.6% prevalence. Similar research reported a 3.3% detection of class 2 integron in isolates of human and animal origins (Cocchi et al., 2007), while 0.7% was reported in isolates from free-ranging food animals (Rehman et al., 2017). The remaining *intI2* genes detected concurrently occurred with *intI1* genes and were classified as class 1+2 integrons. Both *intI1* and *intI2* genes have also been reported in *E. coli* from faecal samples (Odetoyin et al., 2017) and poultry (Dessie et al., 2013).



From the total isolates harbouring the *intI* gene (n=62), *qacEΔ1* was detected in 37.1% (23/62), while the *sul1* gene was detected in 25.8% (16/62). The presence of the *qacEΔ1* and *sul1* genes on the 3′ CS identifies the classification of typical class 1 integrons. Typical class 1 integrons usually harbour quaternary ammonium compounds and sulphonamide resistance genes (Paulsen et al., 1996; Stokes & Hall, 1989). In this study, 10.4% (5/48) of the *intI1* positive isolates harboured both *sul1* and *qacEΔ1* genes and are classified as typical class 1 integrons. However, 28.6% (4/14) of the *intI1* and *intI2* harbouring isolates also harboured

*sul1* and *qacEΔ1* genes on their 3′ CS. Invariably, this indicates the possibility of some typical class 1 integrons being able to harbour the *intI2*, a scenario that has been previously reported (Zhang et al., 2019). The sequence of the *intI2* gene has a 46% similarity with the *intI1* gene and is generally less prevalent in their occurrence within the Enterobacteriaceae family compared with class 1 integrons (Gillings, 2014). Although, the *intI2* gene is interrupted by an early stop codon with a shortened, non-functional 178 amino acid protein product (Hansson et al., 2002), further distinguishing the two genes. The outcomes of our study further support the possibility of the co-occurrence of the *intI1* and *intI2* genes. Although the majority of the *intI1* gene harbouring isolates, 53.2% (33/62), have entirely lost the 3′ CS genes, others harboured at least one of *qacEΔ1* or *sul1*. Some enteric bacteria, including *E. coli,* have been reported to have variations in the 3′ CS, which is inconsistent with most known class 1 integrons. Such integrons have been reported to harbour the transposition gene module (*tni* module), which contains the genes *tniR, tniQ, tniB,* and *tniA* on the 3′ end. Also, the additions and deletions of the *tni module* could occur, giving rise to several insertion sequences within the class 1 integrons (Brown et al., 1996; Kholodii et al., 1995; Rådström et al., 1994; Sajjad et al., 2011). For example, in integron In2, the insertion of IS*1326* and IS*1353* on the 3′ CS gave rise to the deletion of parts of the *tni* module (Brown et al., 1996). Although our present study did not investigate the presence of the *tni* module, we opine that this could be one of the reasons for the variations in the class 1 integrons observed in our study.



### 6.3.3 Characterization of integrons gene cassettes

The gene cassettes that usually harbour ARGs are inserted between the 5′ CS and the 3′ CS. These are the integrons' internal variable regions, and their presence was investigated. The amplification of the *intI1* positive and *intI2* positive isolates revealed that IVRs were present in 36.5% (23/63) of various sizes. Due to the possible variations in class 1 integrons

described earlier, IVRs were not detected in most isolates as they mostly lacked the *qacEΔ1* and/ *sul1* genes.

The amplified IVRs of *intI1* positive isolates ranged from approximately 200 bp to 1700 bp and were present in 30.6% (19/62) of the isolates investigated. Their analysis as shown in Table 6.2 yielded eight amplicons of distinctly different sizes as follows: ≈200bp (n=3), ≈250bp (n=7), ≈400bp (n=1), ≈600bp (n=3), ≈750bp (n=1), ≈800 (n=1), ≈1200 (n=2), and ≈1700 (n=1). Only four separate GC arrays were detected, including *dfrA21-aac-3-Ib*, *dfrA5-aac-3-Ib*, *aadA1-dfrA1*, and *aadA5-dfrA17*, while the remaining IVRs with sequences below 750bp yielded an empty or undetermined GCs. One of the isolates was positive for the IVR; despite the absence of both *qacEΔ1* and *sul1* genes, its sequences yielded an undetermined GC. Some of the isolates having both *qacEΔ1* and *sul1* genes did not harbour any IVR. It could be that these isolates lacked any GCs, but the presence of the integrase gene implies that the isolates already have a platform that can easily enable them to acquire any GCs within their environment. Meanwhile, the analysis of the IVRs of *intI2* positive isolates revealed their presence in 26.7% (4/15). They all possessed a single amplicon size of approximately 1400bp with a GC array of *dfrA1-sat2*.



The *dfrA* variants include genes which encode the enzyme dihydrofolate reductase, which confers resistance against trimethoprim and are the most frequently detected GCs in this study. These include *dfrA1*, *dfrA17*, *dfrA5*, and *dfrA21*. The *aadA1* and *aadA5* belong to aminoglycoside adenyltransferase encoding resistance against aminoglycosides, including streptomycin and spectinomycin (Kaushik et al., 2018; White et al., 2000). Although the *aac(3)-Ib* gene also confers resistance against aminoglycosides, it belongs to the aminoglycoside acetyltransferases. A streptothricin (*sat2*) resistance gene was found in the *intI2* positive isolates, and they occurred in combination with a *dfrA1* gene. Several animals and human-derived *E. coli* isolates showed similar results (Rehman et al., 2017; Zhang et al.,

2020). Our results indicate the occurrence of the combination of an *aadA* GC with a *dfrA* GC. Other studies have reported the association of the *aadA* and *dfrA* genes (Kaushik et al., 2019; Lorestani et al., 2018). It thus indicates co-transmission and subsequent integration of *dfrA* and *aadA* GCs (Chang et al., 2007). A schematic representation of some of the isolates with their GC arrays is depicted in Figure 6.1.

**Table 6. 2. The content and arrangement of genes within the integron's variable regions (n=23).**

| No. of Isolates | Cassette array | Approximate size (bp) | *qacEΔE+sul1* |
|---|---|---|---|
| 1 | *dfrA21-aac(3)-Ib* | 750 | +1 |
| 3 | _[a] | 200 | 1[b] |
| 3 | _[a] | 600 | 1[c] |
| 2 | *aadA1-dfrA1* | 1200 | 1[d] |
| 4 | _[a] | 250 | 1[e] |
| 1 | *drfA5-aac(3)-Ib* | 800 | 1[b] |
| 1 | *aadA5-dfrA17* | 1700 | +1 |
| 4 | *dfrA1-sat2* | 1500 | 1[f] |
| 1 | _[a] | 400 | 1[b] |
| 3 | _[a] | 250 | 1[b] |

[a]The amplicon yielded an undetermined/empty cassette array.
[b]All the isolates harboured only the *qacEΔ1* gene.
[c] Except for one isolate that was positive for *sul1* only while another lacked both *qacEΔ1+sul1*.
[d] The isolates harboured only the *sul1* gene.
[e]One isolate was positive for the *qacEΔ1* gene, while others harboured only the *sul1* gene.
f One isolate harboured only *qacEΔ1;* another harboured only *sul1*, while the remaining two isolates lacked both genes.
*Not applicable.

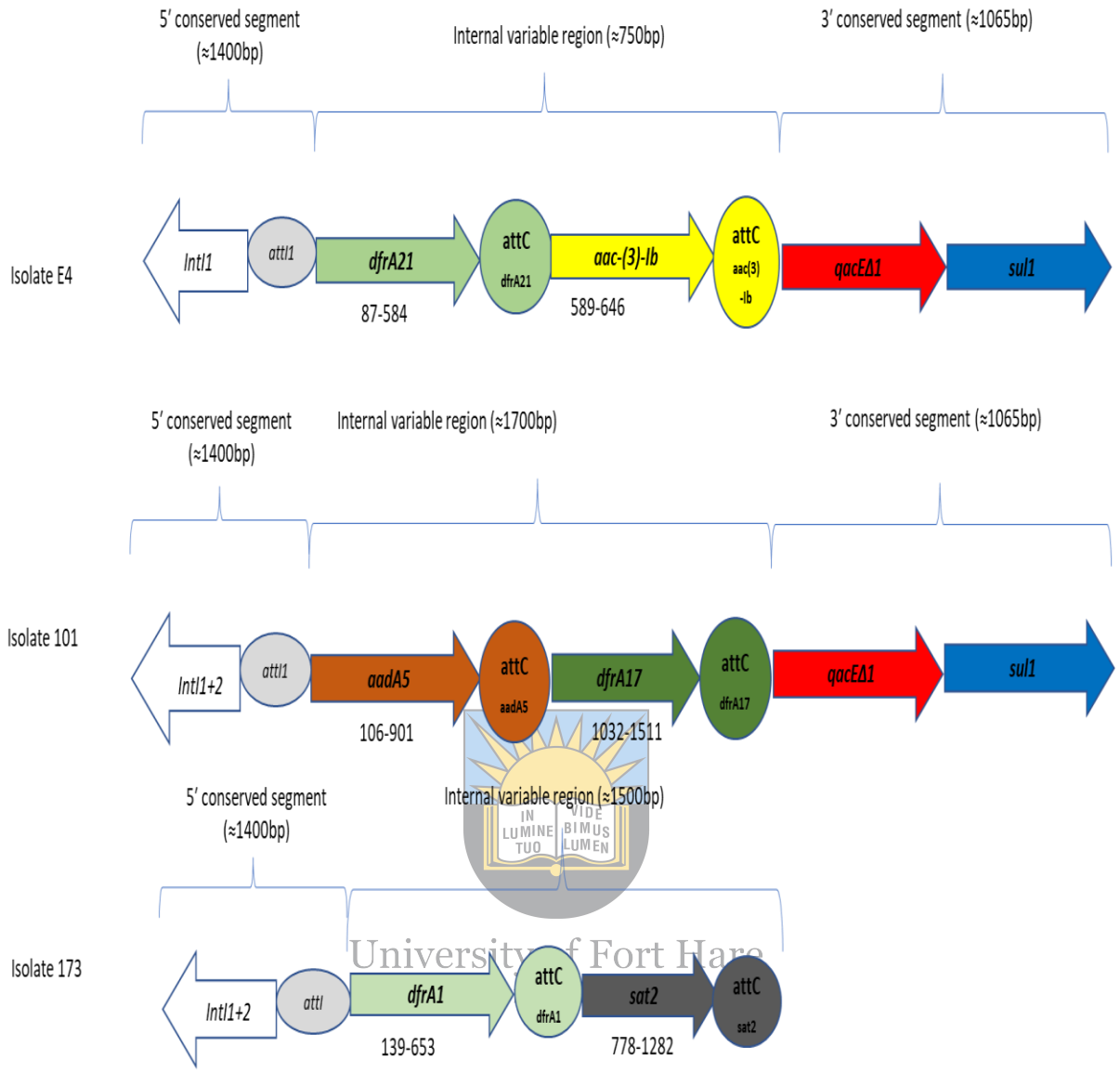


**Figure 6. 1. Schematic representation of some integron-positive *E. coli* with their associated gene cassette arrays.** These isolates were positive for *intI1* and *intI1+2* for class 1 and class 1+2 integrons, respectively. The *sul1*and *qacEΔ1* genes found on the 3′ conserved segment confer resistance against sulphonamides and quaternary ammonium compounds. The cassette arrays include the *aadA5* and *aac(3)-Ib* gene, which encodes resistance against aminoglycosides. The *dfrA* variants and *sat2* genes confer resistance against trimethoprim and streptothricin. The start and stop nucleotide sequences were also depicted, while the arrows indicate the transcription direction.

6.3.4 Antimicrobial susceptibilities

The antibiotic fingerprints of the integron-bearing isolates indicated that all the isolates exhibited resistance against multiple antibiotics and were considered multidrug-resistant, apart from an isolate which displayed resistance against only two antibiotics. Here, isolates with intermediate resistance were considered resistant, and an isolate was defined as MDR when resistance was observed against a minimum of three different antibiotics. In respect of individual antibiotics, all the isolates were resistant against colistin, keenly followed by resistance frequencies of 97% and 94% against cefotaxime and ceftazidime, respectively. The least resistance was observed against imipenem, considered one of the last resort drugs. The percentage frequencies of the other antibiotics assayed include tetracycline (77%), nalidixic acid (66%), ampicillin (58%), cefoxitin and amoxicillin/clavulanic acid (50% each), ciprofloxacin (32%), trimethoprim-sulfamethoxazole (31%), gentamicin (24%), and chloramphenicol (21%). The percentage resistance frequencies of each species are shown in Figure 6.2.

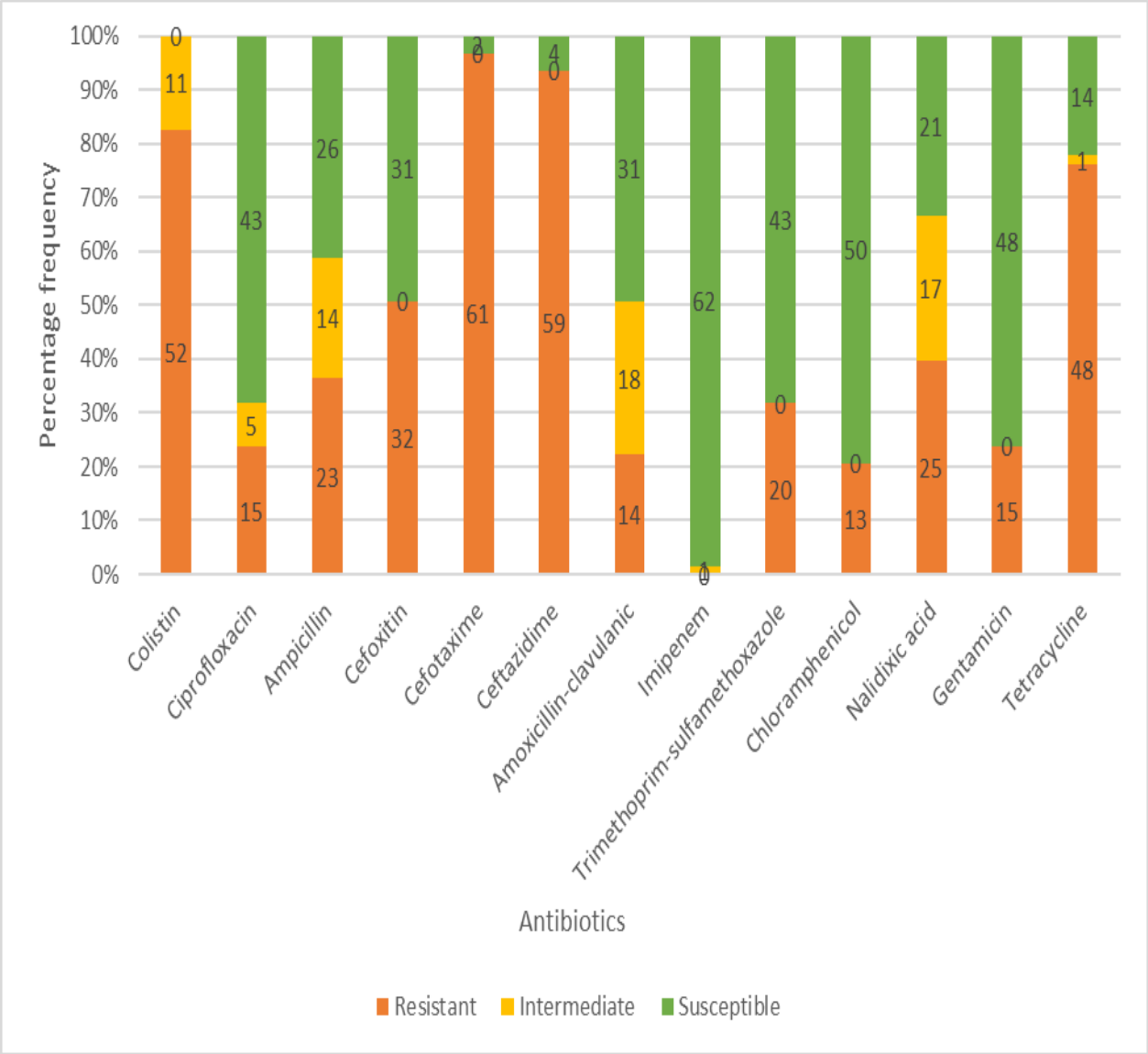


**Figure 6. 2. The antibiotic susceptibility patterns of integron-harbouring *E. coli* isolates (n=63).**

6.3.5 Beta-lactamase production

The production of β-lactamase by *E. coli* is one of their resistance mechanisms against β-lactam antibiotics (Rawat & Nair, 2010). Our assessment of DDST investigated revealed that 7.9% (5/63) of the isolates were ESBL producers. For the MBL production, only 9.5% (6/63) of the isolates were positive. Notably, one of the isolates co-produced ESBL and MBL (Isolate 155 recovered from sheep, data not shown). Two ESBL producers recovered from cattle, and WWTP displayed resistance against ten of the assayed antibiotics and belonged to class 1+2 integrons. The remaining three exhibited resistance against eleven out of 13

antibiotics, where two isolates from sheep were class 1 integrons and one from WWTP harbouring class 1+2 integrons. The presence of these β-lactamases is also associated with co-resistance against other classes of antibiotics by bacterial species harbouring them, further limiting possible treatment options. These β-lactamases are usually carried on mobile genetic elements such as integrons, which aid in the horizontal spread of ARGs among diverse bacterial species (Cantón et al., 2012; Martin & Bachman, 2018; Rawat & Nair, 2010).

### 6.3.6 Correlation between integrase genes and resistance

Pearson's correlation coefficients evaluated the correlation matrix between the antibiotic-resistant phenotypes and the occurrence of integrase genes (*intI1*, *intI2*). The outcome indicated that the *intI2* gene indicated a negative correlation with *intI1*. The cephalosporin antibiotics, cefoxitin and cefotaxime, showed a negative correlation with both *intI1* and *intI2* genes, nalidixic acid and tetracycline showed a negative correlation with the *intI1* gene, while ceftazidime and imipenem displayed a negative correlation with *intI2* gene. The results show significant correlations between antibiotic-resistant phenotypes and integrons, indicating that integrons are critical for antimicrobial resistance and possible dissemination between different bacterial species (Table 6.3). This conclusion has been supported by the current findings and other earlier investigations (Chaturvedi et al., 2021).

| | *intI1* | *intI2* | CIP | AP | FOX | CTX | CAZ | AUG | IMI | TS | C | NA | GM | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *intI1* | 1 | | | | | | | | | | | | | |
| *intI2* | -0.218 | 1 | | | | | | | | | | | | |
| CIP | 0.087 | 0.229 | 1 | | | | | | | | | | | |
| AP | 0.152 | 0.045 | .364** | 1 | | | | | | | | | | |
| FOX | -0.125 | -0.228 | 0.194 | -0.116 | 1 | | | | | | | | | |
| CTX | -0.023 | -.310* | -0.071 | 0.032 | 0.003 | 1 | | | | | | | | |
| CAZ | .488** | -0.147 | 0.038 | 0.046 | -0.126 | .324** | 1 | | | | | | | |
| AUG | 0.129 | 0.137 | .398** | .529** | 0.047 | 0.003 | 0.134 | 1 | | | | | | |
| IMI | 0.016 | -0.074 | 0.186 | -0.152 | 0.125 | 0.023 | 0.033 | 0.125 | 1 | | | | | |
| TS | 0.087 | 0.150 | .414** | .502** | 0.126 | 0.123 | 0.178 | .467** | -0.087 | 1 | | | | |
| C | 0.065 | 0.063 | 0.074 | 0.188 | -0.047 | 0.092 | 0.133 | .267* | -0.065 | .326** | 1 | | | |
| NA | -0.090 | 0.180 | 0.193 | 0.228 | 0.045 | 0.064 | -0.046 | .382** | 0.090 | 0.121 | 0.194 | 1 | | |
| GM | 0.071 | 0.188 | .339** | 0.242 | 0.028 | 0.101 | 0.146 | .401** | 0.227 | .419** | -0.009 | .316* | 1 | |
| T | -0.068 | 0.049 | 0.200 | .327** | 0.085 | 0.121 | -0.139 | 0.161 | 0.068 | 0.036 | -0.105 | 0.189 | 0.209 | 1 |

**. Correlation at 0.01(2-tailed)

*. Correlation at 0.05(2-tailed)

Antibiotics code: CTX-cefotaxime, CIP-ciprofloxacin, TS-trimethoprim/sulfamethoxazole, FOX-cefoxitin, NA-nalidixic acid, AP-ampicillin, AUG-amoxicillin/clavulanic acid, IMI-imipenem, C-chloramphenicol, GM-gentamicin, CAZ-ceftazidime, and T-tetracycline.

*Table 6. 3. Correlation matrix of antibiotic-resistant phenotypes and integron occurrence in E. coli.*

6.3.7 Genetic fingerprinting of confirmed *E. coli* isolates

DNA fingerprinting assays are used to compare DNA patterns, examining genetic similarities across samples and their classification into clusters (Heras et al., 2015). The ERIC-PCR study was utilized to amplify distinct DNA sections to produce exact genetic patterns for individual isolates (Ateba & Mbewe, 2014). The results of the DNA fingerprinting using ERIC-PCR amplification obtained in this study showed that genetic fingerprints of the isolates from rivers, WWTP, cattle, sheep, and goats differed in respect of the distribution of the polymorphic bands generated. The fingerprints were analyzed based on the migration patterns of the amplified bands. Seven isolates that did not generate any polymorphic bands were consequently excluded from the analysis. As shown in Figure 6.3, the dendrogram was generated based on the 57 isolates that produced polymorphic bands. These were further categorized into fourteen clusters at a similarity cut-off of 55%. The highest ERIC-genotype cluster was observed at cluster N, which comprises ten isolates. It was keenly followed by clusters A and G with nine and eight isolates, respectively. Only one isolate was represented in cluster E. Others include cluster F with six isolates, cluster B composed of four isolates, clusters C, H, and J composed of three isolates each while two isolates were found in clusters D, I, K, L, and M.



The dendrogram image reveals the diversity of the *E. coli* strains irrespective of the sources of the isolates. The highest level of genetic relatedness was observed at 70%. Two of the four isolates clustered at B from a similar source (goats) indicated it. Others include isolates from different sources, as shown in clusters N (WWTP and river) and cluster F (swabs from cattle and goat). Since none of the isolates has a 100% genetic similarity, the isolates selected for these assays were unrepetitive and unique. Therefore, detecting integrons harbouring similar

GCs in these genetically unrelated isolates recovered from various sources suggests the widespread ARGs.

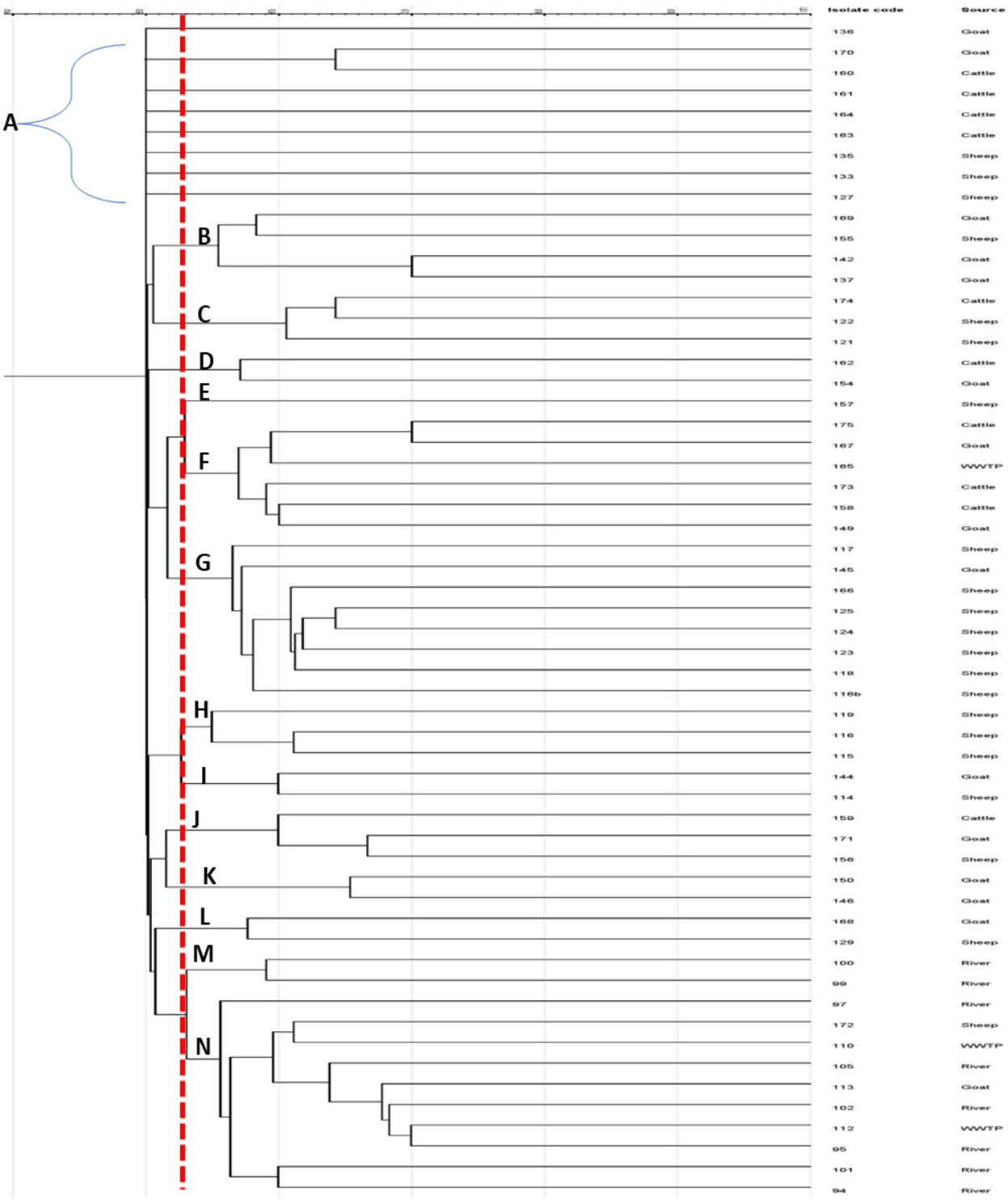


**Figure 6. 3. Cluster analysis using Gel J of ERIC-PCR fingerprints of *E. coli* isolates (n=57).** The dendrogram properties are based on the Dice similarity method using the unweighted pair group algorithm linkage at a tolerance limit of 0.0. Fourteen clusters labelled A-N were defined from groups of closely related strains with an average of 55% genotype similarity. The numbers in parenthesis show the number of isolates within the genotype cluster.

## 6.4 Conclusion

Our report is the first study in South Africa to look at the baseline integron features of *E. coli* in food animals (goats, sheep, and cattle) and water sources (rivers and wastewater treatment plants). Our findings revealed that these animals and water sources could contribute to the spread of antibiotic resistance through integrons in MDR-associated bacteria. As a result, relevant interventions targeted at combating AMR must seek innovative means to prevent the spread of these species harbouring mobile genetic determinants. Other variables implicated in transferring such resistance genes, particularly in livestock, need further investigation.

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**CHAPTER SEVEN**

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**Multidrug-resistant *Enterobacter cloacae* harbouring integrons recovered from diverse environmental matrices in South Africa (Submitted to *International Journal of Environmental Sciences & Technology*)**

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**Abstract**

This study investigated the prevalence and diversity of integrons and their associated gene cassettes in multidrug-resistant (MDR) *Enterobacter cloacae* recovered from rivers, effluents of hospitals and wastewater treatment plants, and animal droppings. The recovered isolates were identified using a conventional polymerase chain reaction targeting the heat shock protein, the *hsp60* gene. The antimicrobial phenotypic testing of the isolates using the disk diffusion technique revealed that all the isolates were resistant against more than two antimicrobial classes and were classified as MDR. The outcomes of the double-disk synergy testing and the ethylenediaminetetraacetic acid tests revealed that 20.5% (9/44) and 22.7% (10/44) of the isolates produced extended-spectrum and metallo-β-lactamases, respectively with co-production observed in one of the isolates. Integrons was detected in 97.7% (43/44) and were classified as follows: Class 1 integrons [88.4%, 38/43], class 2 integron [2.3%, 1/43], and class 1+2 integrons [9.3%, 4/43]. The *qacEΔ1* genes were present in 61.4% (27/44), and a *P2R2* promoter gene was detected in 25% (11/44) of the isolates. The sizes of the integrons' variable regions (IVRs) of the *intI1* positive integrons ranged from 160 bp to 800bp with sizes as follows: ≈160bp (n=8), ≈300bp (n=2), ≈350 (n=1), 600bp (n=1), ≈750 (n=1), and ≈800 (n=3), while the IVR for the isolate harbouring only the *intI2* gene was ≈1500 (n=1). The DNA sequencing of these amplicons revealed the presence of five different GC arrays. These include, *aadA1-dfrA1*, *dfrA7*, *dfrA21*, *dfrA5-aac-3-Ib*, and *dfrA1-sat2*. The study is the first report of an abundance of integrons in MDR *E. cloacae* from various environmental niches in the Eastern Cape Province, South Africa. The high detection rate of this mobile genetic element within the environment is quite worrisome as it can further increase the spread of antibiotic resistance with its attendant unpleasant public-health consequences.

**Keywords:** Integrons, multidrug-resistance, *Enterobacter cloacae*, gene cassettes, environmental matrices

## 7.1 Introduction

The species of Enterobacter are Gram-negative, facultative anaerobes, with their taxonomy continuously updated. As commensals, they are found in the gastrointestinal tracts of mammals and humans. As saprophytes are found in soil and sewage and can act as pathogens in humans, plants, and animals (Akbari et al., 2016; Annavajhala et al., 2019; Mezzatesta et al., 2012). In intensive care units, *E. cloacae* have been identified as one of the ten most common species causing hospital-acquired wounds, pneumonia, urinary tract infections, and sepsis, with antibiotics therapy being the gold standard. However, Enterobacter infections have recently become more common due to the widespread use of broad-spectrum antibiotics. A notable feature among *Enterobacter* spp. is their high likelihood of developing resistance against β-lactam antibiotics during therapy (Choi et al., 2008; Santos et al., 2020). Although several gram-negative bacteria harbour the AmpC chromosomal genes, which allows them to hydrolyze penicillins and cephalosporins, they usually produce these AmpC β-lactamases in low quantities, unable to drive resistance. However, a few gram-negative bacteria, such as *Enterobacter* spp., can produce AmpC β-lactamases in high quantities, driving resistance against these essential antibiotics (Annavajhala et al., 2019; Chow et al., 1994; Kim et al., 2009).



Extended-spectrum β-lactamases (ESBLs) encoding resistance against most β-lactam drugs, including resistance against cephalosporins, were initially reported in *E. cloacae* in 1989 (De Champs et al., 1989), and their occurrence has significantly increased, especially within the clinical context and among patients who were previously on antibiotic therapy (Annavajhala et al., 2019; Kim et al., 2009; Peirano et al., 2018). Apart from β-lactamases, *E. cloacae* also harbour resistance against other antibiotic classes. An example is aminoglycoside resistance

due to plasmids or other mobile genetic elements (MGEs) harbouring aminoglycoside 6′-N-acetyltransferase type I, *aac(6')-I*. In addition, the plasmid-borne *qep* or *qnr* genes conferring resistance against quinolones have been reported (Gomez-Simmonds et al., 2016; Neonakis et al., 2003; Park et al., 2007).

Resistance markers are harboured on MGEs such as bacteriophages, plasmids, and integrons. Integrons are bacterial genetic elements that can acquire, rearrange, and express genes harboured in gene cassettes (GCs). The integron structure consists of three principal elements. These are the integron-integrase gene (*intI*), the recombination point, *attI*, and a constitutive promoter, $P_c$ (Ghaly et al., 2021; Gillings, 2014; Okoh & Fadare, 2022). These essential properties ensure that newly generated variations can express genes likely to confer phenotypic advantages due to the presence of the promoter. The level of expression of the GCs is determined by their proximity to the promoter, Pc, with the GC closest to the promoter having the highest level of expression (Hall, 2012; Hall & Collis, 1995; Kaushik et al., 2018). Therefore, integrons are genetic elements with the capacity to integrate and express a variety of rearrangeable GCs, including diverse antimicrobial resistance genes (ARGs).



Integrons have been classified into various classes based on the amino acid sequences of the integrase (Cambray et al., 2010; Deng et al., 2015). Class 1 and 2 integrons have been the most reported among the Enterobacteriaceae family, while class 3 integrons seem far scarce and infrequently implicated in antibiotic resistance. Members within the same class have the same integrase but can be further differentiated based on their GCs (Kaushik et al., 2018). A class 3 integron recovered from hospital effluent has been reported in *E. cloacae* (Barraud et al., 2013) and is one of the nine fully characterized class 3 integrons; however, their prevalence remains generally low (Barraud et al., 2013; Simo Tchuinte et al., 2016).

Integrons have been found in various environmental sources, including estuarine, stream water sediments and biofilms, creek, and lake sediments (Hardwick et al., 2008; Koczura et al., 2014; Roe et al., 2003), using metagenomic and culture-dependent approaches. In some of these reports, the integrons harboured one to three GCs with many unknown functions. Water has received much attention since it is the primary vector of environmental contaminants. However, the characterization of integrons in MDR *E. cloacae* from various environmental matrices has not been investigated in the Eastern Cape Province. Therefore, this study investigates the prevalence and characterization of integrons in MDR *E. cloacae* from rivers, animal droppings, and hospital and wastewater treatment plants effluents.

## 7.2 Materials and methods

### 7.2.1 Characterization of bacterial strains

#### 7.2.1.1 Bacterial strains



The sources of the isolates selected for this study include animal faecal droppings (FD), rivers, effluents from wastewater treatment plants (WWTPs) and hospital wastewater (HWW). These were recovered between October and November 2017 from Chris Hani and Amathole District Municipalities. The isolates recovered were preserved in 20% glycerol stock at -80 ℃ as part of the AEMREG culture collection. Ethical clearance was obtained with project number OKO011SFAD01 to access the archived bacterial cultures.

#### 7.2.1.2 Recovery and DNA extraction of study isolates

Glycerol stocks were resuscitated in Brain Heart Infusion (BHI) broth (Merck, South Africa) and incubated for 18 hours at 37 ℃. A loopful was streaked on nutrient agar (Oxoid, UK) and further purified twice. For subsequent analysis, single pure colonies were transferred to 2 ml BHI broth and incubated overnight at 37 ℃. The boiling method (Maugeri et al., 2004) was used to extract the genomic DNA (gDNA) with a few modifications, as previously reported

elsewhere (Fadare et al., 2020). The DNA-containing supernatant was then transferred to sterile microcentrifuge tubes (Eppendorf, Germany) and stored at -20 ℃ for future assays.

7.2.1.3 PCR confirmation of isolates

The molecular confirmation of the *Enterobacter cloacae* was done using primer sets that target the heat-shock protein (*hsp60* gene) (Akbari et al., 2016). The positive control *E. cloacae* ATCC BAA-2341$^{TM}$ (Microbiologics, Medimark, Europe) was used to validate the assays. Each PCR reaction (25 µl) contained the double strength PCR master mix (12.5 µl) (Thermo scientific (EU), Lithuania), forward and reverse primers (1 µl) (Inqaba Biotechnical Industries, South Africa). Furthermore, 5.5 µl of nuclease-free water and previously extracted gDNA (5 µl) were added. The DNA amplification was done in a BioRad thermal cycler (LASEC, South Africa). Amplicons (5 µl) were resolved in a 1.5% (w/v) agarose gel after staining with ethidium bromide (5 µl). The gel was run using Mupid-One (Eurogentec, Belgium) electrophoresis system at 100 volts for 60 min in 0.5X TBE buffer with a 100-base-pair molecular marker (Biolabs, New England). The gel image was captured with UV transillumination (ALLIANCE 4.7, UVtec, London, UK). The primers sequences and conditions for PCR assays are described in Table 7.1.

**Table 7. 1: List of primers and thermocycling conditions used in this study.**

| Primer | Primer Sequence (5′ →3′) | Amplicon size (bp) | Thermocycling conditions | Reference |
|---|---|---|---|---|
| *hsp60*-F<br>*hsp60*-R | GTAGAAGAAGGCGTGGTTGC<br>ATGCATTCGGTGGTGATCAT CAG | 341 | 94 ℃ for 5 mins; 30 [94 ℃, 30 s; 54 ℃, 30 s; 72 ℃, 1 min]; 72 ℃ for 5 min. | (Akbari et al., 2016) |
| *INTI1*-F<br>*INTI1*-R | CAG TGG ACA TAA GCC TGT TC<br>CCC GAG GCA TAG ACT GTA | 164 | 94 °C for 5 mins; 35[94 °C, 60 s; 55 °C, 60 s, 72 °C, 30 s] 72 °C, 10 min | (Koeleman et al., 2001) |
| *INTI2*-F<br>*INTI2*-R | TTATTGCTGGGATTAGGC<br>ACGGCTACCCTCTGTTATC | 232 | 94 °C for 5 mins; 32[94 °C, 60 s; 59 °C, 60 s; 72 °C, 2 mins]; 72 °C for10 mins. | (Goldstein et al., 2001) |
| *INTI3*-F<br>*INTI3*-R | AGTGGGTGGCGAATGAGTG<br>TGTTCTTGTATCGGCAGGTG | 600 | 94 °C for 5 mins; 32[94 °C, 60 s; 48 °C, 60 s; 72 °C, 2 mins]; 72 °C for 10 mins. | (Goldstein et al., 2001) |
| INTF<br>P2R2 | AGTGGGTGGCGAATGAGTG<br>TGTTCTTGTATCGGCAGGTG | 540 | 94 °C for 5 mins; 35[94 °C, 60 s; 55 °C, 60 s; 72 °C, 30 s]; 72 °C for 10 mins. | (Wei et al., 2011) |
| *qacEΔ1*-F<br>qacEΔ1-R | ATC GCA ATA GTT GGC GAA GT<br>CAA GCT TTT GCC CAT GAA GC | 225 | 94 ℃ for 9mins; 30[94 ℃, 30 s; 55 ℃, 30s; 72 ℃, 60s]; 72 ℃ for 10 mins. | (Stokes & Hall, 1989) |
| *Sul1*-F<br>*Sul1*-R | ATGGTGACGGTGTTCGGCAT CTGA<br>CTAGGCATGATCTAACCCTC GGTCT | 840 | 94 ℃ for 9mins; 30 [94 ℃, 30 s; 55 ℃, 30 s; 72 ℃, 1 min]; 72 for 10 mins. | (Grape et al., 2005) |
| 5′CS<br>3′CS | GGCATCCAAGCAGCAAG<br>AAGCAGACTTGACCTGA | Variable | 94 °C, 5 min; 35[94 °C, 1 min; 45 °C, 1 min; 72 °C, 2 min]; 72 °C for 10 mins. | (Lévesque et al., 1995) |
| hep74<br>hep51 | CGGGATCCCGGACGGCATGC ACGATTTGTA<br>GATGCCATCGCAAGTACGAG | Variable | 94 °C, 5 min; 33[94 °C, 1 min; 59 °C, 45 s; 72 °C, 5 min]; 72 °C, 5 min | (White et al., 2001) |



7.2.2 Phenotypic classification of resistance

7.2.2.1 Antibiotic susceptibility testing

The susceptibility against a panel of thirteen antibiotics representing nine classes was carried out using the diffusion technique (DDT) described in our previous report (Fadare & Okoh,

2021a). The antibiotics include imipenem-10 µg, ciprofloxacin-5 µg, ceftazidime-10 µg, ampicillin-10 µg, cefotaxime-30 µg, trimethoprim-sulfamethoxazole-25 µg, colistin-10 µg, nalidixic acid-30 µg, chloramphenicol-30 µg, cefoxitin-30 µg, gentamicin-10 µg, tetracycline-30 µg, and amoxicillin/clavulanic acid-20 µg/10 µg,. The antibiotics were purchased from Mast Diagnostics, South Africa. The results of the diameter of the inhibition zones were read according to the Clinical and Laboratory Standard Institutes (CLSI) guidelines. Isolates were categorized as resistant, intermediate, or susceptible, while those that displayed resistance against more than two antimicrobial classes were considered MDR (CLSI, 2020).

7.2.2.2 Extended-spectrum β-lactamase (ESBL) production

All the isolates were subjected to the double-disk synergy test (DDST) for the phenotypic production of ESBL following guidelines established by CLSI. The antibiotics used include cefoxitin-30 µg, ceftazidime-10 µg, and cefotaxime-30 µg, in combination with amoxicillin/clavulanic acid -20 µg/10 µg.



7.2.2.3 Metallo β-lactamase (MBL) production

The ethylenediaminetetraacetic acid (EDTA) test was used to determine the production of MBL according to CLSI standards. Briefly, the 0.5 McFarland adjusted test isolate evenly spread on a Muller-Hinton agar (Oxoid, UK) plate was exposed to 10 µg imipenem discs A and B. Then, 10 µl of 0.5 M EDTA was added to disc A to obtain a concentration of 750 µg. An increase in inhibitory zone width of 5mm in disc A compared to disc B after 18 hours of incubation at 37 °C was considered positive for MBL generation (CLSI, 2020).

7.2.3 Characterization of integron genetic determinants

The confirmed bacterial strains were screened following PCR protocols described above, with primers targeting specific genes listed in Table 7.1. The presence of *intI1*, *intI2*, and

*intI3* genes located on the 5′-CS was assayed to classify the integrons. In addition, a *P2R2* gene on the 5′-CS of a typical integron was also screened to detect the typical integron's promoter. Furthermore, the *intI1*-positive strains were assessed for genes (*qacEΔ1* and *sulI*) on the ORFs at the 3′-CS. The positive control for a typical class 1 integron used was *Acinetobacter baumannii* ATCC 19606.

7.2.4 Mapping of integrons variable regions (IVRs)

Isolates harbouring the *intI1* and *intI2* genes were assessed in separate PCR assays to detect the IVRs harbouring the GCs. Specific primer sets 5′-CS and 3′-CS, which join the *attI1* site of the 5′-CS and the 3′-CS of *intI1*, were used for *intI1* positive isolates. In addition, the primer set hep74 and hep51, which binds to the *attI2* and the downstream *orfX sites*, were used to map the *intI2* positive isolates. The PCR reactions were carried out in triplicates to ensure reproducibility.



7.2.5 Restriction analysis and DNA sequencing of amplicons

Amplicons of variable regions that appeared similar in size were exposed to the *AluI* restriction enzyme (Biolabs, England) for the restriction fragment length polymorphism (RFLP) to assess if the products were in the same sequence. For the identification of similar GC arrays in the integrons, *AluI* was chosen due to its recognition sequence being only four bases, thereby increasing the likelihood of its activity over the other enzymes that target the six-base sequence. Briefly, 30 µl of the amplicon was exposed to 1.0 µl of 10U/ml *AluI*. The reaction mixture was then incubated for 4 hours at 37 ℃. The products were then run on a 2% agarose gel and visualized. These were characterized according to their distinct restriction profiles, and two randomly selected representative amplicons from each different RFLP class were selected for sequencing. This step was taken to help reduce the risk of sequencing multiple identical variable regions needlessly.

7.2.6 Variable sequence analysis, cassette identification

The content and arrangement of the inserted GCs within the amplified IVRs were analyzed through sequencing. The amplicons were sequenced in both directions on an ABI 3500XL sequencer using the Nimagen, BrilliantDye™ Terminator Cycle sequencing kit V3.1 (Inqaba Biotechnical Industries, South Africa). The sequences were then modified with Chromas 2.7 and pairwise aligned using BioEdit sequence alignment editor software. BLAST nucleotide search analysis (https://blast.ncbi.nlm.nih.gov/Blast) was performed on the generated consensus sequences to identify the contents of the inserted gene cassette. The position of each gene in each cassette was determined using ABRicate 0.8.4 (https://github.com/tseemann/abricate) (ResFinder, ARG-ANNOT, CARD, and NCBI databases).

7.2.7 Statistical analysis

The descriptive statistical software in Microsoft Excel 2016 and the Statistical Package for the Social Sciences (SPSS) version 25 were used to examine the data (SPSS Inc., Chicago, IL). The data was validated, and relationships were calculated with 2 X 2 cross-tabulation tables utilizing exploratory data analysis. The statistical significance of susceptibility and the number of integron-positive isolates was investigated using Pearson's Chi-square test. Significant was defined as a P value of less than 0.05.



**7.3 Results and discussion**

7.3.1 Identification of recovered isolates

All the assayed isolates (n= 44) were positive for the *hsp60* gene for the molecular confirmation of *E. cloacae*. The breakdown of the confirmed isolates and their sources include rivers (n=14), HWW (n=6), WWTP (n=12), and FD (n=12).

7.3.2. Phenotypic Characterization of resistance

7.3.2.1 Antibiotics susceptibility

The antibiotic fingerprints of *E. cloacae* revealed that all the isolates exhibited resistance against multiple antibiotics in different classes and were considered multidrug-resistant. The occurrence of MDR *E. cloacae* from environmental niches is a concern as it further limits therapeutic options. As shown in Figure 7.1, all the *E. cloacae* exhibited resistance against colistin. It was followed keenly by their resistance against cefotaxime and nalidixic acid (98%). The percentage frequencies of resistance observed against the remaining antibiotics assayed are as follows: ceftazidime (91%), ampicillin (84%), cefoxitin and tetracycline (82%), amoxicillin/clavulanic acid (73%), trimethoprim-sulfamethoxazole and chloramphenicol (34%), ciprofloxacin (32%), imipenem (23%), and gentamicin (20%). Here intermediate resistance observed was also classified as resistance. The *Enterobacter* spp. naturally have the chromosomal AmpC gene, which enables them to exhibit resistance against most antibiotics (Annavajhala et al., 2019; Park et al., 2007). Due to the high resistance observed in a majority of the antibiotics assayed in this study, antibiotics such as ciprofloxacin, imipenem, and gentamicin with lower resistance frequency imply that they might still be potent for treating infections caused by MDR *E. cloacae* in South Africa with precautionary guidance to avert an emergence of resistance. Although imipenem is one of the drugs of last resort, it is rarely used as a frontline antibiotic, so the observed low resistance against it, as observed in this study, was not unexpected.

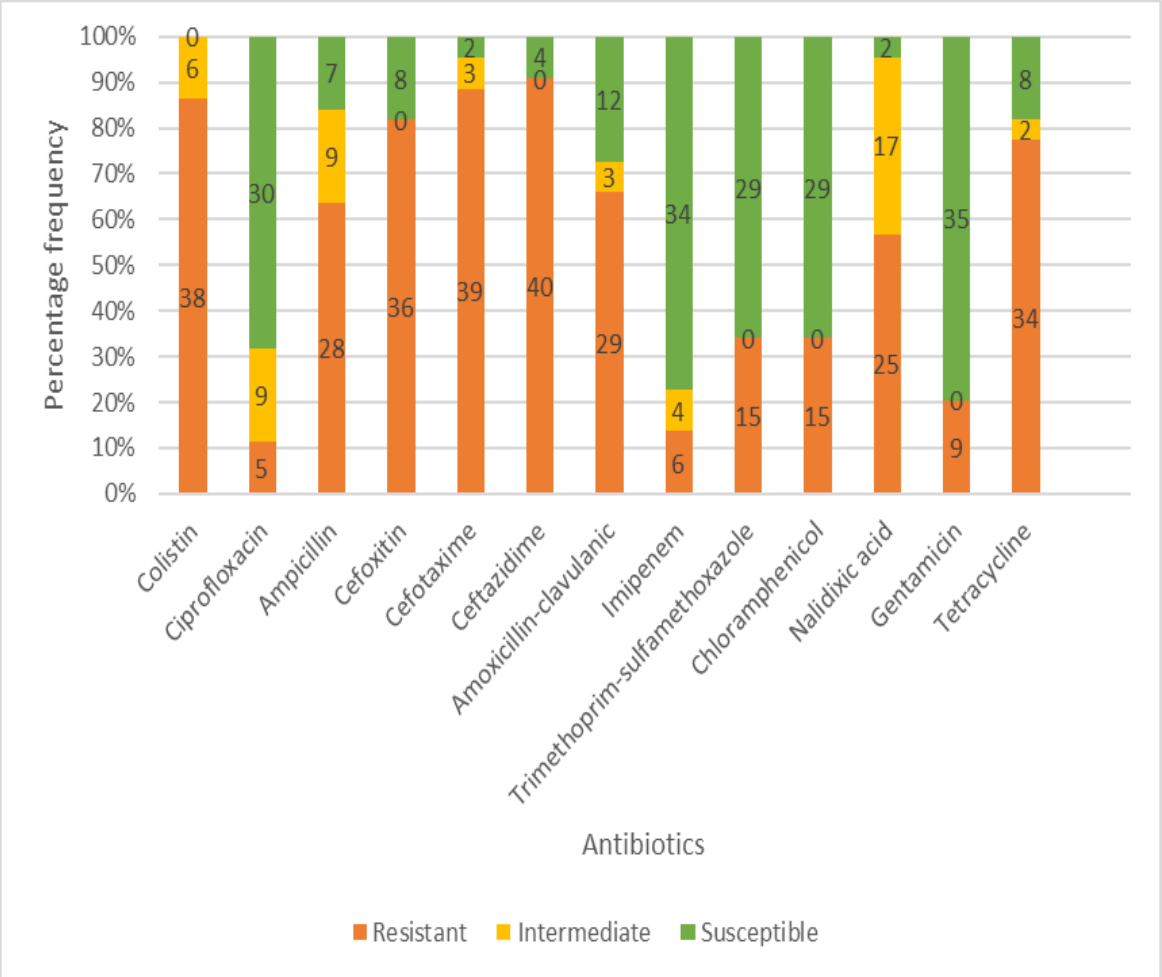




**Figure 7. 1: The antibiotic susceptibility profile of *E. cloacae* (n=44).**

7.3.2.2. Prevalence of β-lactamase production

Antibiotics called β-lactams are among the most effective treatments for infections caused by Enterobacteriaceae (Li et al., 2019). However, the production of β-lactamases enables microbes to hydrolyze these antibiotics. Penicillins, monobactams, penicillin inhibitors, and third and fourth-generation cephalosporins can all be hydrolyzed by ESBLs (Annavajhala et al., 2019; Bush & Jacoby, 2010; Cantón et al., 2012; Fadare & Okoh, 2021b). In this study, Figure 7.2 showed that 20.5% (9/44) of the *E. cloacae* produced ESBL using the DDST protocol. Carbapenemases are another type of essential β-lactamase that can hydrolyze carbapenems and all other β-lactam drugs (Caltagirone et al., 2017; Queenan & Bush, 2007). Veronica integron metallo β-lactamase (VIM) forms are among the most frequent

carbapenemases. The outcome of the EDTA phenotypic test in this study revealed that 22.7% (10/44) of the isolates produced MBL. The detection of MBLs is critical because they can hydrolyze practically any class of antibiotics, including carbapenems, fluoroquinolones, and aminoglycosides, and they may quickly disseminate since they are plasmid-mediated (Kaore et al., 2012; Walsh et al., 2005). In this study, most isolates displayed resistance against all the cephalosporins, as seen in Figure 7.2. A similar study reported cephalosporin-resistant strains harbouring various β-lactamases (Aarestrup et al., 2010).

The presence of these β-lactamases is also associated with co-resistance against other classes of antibiotics by bacterial species harbouring them, further limiting treatment options. These β-lactamases are usually carried on MGEs like integrons, which aid in the horizontal spread of ARGs among diverse bacterial species (Cantón et al., 2012; Martin & Bachman, 2018; Okoh & Fadare, 2022; Rawat & Nair, 2010).

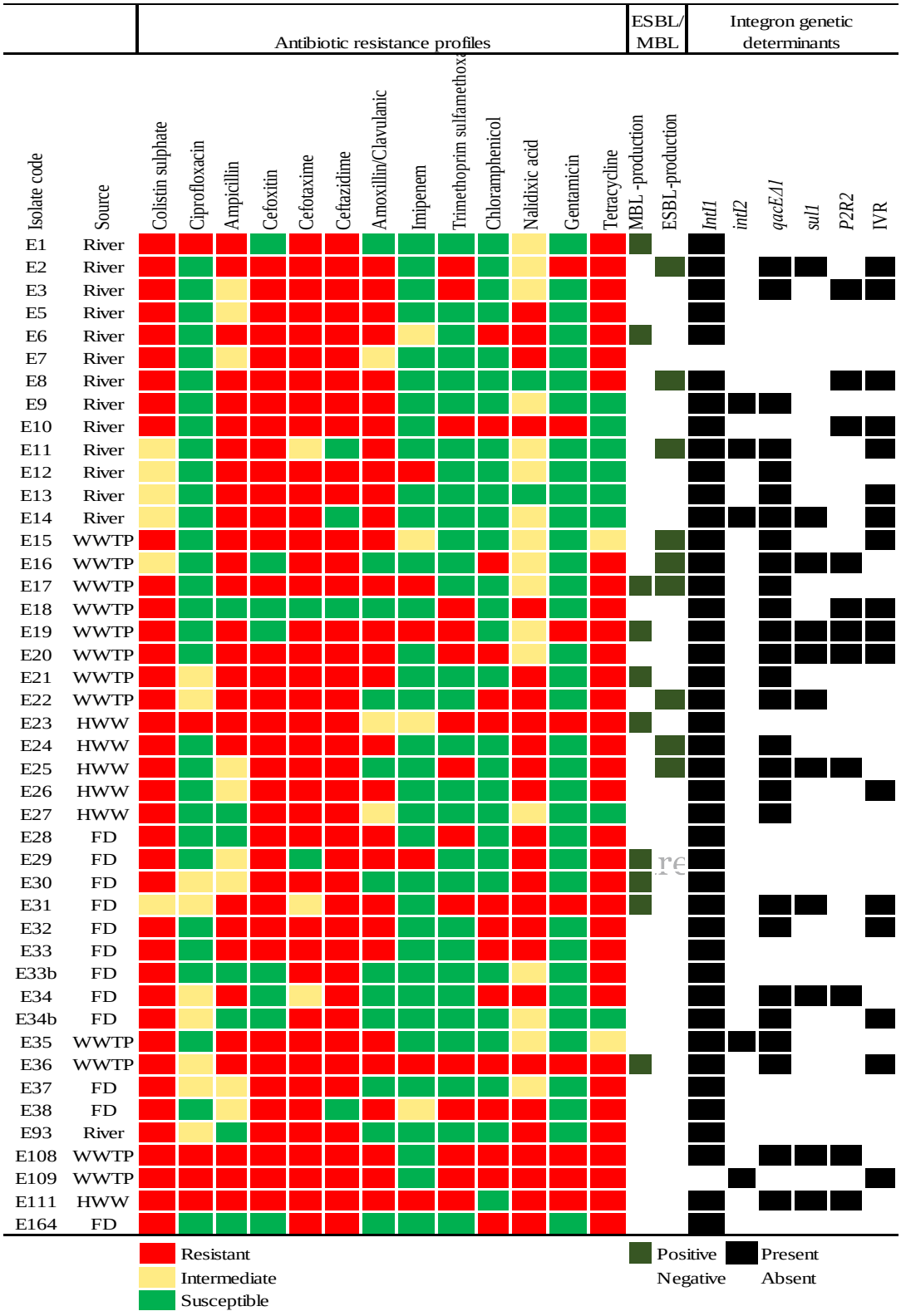


**Figure 7. 2:The phenotypic antibiotic susceptibility profiles, the production of ESBL and MBL, and the detection of integron genetic determinants of isolates from environmental matrices.** Each isolate's susceptibility profiles against certain antibiotics are colour coded to indicate whether it is resistant, intermediate-resistant, or susceptible. The production of ESBL and MBL is shown as negative or positive, while the integron genetic elements are shown as absent or present. WWTP: Wastewater treatment plants effluent, HWW: Hospital wastewater, FD: Animal faecal droppings.

7.3.3. Characterization of integrons

7.3.3.1 Detection of integrase, *qacEΔ1* and *sul1* genes

All but one of the isolates investigated, 97.7% (43/44), harboured an integron-integrase, *intI* gene. The *intI1* gene was found in all the integron-positive isolates except for one isolate, 97.7% (42/43). The *intI2* gene was detected in 11.6% (5/43) and mostly occurred with the presence of *intI1*. Therefore, the isolates were classified as follows: Class 1 integrons [88.4%, 38/43], class 2 integron [2.3% ,1/43], and class 1+2 integrons [9.3%, 4/43]. The *E. cloacae* strain, E109, harboured the only *intI2* gene (Figure 7.2).

The findings of our study are similar to the high rate of detection of the *intI1* gene in other previous studies with an abundance of class 1 integron (Cambray et al., 2010; Chaturvedi et al., 2021), while the *intI2* genes have been lower (Chaturvedi et al., 2021; Koczura et al., 2012; Kotlarska et al., 2015; Mokracka et al., 2012). The *intI1* genes have the highest detection frequency among the other integrase genes due to their capacity to recombine with different *attC* sites (Deng et al., 2015; Kaushik et al., 2018). In addition, the detection of isolates co-harbouring *intI1* and *intI2* genes has also been described in other members of Enterobacteriaceae. For instance, class 1+2 integrons were detected in rivers (Chaturvedi et al., 2021; Kaushik et al., 2019) and waterfowl birds (Zhang et al., 2019). Although we did not detect the presence of any *intI3* gene among the *E. cloacae* investigated in this study, one *intI3* was detected in *E. cloacae* from a hospital effluent in France with a GC array $bla_{\text{OXA-256}}$-*aac(6)-Ib* (Barraud et al., 2013). The *intI3* genes are the least prevalent in colonizing new species even though they have a 60% sequence identity with *intI1* (Collis et al., 2002; Okoh & Fadare, 2022; Simo Tchuinte et al., 2016). They have been reported in only a few studies (Barraud et al., 2013; Simo Tchuinte et al., 2016), occurring in meagre numbers. Fewer than ten class 3 integrons have been described. These are In3-1 and In3-2 recovered from clinical Enterobacteriaceae (Arakawa et al., 1995; Correia et al., 2003; Poirel et al., 2010), In3-3 and

In3-4 from environmental Delfia (Xu et al., 2007), In3-5 from hospital effluent *E. cloacae* (Barraud et al., 2013), In3-6 from hospital effluent *Acinetobacter johnsonii*, In3-7 from sewage sludge *Aeromonas allosaccharophila*, and In3-9 from hospital effluent *Citrobacter freundii* (Simo Tchuinte et al., 2016).

In this study, 61.4% (27/44) of the isolates harboured at least one of the *qacEΔ1* and *sul1* genes. About 26.3% (10/38) of the *intI1* positive isolates also harboured *qacEΔ1* and *sul1* genes and were considered typical class 1 integrons. The presence of the *qacEΔ1* and *sul1* genes on the 3′ CS identifies the classification of typical class 1 integrons. However, these genes were also found in one of the isolates harbouring both *intI1* and *intI2*. The outcome of this study supports the findings of a previous study by Zhang and colleagues (Zhang et al., 2019), where some typical class 1 integrons were able to harbour the *intI2* gene. The *qacEΔ1* is a truncated *qacE* gene conferring resistance against quaternary ammonium compounds and precedes the insertion of a *sul1* gene at the 3′ end, which encodes resistance against the antibiotic sulphonamide (Gillings, 2014). The *qacEΔ1* gene was detected in 38.1% (16/42) of the remaining *intI1* positive isolates and lacked the *sul1* gene. The high prevalence of integrons in these isolates recovered from various environmental matrices indicates the widespread integrons within the environment. As shown in Table 7.2, detecting the integrase gene in each isolate was associated with resistance against two antibiotics assayed at significant levels. These were ceftazidime and tetracycline ($P<0.05$).

**Table 7. 2: The association between resistance against antibiotics and the presence of integrons in multidrug-resistant *Enterobacter cloacae.***

| Antibiotic | No. of resistant isolates | | No. of susceptible isolates | | |
|---|---|---|---|---|---|
| | Total (%) | Integron positive (% of resistant isolates) | Total (%) | Integron-positive (% of susceptible isolates) | *P*-value ($X^2$ test) |
| Colistin | 44 (100) | 43 (100) | 0 (0) | 0 (0) | [a] |
| Ciprofloxacin | 14 (31.8) | 14 (32.6) | 30 (68.2) | 29(67.4) | 0.547 |
| Ampicillin | 37 (84.1) | 36 (83.7) | 7 (15.9) | 7(16.3) | 0.302 |
| Cefoxitin | 36 (81.8) | 35 (81.4) | 8 (18.2) | 8 (18.6) | 0.263 |
| Cefotaxime | 42 (95.5) | 41 (95.3) | 2 (4.5) | 2 (4.7) | 0.604 |
| Ceftazidime | 40 (90.9) | 39 (90.7) | 4 (9.1) | 4 (9.3) | **0.011*** |
| Amoxillin/ Clavulanic acid | 32 (72.7) | 31 (72.1) | 12 (27.3) | 12 (27.9) | 0.146 |
| Imipenem | 10 (22.7) | 10 (23.3) | 34 (77.3) | 33 (76.7) | 0.198 |
| Trimethoprim sulfamethoxazole | 15 (34.1) | 15 (34.9) | 29 (65.9) | 28 (65.1) | 0.480 |
| Chloramphenicol | 15 (34.1) | 15 (34.9) | 29 (65.9) | 28 (65.1) | 0.480 |
| Nalidixic acid | 42 (95.5) | 41 (95.3) | 2 (4.5) | 2 (4.7) | 0.604 |
| Gentamicin | 9 (20.5) | 9 (20.9) | 35 (79.5) | 34 (79.1) | 0.979 |
| Tetracycline | 36 (81.8) | 35 (81.4) | 8 (18.2) | 8(18.6) | **0.010*** |

*Significant values are represented in bold.
[a]No statistics are computed because colistin exhibited a constant resistance attribute



7.3.3.2 Characterization of variable regions and promoters

The occurrence of the IVRs that harbour GCs located between the 5′ and 3′ CS of the integron-positive isolates was 39.5% (17/43). The IVRs were detected in all *intI1* positive isolates, which were also positive for the *qacEΔ1* gene except for two isolates (Isolate No. E8 and 10) (Figure 7.2 and Table 7.3), which lacked the *qacEΔ1* and *sul1* genes. The lack of these 3′ CS genes could result from a mutation in these segments and could be attributed to the failure in their amplification (Koczura et al., 2014; Laroche et al., 2009).

The amplicon sizes of the IVRs of the *intI1* positive integrons ranged from 160 bp to 800bp with sizes as follows: ≈160bp (n=8), ≈300bp (n=2), ≈350 (n=1), 600bp (n=1), ≈750 (n=1),

and ≈800 (n=3), while the IVR for the isolate harbouring only the *intI2* gene was ≈1500 (n=1). The DNA sequencing of these amplicons revealed the presence of five different GC arrays. These include, *aadA1-dfrA1*, *dfrA7*, *dfrA21*, *dfrA5-aac-3-Ib*, and *dfrA1-sat2*. The most frequently detected GC was the *dfrA* variants (*dfrA1*, *dfrA5*, *dfrA7*, and *dfrA21*), which encode the dihydrofolate reductase conferring resistance against trimethoprim. The GCs encoding resistance against aminoglycosides includes the *aadA1* and the *aac-3-Ib*. The *aadA1* gene belongs to aminoglycoside adenyltransferases, while the *aac-3-Ib* gene belongs to the aminoglycoside acetyltransferases. A streptothricin (*sat2*) resistance gene was found in the *intI2* positive isolate, and it occurred in combination with a *dfrA1* gene. The sequences of the remaining amplicons yielded undetermined/empty GCs (Table 7.3). It could be that the region sequences have not been previously reported; therefore, further studies must be done to determine their contents and functions. In this report, we, therefore, classified them as undetermined. Figure 7.3 shows a schematic representation of some integron-harbouring isolates and their associated GC arrays.



Most GCs are promoter-less and rely on an external promoter located on the 5′ CS on the integron to express the genes they harbour (Gillings, 2014). In this study, 25.6% (11/43) of the assayed isolates were positive for the *P2R2* gene investigated for a typical promoter gene. We could not amplify promoters on the remaining integron-positive isolates targeting the *P2R2* gene, which could result from mutation or the integrons harbour a different promoter type. This study's detection of promoter genes suggests that resistance genes in the GCS can be immediately expressed, therefore conferring phenotypic advantages on the isolates harbouring them. Although this research did not investigate the expression of the GCs, some other studies have reported that the maximum expression level usually occurs in the GC closest to the promoter (Hall, 2012; Kaushik et al., 2018).

**Table 7. 3: Characteristics of integrons in *E. cloacae* harbouring IVRs (n=17).**

| Integron type | No. of Isolates | Cassette array | Approximate size (bp) | *qacEΔE+sul1* |
|---|---|---|---|---|
| Class 1 | 8 | _[a] | 160 | +[b] |
| | 1 | *aadA1-dfrA1* | 750 | +[c] |
| | 2 | *dfrA7* | 300 | +[d] |
| | 2 | *dfrA21* | 800 | +[e] |
| | 1 | _[a] | 350 | +[c] |
| Class 1+2 | 1 | _[a] | 600 | +[c] |
| | 1 | *dfrA5-aac-3-Ib* | 800 | + |
| Class 2 | 1 | *dfrA1-sat2* | 1500 | NA |

[a]The amplicon yielded an undetermined/empty cassette array.
[b]Except for five isolates that harboured only the *qacEΔ1* gene.
[c] The isolate harboured only *qacEΔ1*.
[d] The isolates lacked both genes.
[e]Except for one isolate that harboured only the *qacEΔ1* gene.
NA means Not applicable.

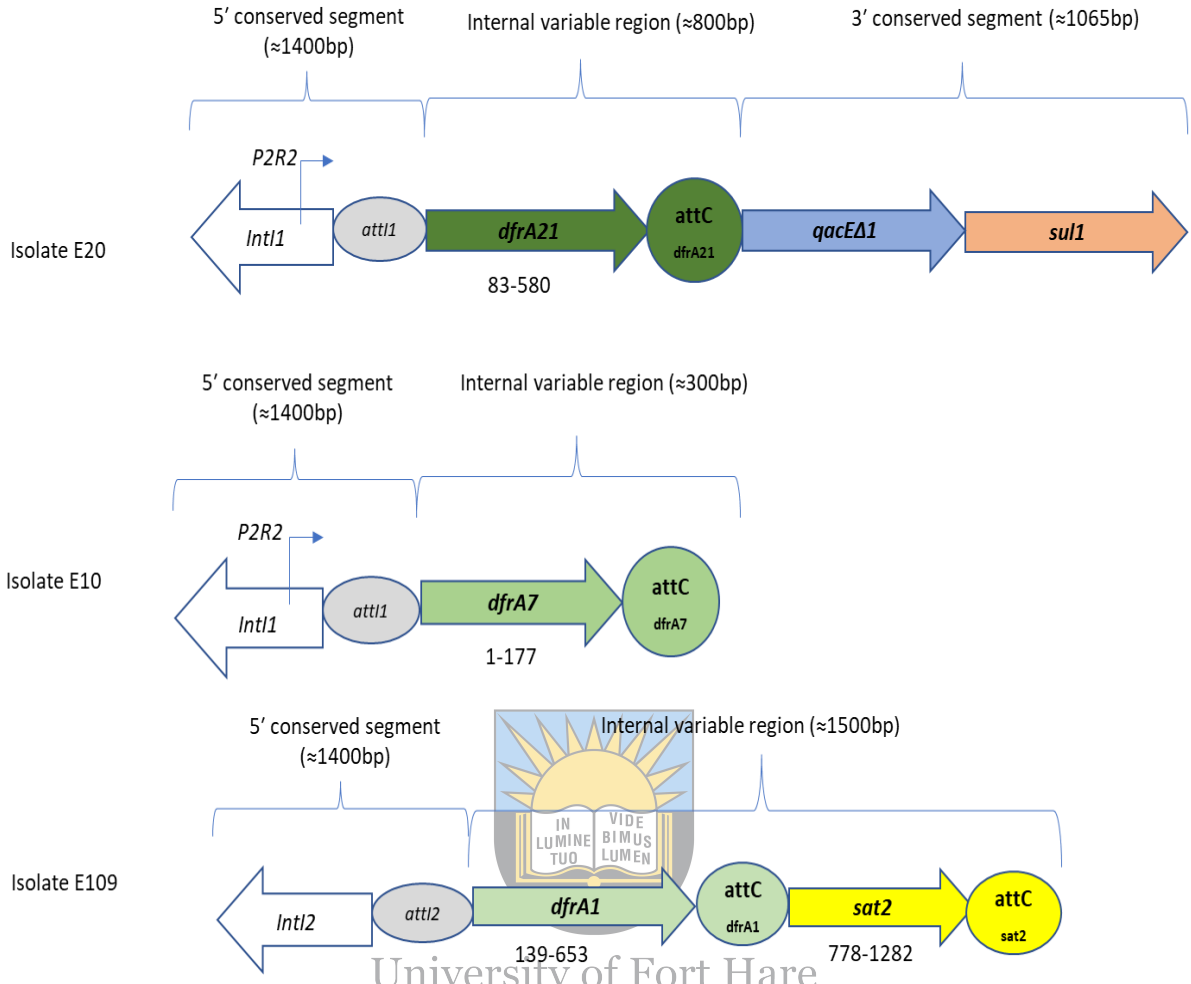




**Figure 7. 3: Schematic representation of class 1 and 2 integrons in *E. cloacae*.** The *intI1*, *intI2*, and *P2R2* genes are on the 5′ conserved segment. Typical class 1 integron harboured *qacEΔ1* and *sul1* genes on the 3′ conserved segment conferring resistance against quaternary ammonium compounds and sulphonamides. The internal variable regions harbour gene cassettes *dfrA* variants and *sat2* genes conferring resistance against trimethoprim and streptothricin, respectively. The start and stop nucleotide sequences were also depicted, while the arrows indicate the transcription direction.

## 7.4. Conclusion

Multidrug-resistant isolates producing β-lactamases in *E. cloacae* recovered from varied environmental niches are a tremendous public health concern. The high occurrence of integrons harbouring diverse gene cassettes with promoters in these isolates further heightens our concern over the dissemination of multidrug-resistant bacterial species.

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**CHAPTER EIGHT**

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***In vitro* assessment of the combination of antibiotics against some integron-harbouring Enterobacteriaceae from environmental sources (Published in *Antibiotics*)**

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**Abstract**

One strategy for combating antimicrobial resistance in many infections is to combine antibacterial compounds to create combinations that outperform each molecule alone. In this study, we examined and studied the inhibitory effect of combining two drugs belonging to different antibiotic classes to obtain a possible potentiating effect against some Enterobacteriaceae isolates harbouring integrons recovered from rivers and effluents of hospital and wastewater treatment plants in Eastern Cape Province, South Africa. These integrons could easily enable the isolates to acquire genes that confer additional resistance against conventional antibiotics. The minimum inhibitory concentration of the various antibiotics was determined using the broth microdilution, while the checkerboard method was used to determine the fractional inhibitory concentration indices (FICIs). A total of 26.3% (10/38) of the interactions were categorized as synergistic, while 73.7% (28/38) were indifferent. None of the combinations was antagonistic. The time-kill assays revealed all the synergistic interactions as bactericidal. Therefore, the combinations of gentamicin with tetracycline, ciprofloxacin, and ceftazidime against multidrug-resistant (MDR) *Klebsiella pneumoniae*; tetracycline-ceftazidime combination against MDR *Escherichia coli*, colistin combinations with ceftazidime and gentamicin, and tetracycline-gentamicin combinations against MDR *Citrobacter freundii* may be future therapeutic alternatives. Hence, the synergistic combinations reported in this study must be further assessed *in vivo* before their clinical applications.



**Keywords:** Enterobacteriaceae, *in vitro* activity, combination therapy, time-kill assay, checkerboard

## 8.1 Introduction

Antibiotic misuse in clinical and agricultural settings has exacerbated the dissemination of antibiotic-resistant bacteria (ARB) and their resistance determinants in clinical settings and the environment (Chaturvedi et al., 2021; Fletcher, 2015; Singh et al., 2021). Although much research has focused on how antibiotic resistance genes (ARGs) propagate in clinical settings, many papers have also explored how they spread in the environment (Chaturvedi et al., 2021; Fadare & Okoh, 2021a, 2021b; Liu et al., 2021; Teixeira et al., 2020). As a result, various microorganisms, particularly gram-negative bacteria, acquire resistance against various antibiotics from various antimicrobial classes used to treat the diseases they cause, thus leading to multidrug resistance, which poses a problem for treating future bacterial infections. These ARGs are passed down to offspring or, more typically, through horizontal gene transfer via mobile genetic elements such as plasmids, bacteriophages, integrons, and transposons. Integrons have a well-established and confirmed involvement in the spread of resistance. They are genetic elements that can capture gene cassettes which carry various ARGs and serve as expression systems for the genes they harbour (Gillings, 2014; Kaushik et al., 2019; Okoh & Fadare, 2022).



The emergence of multidrug resistance in Enterobacteriaceae is a critical public health issue that has attracted the attention of the World Health Organization (WHO). They have been classified as one of the critical priority pathogens urgently requiring new antibiotics (WHO, 2017). The resistance phenomenon has proven most of the current antibiotics ineffective, compounded by the slow pace of discovering new antibiotics, necessitating the hunt for new and practical remedies (Brennan-Krohn & Kirby, 2019; Spellberg, 2014). One of such is the exploration of synergy among existing antibiotics. Two medications combined have a higher impact, thereby allowing current antibiotics to be salvaged for use in treating multidrug-

resistant (MDR) bacteria, even if the bacteria are resistant against one or both antibiotics separately.

Recognizing that no antibiotic compound is universally effective for all illnesses, one of the primary motivations for combining antibiotics was the potential for greater efficacy than single antibiotics. Antibiotics are combined to achieve a variety of goals. The first is the capacity to broaden the antibacterial range during empirical therapy when the pathogen's identification is still unclear. The second goal is to achieve synergistic effects, improving therapeutic efficacy. Other goals include preventing the formation of resistance and reducing host toxicity (Moellering, 1983; Silver, 2007, 2016). The earliest drugs combined were streptomycin and penicillin in 1950 (Jawetz et al., 1952), while trimethoprim and sulphonamides were combined in 1968 (Bushby & Hitchings, 1968). These combinations enhanced the antibiotics' effectiveness and antibacterial spectrum. Colistin, which at present is considered a last-resort drug, functioned well when combined with protein synthesis inhibitors such as linezolid, fusidic acid, and clindamycin, which have minimal effect on gram-negative bacteria on their own (Brennan-Krohn et al., 2018). Now backed by rigorous mechanistic, clinical, and epidemiological data, such combinations remain in frontline use today (Laxminarayan et al., 2013; Silver, 2016; Tyers & Wright, 2019). Combinations should be applied against specific life-threatening infections as it has been reported that combinations of antibiotics can also facilitate the spread of resistance. Combining drugs that are not inhibitory but, when combined, results in an impact that exceeds the activity of individual drugs owing to complementary activities or various targets of action in microbial cells. Such combinations are effective ways of tackling pathogen-caused diseases. Therefore, our research aimed to assess the *in vitro* activities of various antibiotics from different classes in combination with different antibiotics against environmental strains of integrons-harbouring Enterobacteriaceae.

## 8.2 Materials and methods

### 8.2.1 Bacterial isolate characterizations

Enterobacteriaceae isolates were selected from our previous studies (Fadare et al., 2020; Fadare & Okoh, 2021a, 2021b), with the various sources indicating the diversity of the environmental isolates assessed (Table 8.1). These isolates were deposited at the Applied and Environmental Microbiology Research Group (AEMREG) culture collection. The bacterial strains were resuscitated in Brain Heart Infusion (BHI) broth (Merck, Johannesburg, South Africa) and incubated at 37 ℃ for 18 hours. A loopful was streaked on Violet Red Bile Glucose (VRBG) agar and incubated overnight at 37 ℃. Isolates were purified further by streaking twice on nutrient agar (Oxoid, Basingstoke, UK). Single pure colonies were transferred to 2 mL BHI broth, and genomic DNA was extracted using the boiling method previously described (Maugeri et al., 2004). The identities of the isolates were confirmed using conventional polymerase chain reaction (PCR). The integrase genes (*intI1* and *intI2*) were assayed to classify the integrons present in the confirmed isolates. The list of primers and thermocycling conditions for the PCR assays is presented in Appendix 2.



### 8.2.2 Preparation of antibiotics and media used

Standard laboratory powder assays included ceftazidime, gentamicin, tetracycline, ciprofloxacin, colistin sulphate, ampicillin, meropenem, and amikacin. These were purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions were prepared using the potency (µg per mg powder) of each antibiotic as supplied by the manufacturer, following the formula below (Brennan-Krohn & Kirby, 2019; Wiegand et al., 2008):

$$W = \frac{C \times V}{P}$$

Where 'W' is the weight of the antibiotics to be dissolved (mg), C is the desired concentration of the stock solution to be prepared (µg/mL), V is the desired volume (mL), and *P* is the potency of the antibiotic powder as supplied by the manufacturer (µg/mg). The diluent of all

antibiotics used was sterilized distilled water, while anhydrous sodium carbonate at 10% weight was added to ampicillin and ceftazidime stock solutions (CLSI, 2020). We used double-strength Muller Hinton II Broth (2X MHB) (Oxoid, Basingstoke, UK).

8.2.3 Standardization of inoculum

Following the guidelines recommended by the Clinical and Laboratory Standard Institute (CLSI), the inoculum was prepared by adjusting the turbidity of the test microorganisms in sterilized normal saline to 0.5 McFarland using the spectrophotometer (Merck), with a wavelength set at 600 nm. The absorbance of the test microorganisms ranged from an optical density of 0.08-0.1 to give an approximate 1 $\times 10^8$ CFU/mL inoculum size. Then 0.1 mL of the adjusted 0.5 McFarland standard inoculum was transferred to 9.9 mL 2X MHB to give an approximate 1 $\times 10^6$ CFU/mL inoculum size used within 30 mins to avoid change in cell number (CLSI, 2020).-

8.2.4 Antimicrobial susceptibility testing



The minimum inhibitory concentration (MIC) of the antibiotics was determined using the round-bottomed 96-well microtiter plates (Greiner Bio-one, Monroe, NC, USA) following the broth microdilution procedure described by Wiegand and colleagues (Wiegand et al., 2008). Briefly, 50 µL of sterile distilled water was aliquoted into wells 2 to 10, which served as the antibiotics' diluent. Subsequently, 100 µL of the highest concentration of the antibiotics to be investigated was dispensed into well 1. It was serially diluted by transferring 50 µL of the antibiotics from well 1 through well 10 and finally discarded after dilution in the last well allowing for the geometric serial dilution of the antibiotics across the rows. Each well containing the antibiotic solution was inoculated with 50 µL of the test organism earlier standardized. Wells 11 served as the growth control, containing only the inoculum, while wells 12 served as the sterility control (SC), containing only the assayed antibiotics.

The microtiter plates were covered and incubated at 35 ℃ for 16 -20 hours. The results were read after the addition of the 30 µL resazurin dye (w/v, 0.015%) (Glentham Life Sciences, Corsham, UK) or the 2,3,5, triphenyl tetrazolium chloride (Merck, Darmstadt, Germany), depending on the availability of the dyes, with a further 2-hour incubation period for the observation of a colour change. The well with the lowest concentration of the antibiotics that completely inhibited the growth of the bacteria, as indicated with no observable colour change, was read as the MIC value, which was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI breakpoints (CLSI, 2020; EUCAST, 2018). The tests were carried out in triplicates.

8.2.5 Quality Control

Quality control was done to validate the methods employed in this study. The performance of all the antibiotic stock solutions was validated against referenced organisms *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. The reference strains were purchased from the American Type Culture Collection (Manassas, VA, USA) to determine the MIC values. The results were then compared with EUCAST values (EUCAST, 2018).



Immediately after inoculation of the microtiter plates with the inoculum for the MIC studies, 10 µl of the bacterial inoculum were obtained from the growth control columns (columns without antibiotics) and aliquot into sterile 990 µL 2X MHB vortexed to ensure they were thoroughly mixed; from this, another 1:100 dilution was produced. Then, 100 µL from each dilution were aseptically spread onto sterile Muller Hinton agar (MHA) plates and incubated at 35 ℃. Colonies were counted after 18 hours, and values obtained around 50 colonies on the lower dilution indicate that bacterial inoculum was accurately standardized (Wiegand et al., 2008).

8.2.6 Checkerboard assay

Antibiotics from different classes whose breakpoints were non-susceptible were combined in a checkerboard style for this assay. First, each antibiotic was prepared by serially diluting in water to obtain the desired dilution folds starting from double the MIC values earlier obtained. A total of 50 µL of drug A was dispensed down each column starting from the highest concentration except for column 12. Similarly, drug B was dispensed along the rows except for row H. Then, 50 µL of each adjusted 0.5 McFarland standard was transferred into 15 mL 2X MHB and aliquoted to all the wells to obtain a final concentration of $5\times10^5$ CFU/mL with a final volume of 150 µL per well. The last well, H12, served as the growth control. Results were obtained after 24 hours of incubation at 35 ℃ as described earlier. The fractional inhibitory concentration (FIC) index of the combined drugs was calculated as follows:

$$\frac{MIC\ A(combination)}{MIC\ A(alone)} + \frac{MIC\ B(combination)}{MIC\ B(alone)} = FIC\ A + FIC\ B = FIC\ Index$$



Synergy was defined as an FIC index value less than 0.5, while antagonism was defined for values greater than 4, and values in between were interpreted as indifferent (Brennan-Krohn et al., 2018; Odds, 2003). The assays were duplicated, and synergy was determined when the FIC index yielded values less than 0.5. When a skipped well occurred, the higher FIC index was used to prevent false-positive synergy interpretations. The data were discarded if there were more than two skipped wells in a single grid or if the MIC was more than 2-fold dilution above or below the modal MIC for that isolate, followed by a repeat of the experiment. However, the antibiotic combination for the isolate was eliminated from further investigation if the same error persisted (Brennan-Krohn et al., 2018).

8.2.7 Time-kill assays

The time-kill assays (TKAs) were performed on all the synergistic combinations from the checkboard assays. For different isolates that demonstrated synergy to the same antibiotic combinations, only one was selected for the TKA. The rate of kill (ROK) was determined by enumerating the viable cell counts at specific intervals over 24 hours. The MICs of each antibiotic alone and the combined antibiotics at 20 mL each were investigated in a 100 mL conical flask. Then 200 µL of the adjusted 0.5 McFarland inoculum was added to 20 mL of 2X MHB to give a final concentration of 5 $\times 10^5$ CFU/mL when added to the antibiotics to be assayed. The cultures were incubated at 35 ℃ with shaking at 120 rpm. Aliquots were removed from the cultures at 0, 1, 2, 4, 6, 8, 12, and 24 hours and a 10-fold dilution series were performed in sterile 2X MHB. A 100 µL of each appropriate dilution was spread on MHA plates in triplicates. The plates were incubated at 35 ℃, and colony counts were recorded after 24 hours. A growth control was run in parallel with each experiment. The time-kill curves were determined by plotting mean colony counts (log CFU/mL) against the incubation time (hours). The combination's efficacy was synergistic when viable bacteria were reduced by ≥2 log10 CFU/mL compared to the most active single antibiotic. The combination therapy's efficiency was also evaluated as bactericidal when there was a ≥3 log10 CFU/mL reduction compared to the initial inoculum at 24 hours.



8.2.7 Data analysis

Data was entered on Microsoft Excel 2016, and Statistical analysis was performed using descriptive analysis.

**8.3 Results**

The MIC result for each antibiotic assayed against each isolate is presented in Table 8.1. Sixty-nine percent (18/26) of the isolates investigated were considered MDR as they exhibited resistance against antibiotics in over two different classes. The highest resistance

was observed against ampicillin with a resistance frequency of 73% (19/26), followed by resistance against ceftazidime with 65% (17/26). Others included tetracycline and colistin (58%, 15/26), ciprofloxacin (54%, 14/26), gentamicin (42%, 11/26), and amikacin (15%, 4/26). None of the isolates exhibited resistance against meropenem, considered one of the drugs of last resort, with low MIC values.

All the MDR *Citrobacter* spp. were exposed to various antibiotics for the checkerboard assays, with the combination outcomes shown in Table 8.2. The outcome of the interactions of the checkboard assays showed that 85% (17/20) of the combinations were indifferent, while 15% (3/20) were synergistic. The synergistic combinations were observed in the combinations of colistin with ceftazidime and gentamicin and between gentamicin and tetracycline. Table 8.3 shows the outcomes of various antibiotic combinations with interpretable results for *E. coli*, *K. pneumoniae*, and *K. oxytoca*. The most synergistic relationship was observed when gentamicin was combined with tetracycline. The combination of gentamicin with ceftazidime and ciprofloxacin showed a synergistic effect in a *K. pneumoniae* isolate. An *E. coli* isolate also showed synergism with tetracycline and ceftazidime. The FIC index ranged from 0.19 to 1.0. About 9% (7/18) of these exhibited synergistic interactions, while 61% (11/18) were indifferent. No antagonistic reaction was observed in any of the combinations in this study.



The efficacy of these synergistic combinations was further demonstrated in the time-kill curves in Figures 8.1 and 8.2. These combinations were all bactericidal starting as early as 2 hours and maintained throughout the 24-hours assay, while synergistic effects were only seen in Figure 8.1 time-kill curves. For Figure 8.2, the time-kill curves show the single active agents being bactericidal, although later compared with the combined drugs.

**Table 8. 1: Bacterial species characterization including Minimum Inhibitory Concentration (MIC).**

| Isolate Identifier | Species | Source[b] | Integrase gene | MIC[a] (µg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | GEN | MEM | CIP | TET | CAZ | AMP | COS | AMK |
| C1 | *C. braakii* | WWTP | *intI1+intI2* | 1 | 0.03 | 1 | 1024 | 64 | >4096 | 4 | 1 |
| C2 | *C. freundii* | River | *intI1* | 2 | 0.015 | 0.25 | 1 | 2 | 16 | 4 | 4 |
| C3 | *C. freundii* | HWW | *intI1* | 64 | 0.03 | 64 | 128 | 128 | >4096 | 4 | 4 |
| C4 | *C. freundii* | HWW | *intI1* | 128 | 0.015 | 128 | 1024 | 128 | >4096 | 2 | 16 |
| EC1 | *E. cloacae* | River | *intI1* | 1 | 0.015 | 0.06 | 4 | 0.05 | 4 | 0.125 | 2 |
| EC2 | *E. cloacae* | WWTP | *intI1* | 1 | 0.015 | 0.06 | 2 | 0.05 | 4 | 0.25 | 2 |
| EC3 | *E. cloacae* | WWTP | *intI1+intI2* | 2 | 0.25 | 2 | 64 | 8 | ≤8 | 4 | 8 |
| E1 | *E. coli* | River | *intI1+intI2* | 32 | 0.015 | 0.03 | 512 | 32 | >4096 | 8 | 8 |
| E2 | *E. coli* | WWTP | *intI1+intI2* | 128 | 0.007 | 1 | 1024 | 128 | >4096 | 4 | 8 |
| E3 | *E. coli* | HWW | *intI1+intI2* | 64 | 0.007 | 2 | 512 | 64 | 1024 | 8 | 64 |
| E4 | *E. coli* | WWTP | *intI1+intI2* | 1 | 0.007 | 0.06 | 4 | 0.5 | 8 | 0.125 | 4 |
| KO1 | *K. oxytoca* | WWTP | *intI1* | 1 | 0.125 | >32 | 8 | 8 | >4096 | 1 | 1 |
| KO2 | *K. oxytoca* | HWW | *intI1* | 1 | 0.015 | ≤0.06 | 4 | ≤0.5 | 16 | 1 | 4 |
| KO3 | *K. oxytoca* | HWW | *intI1* | 128 | 0.06 | 16 | 64 | >256 | >4096 | 64 | 32 |
| KO4 | *K. oxytoca* | HWW | *intI1* | 8 | 0.125 | 64 | 512 | 64 | 8192 | 0.5 | 0.5 |
| KO5 | *K. oxytoca* | HWW | *intI1* | 0.5 | 0.03 | ≤0.003 | 2 | 0.25 | 8 | 1 | 0.25 |
| KO6 | *K. oxytoca* | WWTP | *intI1+intI2* | 0.25 | 0.015 | ≤0.0019 | 1 | 0.06 | 64 | 2 | 0.125 |
| KP1 | *K. pneumoniae* | WWTP | *intI1* | 128 | 0.125 | 32 | 4096 | 128 | >4096 | 8 | 8 |
| KP2 | *K. pneumoniae* | WWTP | *intI1* | 0.5 | 0.015 | 0.03 | 4 | 0.5 | 8 | 4 | 0.5 |
| KP3 | *K. pneumoniae* | River | *intI1+intI2* | 1 | 0.015 | 0.5 | 2 | 32 | 1024 | 4 | 4 |
| KP4 | *K. pneumoniae* | River | *intI1+intI2* | 1 | 0.015 | 0.125 | 2 | 1 | 32 | 1 | 2 |
| KP5 | *K. pneumoniae* | WWTP | *intI1* | 32 | 0.25 | 16 | 1024 | 512 | >16384 | >4096 | 1 |
| KP6 | *K. pneumoniae* | HWW | *intI1* | 16 | 0.03 | 64 | 512 | 128 | 8192 | 0.5 | 8 |
| KP7 | *K. pneumoniae* | HWW | *intI1* | 1 | 0.06 | 0.015 | 54 | 0.5 | 8 | >4096 | 32 |
| KP8 | *K. pneumoniae* | HWW | *intI1* | 1 | 0.015 | 0.125 | 1 | 64 | >4096 | 8 | 2 |
| KP9 | *K. pneumoniae* | HWW | *intI1+intI2* | 128 | 0.03 | 2 | 128 | 64 | >4096 | 4 | 2 |

[a]The shaded portions indicate MIC values classified as resistant, while the unshaded areas indicate those classified as susceptible. GEN: gentamicin, MEM: meropenem, CIP: ciprofloxacin, TET: tetracycline, CAZ: ceftazidime, AMP: ampicillin, COS: colistin, and AMK: amikacin.
[b]Source of bacterial isolation includes WWTP: wastewater treatment plant effluents, HWW: hospital wastewater effluents.

**Table 8. 2: The minimum inhibitory concentration (MIC) of various antibiotics alone and the results of the checkerboard assays for multidrug-resistant integron-harbouring *Citrobacter* spp.**

| Isolate identifier | Antibiotic[a] | MIC alone | MIC in combination | FIC[b] | FICI[c] | Interpretation |
|---|---|---|---|---|---|---|
| C1 | CAZ | 128 | 64 | 0.50 | 1.00 | Indifferent |
| | COS | 2 | 1 | 0.50 | | |
| C2 | CAZ | 4 | 1 | 0.25 | 0.50 | Synergy |
| | COS | 4 | 1 | 0.25 | | |
| C3 | CAZ | 128 | 64 | 0.50 | 1.00 | Indifferent |
| | COS | 2 | 1 | 0.50 | | |
| C4 | CAZ | 128 | 32 | 0.25 | 0.75 | Indifferent |
| | COS | 2 | 1 | 0.50 | | |
| C1 | TET | 1024 | 512 | 0.50 | 0.75 | Indifferent |
| | GEN | 1 | 0.25 | 0.25 | | |
| C2 | TET | 2 | 1 | 0.50 | 1.00 | Indifferent |
| | GEN | 2 | 1 | 0.50 | | |
| C3 | TET | 256 | 16 | 0.06 | 0.56 | Indifferent |
| | GEN | 32 | 16 | 0.50 | | |
| C4 | TET | 1024 | 128 | 0.13 | 0.25 | Synergy |
| | GEN | 64 | 8 | 0.13 | | |
| C1 | GEN | 1 | 0.5 | 0.50 | 0.75 | Indifferent |
| | COS | 2 | 0.5 | 0.25 | | |
| C2 | GEN | 1 | 0.25 | 0.25 | 0.75 | Indifferent |
| | COS | 4 | 2 | 0.50 | | |
| C3 | GEN | 64 | 32 | 0.50 | 0.63 | Indifferent |
| | COS | 2 | 0.25 | 0.13 | | |
| C4 | GEN | 128 | 32 | 0.25 | 0.38 | Synergy |
| | COS | 2 | 0.25 | 0.13 | | |
| C1 | TET | 2048 | 1024 | 0.50 | 0.75 | Indifferent |
| | COS | 2 | 0.5 | 0.25 | | |
| C2 | COS | 4 | 4 | 1.00 | 2.00 | Indifferent |
| | TET | 2 | 2 | 1.00 | | |
| C3 | TET | 128 | 64 | 0.50 | 1.00 | Indifferent |
| | COS | 2 | 1 | 0.50 | | |
| C4 | TET | 2048 | 1024 | 0.50 | 0.75 | Indifferent |
| | COS | 2 | 0.5 | 0.25 | | |
| C1 | AMP | 8192 | 4096 | 0.50 | 1.00 | Indifferent |
| | CIP | 0.5 | 0.25 | 0.50 | | |
| C2 | AMP | 8 | 4 | 0.50 | 1.00 | Indifferent |
| | CIP | 0.25 | 0.125 | 0.50 | | |
| C3 | AMP | 8192 | 4096 | 0.50 | 1.00 | Indifferent |
| | CIP | 2 | 1 | 0.50 | | |
| C4 | AMP | 8192 | 4096 | 0.50 | 1.00 | Indifferent |
| | CIP | 128 | 64 | 0.50 | | |

[a]Antibiotic codes:GEN: gentamicin, MEM: meropenem, CIP: ciprofloxacin, AMP: ampicillin, TET: tetracycline, COS: colistin, CAZ: ceftazidime, and AMK: amikacin.

[b] FIC: Fractional Inhibitory Concentration of each drug calculated as MIC in combination / MIC alone.

[c] FICI: Fractional Inhibitory Concentration Index of both drugs calculated by adding the FIC of the two drugs.

**Table 8. 3: The minimum inhibitory concentration (MIC) of antibiotics singly and in combination as derived from the checkerboard assays for multidrug-resistant integron-harbouring *E. coli, K. pneumoniae,* and *K. oxytoca*.**

| Organism (Isolate code) | Antibiotic[a] | MIC alone | MIC in combination | FIC[b] | FICI[c] | Interpretation |
|---|---|---|---|---|---|---|
| *E. coli* (E3) | GEN | 32 | 8 | 0.25 | 0.50 | Synergy |
| | TET | 512 | 128 | 0.25 | | |
| *E. coli (E2*) | GEN | 64 | 16 | 0.25 | 0.38 | Synergy |
| | TET | 512 | 64 | 0.13 | | |
| *E. coli* (E1) | GEN | 64 | 16 | 0.25 | 0.31 | Synergy |
| | TET | 1024 | 64 | 0.06 | | |
| *K. pneumoniae* (KP1) | GEN | 128 | 16 | 0.13 | 0.38 | Synergy |
| | TET | 4096 | 1024 | 0.25 | | |
| *K. oxytoca* (KO1) | GEN | 0.5 | 0.06 | 0.12 | 0.62 | Indifferent |
| | TET | 4 | 2 | 0.50 | | |
| *E. coli* (E3) | GEN | 32 | 16 | 0.50 | 0.63 | Indifferent |
| | CAZ | 128 | 16 | 0.13 | | |
| *E. coli (E2*) | GEN | 64 | 16 | 0.25 | 0.75 | Indifferent |
| | CAZ | 64 | 32 | 0.50 | | |
| *E. coli* (E1) | GEN | 64 | 32 | 0.50 | 0.75 | Indifferent |
| | CAZ | 64 | 16 | 0.25 | | |
| *K. pneumoniae* (KP1) | GEN | 128 | 16 | 0.13 | 0.19 | Synergy |
| | CAZ | 128 | 8 | 0.06 | | |
| *K. pneumoniae* (KP1) | GEN | 128 | 16 | 0.13 | 0.38 | Synergy |
| | CIP | 32 | 8 | 0.25 | | |
| *K. pneumoniae* (KP1) | CAZ | 128 | 64 | 0.50 | 1.00 | Indifferent |
| | CIP | 32 | 16 | 0.50 | | |
| *K. pneumoniae* (KP1) | AMP | 16384 | 8192 | 0.50 | 1.00 | Indifferent |
| | CIP | 32 | 16 | 0.50 | | |
| *E. coli* (E3) | TET | 512 | 256 | 0.50 | 0.63 | Indifferent |
| | CAZ | 128 | 16 | 0.13 | | |
| *E. coli (E2*) | TET | 512 | 256 | 0.50 | 1.00 | Indifferent |
| | CAZ | 64 | 32 | 0.50 | | |
| *E. coli* (E1) | TET | 512 | 64 | 0.13 | 0.38 | Synergy |
| | CAZ | 64 | 16 | 0.25 | | |
| *K. oxytoca* (KO1) | TET | 4 | 1 | 0.25 | 0.75 | Indifferent |
| | COS | 0.5 | 0.25 | 0.50 | | |
| *K. oxytoca* (KO1) | CAZ | 8 | 2 | 0.25 | 0.75 | Indifferent |
| | COS | 0.5 | 0.25 | 0.50 | | |
| *K. oxytoca* (KO1) | CIP | 256 | 128 | 0.50 | 0.75 | Indifferent |
| | COS | 0.5 | 0.125 | 0.25 | | |

[a]Antibiotic codes: GEN: gentamicin, MEM: meropenem, CIP: ciprofloxacin, TET: tetracycline, COS: colistin, AMP: ampicillin, CAZ: ceftazidime, and AMK: amikacin.
[b] FIC: Fractional Inhibitory Concentration of each drug calculated as MIC in combination / MIC alone.
[c] FICI: Fractional Inhibitory Concentration Index of both drugs calculated by adding the FIC of the two drugs.

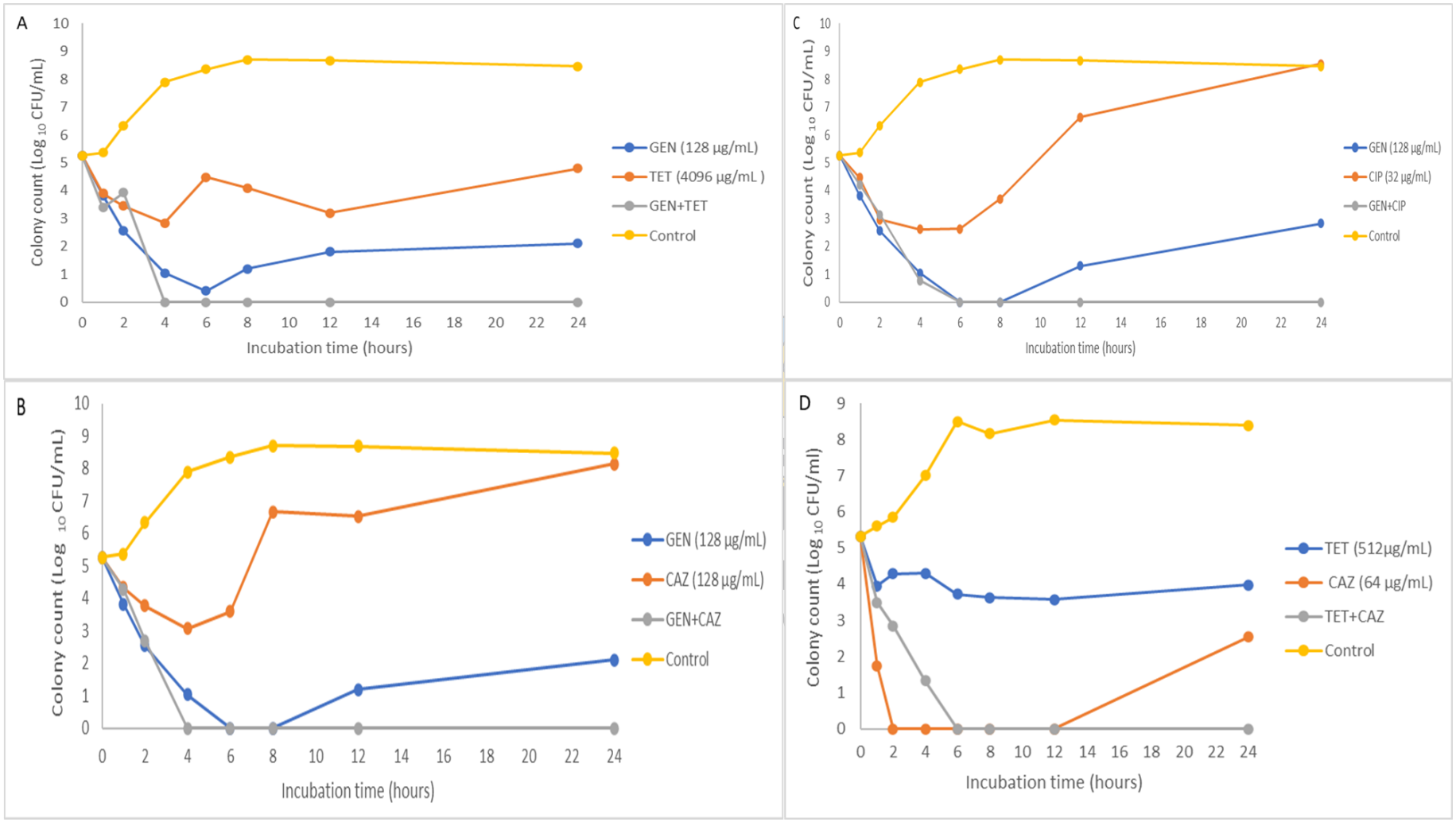


**Figure 8. 1: Time-Kill curves for antimicrobials in combination at various MIC values.** A, B, and C show the combination of gentamicin at MIC with tetracycline, ceftazidime, and ciprofloxacin against MDR *K. pneumoniae* (KP1). D shows drug combination against MDR *E. coli* (E1).

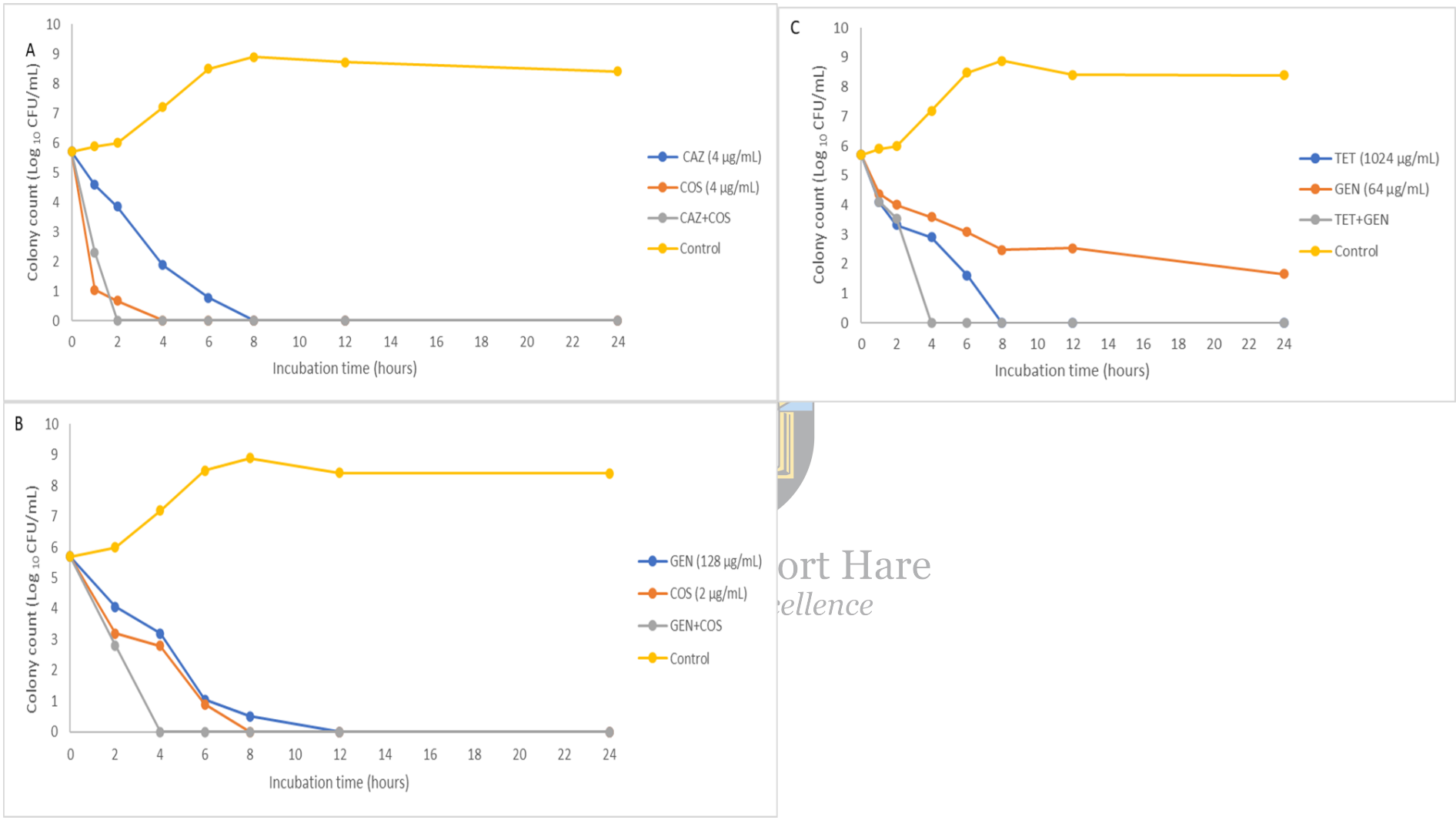


**Figure 8. 2: Time-Kill curves for antimicrobials in combination at various MIC values against MDR *C. freundii*.** A and B show the combination of colistin at MIC in combination with ceftazidime and gentamicin against isolates C2 and C4, respectively. C shows a drug combination between gentamicin and tetracycline against isolate C4.

## 8.4 Discussion

A multidrug-resistant organism displays resistance against a minimum of one antibiotic in more than two different classes (Fadare et al., 2020; Magiorakos et al., 2012). Novel approaches to antimicrobial therapy for MDR bacteria have become increasingly crucial as resistance rates to last-resort antibiotics rise. The few antibiotics that are effective against these bacteria have severe clinical limitations, such as hazardous side effects in the case of colistin (Ordooei Javan et al., 2015). Even new agents such as ceftazidime-avibactam are susceptible to resistance development (Shields et al., 2017). Combination medication regimens are one method of treating MDR Gram-negative bacteria, but little research has been done to explore their potency against infections.

All the selected isolates harboured at least one integrase gene, *intI*, and were thus classified as integron-harbouring. Integrons are mobile genetic elements considered efficient gene expression systems that allow bacterial species to capture gene cassettes within their environment and immediately express the ARGs on them due to the presence of inherent promoters (Hall, 2012; Kaushik et al., 2018; Okoh & Fadare, 2022). The presence of integrons with possible ARGs on the gene cassettes further fortifies the bacterial species against the usual antibiotics administered against them. In previous research by Li and colleagues, integron harbouring isolates demonstrated resistance against a substantially greater number of antibiotics than negative isolates (Li et al., 2013). Integrons present a selective advantage to bacteria in settings where antibiotic use causes selective pressures, which may explain the high occurrence of multidrug resistance observed in this study.



In this study, meropenem exhibited the lowest MIC values against all the isolates. An outcome that was not unexpected since meropenem is not one of the frontline drugs usually administered against bacterial infections (Fadare & Okoh, 2021b; Teixeira et al., 2020). Most of our isolates displayed resistance against more than two different antibiotic classes and

were thus classified as multidrug-resistant. MDR bacteria are usually a concern when recovered in clinical settings; however, their high detection rate in environmental settings, as with this study, even poses a greater risk to the public. More commonly now, organisms acquire various adaptability mechanisms, such as the acquisition of integrons to survive or evade the arsenal of antibiotics designed against them and therefore, other means of overcoming this ARB must also be devised. Therefore, it behoves us to explore the possibilities of combining drugs that can be used simultaneously to combat or reduce the possibility of developing resistance.

The antibiotics with MIC values categorized as resistant were combined in a checkboard style, and the outcomes with interpretable results are shown in Table 8.2 and Table 8.3. The various combinations explored yielded synergistic or indifferent interactions of different classes of drugs. As shown in Figure 8.1A-C, the killing rate of *K. pneumoniae* (KP1) by gentamicin is faster than tetracycline, ciprofloxacin, and ceftazidime. The higher kill rate by gentamicin observed in this study is similar to the results of another *in vitro* experiment of the ciprofloxacin-gentamicin combination (Lemaître et al., 2012). Our study's combinations of drugs against MDR *K. pneumoniae* and *E. coli* yielded synergistic and bactericidal outcomes. In Figure 8.1D, ciprofloxacin's ROK was faster than tetracycline's ROK against *E. coli* (E1). Ciprofloxacin activity was noted to have reduced the cell count to zero as early as 2 hours after exposure and was maintained till 12 hours, and the viable cells re-emerged at 24 hours, suggesting that the drug was a bacteriostatic agent. However, when ciprofloxacin was combined with tetracycline, there were no viable cells from 6 hours until the end of the assay, indicative of a bactericidal effect. The synergism observed in the isolates' TKAs further confirms the synergism obtained in the checkerboard assays.



In Figure 8.2, the ROK of the combined drugs against *C. freundii* isolates were all bactericidal. However, the synergistic interactions obtained in duplicate checkerboard assays

were not observed in the TKAs. Except for Figure 8.2C, where one of the drugs (gentamicin) was not bactericidal throughout the time, most of the single agents were bactericidal at the MIC values. The effectiveness of the combinations was seen at a shorter time to attain the bactericidal effect than the single agents.

The most used antibiotic in the combination studies with interpretable results was gentamicin in this study, as seen in Table 8.2 and Table 8.3. It belongs to the aminoglycoside class of antibiotics and is used in treating MDR bacteria. Although they have been used for several decades to treat infections caused by non-fastidious gram-negative bacteria (Castanheira et al., 2018), the most prevalent bacterial resistance mechanisms in this antibiotic class are the enzymatic modification aminoglycoside antibiotics (Wachino et al., 2020). The enzymes belong to families such as aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside adenyl transferases (ANTs) (Sparo et al., 2018; Wang et al., 2022). These enzymes are often encoded on gene cassettes of integrons, also present in the isolates investigated in this study.



In this study, one of the combinations of gentamicin with ceftazidime yielded a synergistic interaction, and further investigation in the TKA revealed the bactericidal and synergistic effects of the combination compared to the individual agents. Here, *K. pneumoniae* (KP1) was resistant against ceftazidime, while gentamicin showed better activity but was not bactericidal against the isolate. It has been reported that β-lactams such as ceftazidime are known to break the bacterial cell wall in a non-fatal way, allowing aminoglycosides such as gentamicin to enter bacteria and increase their killing effectiveness (Davis, 1982; Wang et al., 2022). In another study, aminoglycosides, due to their synergistic antibacterial properties, were combined with β-lactam antibiotics, which broadened the scope of treatment, accelerated bacterial clearance and enhanced antibiotic resistance (Le et al., 2011). Several other studies have reported the combination of aminoglycosides with β-lactams for treating

MDR bacteria species (Sick et al., 2014; Tamma et al., 2012; Wang et al., 2022). In this study, the combination of gentamicin (aminoglycoside) with colistin (polymyxins) against *C. freundii*, which yielded a synergistic interaction as shown in Table 8.2, is similar to the reports of Hussein and colleagues, where amikacin was combined with polymyxins with a synergistic antibacterial effect (Hussein et al., 2020).

*In vitro* assessments of antimicrobial synergy are naturally limited in their ability to predict *in vivo* outcomes accurately; hence, necessary precautions must be taken to apply such combination therapy in clinical applications. First, the concentrations tested may be above the tolerable threshold for the actual serum levels, and a pharmacokinetic/pharmacodynamic simulation is needed. As far as *in vivo* study is concerned, higher MIC levels for the antibiotics tested may not be clinically beneficial. Another critical concern is that the inoculum size used for these *in vitro* assays may differ significantly *in vivo* vis-a-vis host defence mechanisms, and the checkerboard results and rate of kill assays obtained in this study may not reflect the accurate outcome when utilized in clinical settings. Therefore, future studies in which the synergistic and bactericidal relationships observed in the combinations in this present study need to be tested in animal models, pharmacokinetic/pharmacodynamic studies, and human subjects will be essential in determining the possible clinical outcomes applications of our findings. Although, within the confines of *in vitro* studies, specific steps were taken to increase the robustness of our results by testing different isolates in the checkerboard array and then further assessing the synergistic combinations through the TKAs. In most cases, synergy was also present in the ROK studies, and all the synergistic relationships from the checkboard assays were bactericidal (Figures 8.1 and 8.2).

## 8.5. Conclusion

According to the findings of this study, some *in vitro* combinations of different classes of antibiotics targeting different mechanisms of action can be effective against MDR Enterobacteriaceae infections. However, further studies, including pharmacokinetics, pharmacodynamics, and clinical trials, are needed on the synergistic combinations to confirm their advantages over monotherapy.

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# CHAPTER NINE

# GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

## 9.1 General discussion

Antibiotics have prevented millions of deaths since being developed decades ago to treat bacterial infections and illnesses. This victory, however, was short-lived because the number of microbes resistant against antibiotics has increased at alarming rates, far faster than the expected average pace of microbial evolution (Cohen, 2000; Czekalski et al., 2014; Fadare et al., 2020). The rapid increase rate of resistance has been ascribed to the use and misuse of antibiotics as prophylactics, therapeutics, and growth enhancers. The antibiotic-resistant bacteria (ARB) and the genes they harbour bestow resistance phenotypes on the ARB (Founou et al., 2016; Rahimi, 2019; Tärnberg, 2012; Thanner et al., 2016). The antimicrobial resistance scourge has become a global public health challenge primarily due to the ability of ARB in a particular niche to transfer antibiotic-resistance genes (ARGs) to other organisms, even in widely varied niches, through genetic elements such as integrons. After obtaining these genetic components, previously susceptible bacteria can be transformed into more virulent and resistant species (Czekalski et al., 2014; Fadare et al., 2020; Tärnberg, 2012).



Integrons are mobile genetic elements found on pathogenicity islands, transposons, and plasmids, easing their distribution among various bacteria. They are considered efficient gene expression systems that naturally capture, integrate gene cassettes (GCs), and immediately express the captured ARGs due to the inherent promoters located on their structures (An et al., 2018; Gillings, 2014; Hall & Collis, 1995; Sandoval-Quintana et al., 2022; White et al., 2001). Gene cassettes are variable sequences that can occur as free, circular, nonreplicating DNA molecules and are usually linear when integrated into integrons (Cambray et al., 2010; Sandoval-Quintana et al., 2022). Their presence confers resistance against most antibiotic classes. These include all known aminoglycosides, erythromycin, antiseptics of the quaternary ammonium compounds, β-lactams, chloramphenicol, fosfomycin, trimethoprim,

lincomycin, quinolones, rifampicin, and streptothricin (Deng et al., 2015; Gillings, 2014; Kaushik et al., 2019; Mazel, 2006; Okoh & Fadare, 2022).

In this research, a high occurrence of integrons and their associated GCs were detected across the members of Enterobacteriaceae (*Citrobacter* spp., *Klebsiella* spp., *Enterobacter cloacae* and *Escherichia coli*), recovered from diverse sources such as rivers, animal droppings, effluents of hospital and wastewater treatment plants. Integrons give a selective advantage to bacteria in settings where antibiotic use causes selective pressures, which may explain the high occurrence of multidrug-resistant (MDR) among the isolates harbouring integrons.

Among the fifty-two Klebsiella species investigated, *K. pneumoniae* (63%) was more frequently detected than *K. oxytoca* (37%). It agrees with the reports that *K. pneumoniae* is the most prevalent species of the genus as it is the causative agent of most nosocomial Klebsiella infections, thereby referred to as the most medically significant species of the genus (Magill et al., 2014; Podschun & Ullmann, 1998). All the *K. pneumoniae* harboured an integron integrase gene while it was absent in only one *K. oxytoca* isolate. The high rate of detection of integrons (98%, 51/52) in the genetically unrelated isolates indicates the widespread of these mobile genetic elements. The Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR fingerprints of the isolates categorized all isolates into nine clusters at a similarity index of 60%. All the integron-positive isolates were classified as MDR because they exhibited a phenotypic resistance against antibiotics in a minimum of three different classes. The integron-positive isolates were classified as class 1 integrons (80%) and class 1+2 integrons (20%) due to the isolates harbouring both *intI1* and *intI2* genes. The studies of (Firoozeh et al., 2019) and (Lima et al., 2014) have stated that all the MDR *K. pneumoniae* in their studies harboured the *intI1* gene, although these isolates were from clinical samples. Other research from environmental origins has also reported the detection of the *intI1* gene as the most prevalent in similar studies (Kaushik et al., 2018; Zhang et al.,

2019). The integrons with gene cassettes harboured three different GC arrays *aac(6')-Ib, aadA1-dfrA1,* and *dfrA1-sat2*.

Out of 71 *E. coli* isolates assessed for the detection of integrons, the integron integrase genes were detected in 88.7%. These were classified into class 1, 2 and 1+2 integrons with 76.2% (48), 1.6% (1), and 22.2% (14), respectively. The *intI1* internal variable regions (IVRs) were detected in 19 isolates with GCs such as *dfrA1*, *dfrA21*, *dfrA5*, *dfrA17*, *aadA5*, *aadA1*, *aadA5*, and *aac-3-Ib*, while the remaining IVRs contained an empty or undetermined GCs. The *intI2* IVRs were detected in 4 isolates with a constant GC array of *dfrA1-sat2*. The outcome of the ERIC-PCR revealed that the isolates were characterized into fourteen clusters at a similarity cut-off of 55%, indicating that the isolates had unique DNA fingerprints and were unrelated. The high detection of integrons from the various sources of the unique isolates signifies a widespread distribution of integrons in *E. coli*, even in non-clinical sources. The most frequently detected integrase was the *intI1*, similar to the high occurrence rate detected in similar studies (Sáenz et al., 2004; Soufi et al., 2009; Su et al., 2012; Zhang et al., 2019).



All the *Citrobacter* spp. assessed harboured the *intI1* gene, of which 18.8% (3/16) were concurrently positive for the *intI2* genes. About 62.5% (10/16) of the isolates were typical class 1 integrons due to the presence of *qacEΔ1* and *sulI* genes. Here, all the isolates except one were classified as MDR. Although the reports of the detection of *Citrobacter* spp. have not been commonplace in environmental sources, this has limited the detection of integrons in this genus. Some studies with clinical isolates in Egypt and Iran have stated over 50% occurrence of *intI1* gene in *Citrobacter* spp. (Lorestani et al., 2018; Malek et al., 2015). The analysis of the sequences of the integrons variable regions of the *Citrobacter* spp in this study revealed various GC arrays. These include *dfrA*5-*aac3-Ib*, *aac(6')-Ib*, *aadA1-dfrA1-aadA1*, *aadA1-dfrA1*, *aadA5-dfrA17,* and *dfrA*21- *aac3-Ib*.

The *Enterobacter cloacae* recovered from the various environmental matrices were all classified as MDR. All but one of the isolates, 97.7% (43/44), harboured an integron-integrase, *intI* gene. The *intI1* gene was found in all the integron-positive isolates except for one isolate [97.7% (42/43)]. The *intI2* gene was detected in 11.6% (5/43) and mostly occurred with the presence of *intI1*. Therefore, the isolates were classified as follows: Class 1 integrons [88.4%, 38/43], class 2 integron [2.3% ,1/43], and class 1+2 integrons [9.3%, 4/43]. These integrons harboured the following GC arrays: *aadA1-dfrA1*, *dfrA7*, *dfrA21*, *dfrA5-aac-3-Ib*, and *dfrA1-sat2*. Here, 61.4% (27/44) of the isolates harboured at least one of the *qacEΔ1* and *sul1* genes. About 26.3% (10/38) of the isolates that were *intI1* positive also harboured both *qacEΔ1* and *sul1* genes and were considered typical class 1 integrons. The presence of the *qacEΔ1* and *sul1* genes on the 3′ conserved segment of an integron identifies the classification of typical class 1 integrons. Nevertheless, these genes were also found in one of the isolates harbouring both *intI1* and *intI2*. The result supports a previous study by Zhang and colleagues (Zhang et al., 2019), where some typical class 1 integrons also harboured the *intI2* gene. The *qacEΔ1* is a truncated *qacE* gene conferring resistance against quaternary ammonium compounds and precedes the insertion of a *sul1* gene at the 3′ end, which encodes resistance against the antibiotic sulphonamide (Gillings, 2014). The *qacEΔ1* gene was detected in 38.1% (16/42) of the remaining *intI1* positive isolates and lacked the *sul1* gene.



Across the genus, none of the isolates harboured *intI3* genes for the class 3 integrons. The *intI3* genes are the least prevalent in colonizing new species even though they have a 60% sequence identity with *intI1* (Collis et al., 2002; Okoh & Fadare, 2022; Simo Tchuinte et al., 2016) and have been reported only in a few studies (Barraud et al., 2013; Simo Tchuinte et al., 2016). Fewer than ten class 3 integrons have been described. These are In3-1 and In3-2 recovered from clinical Enterobacteriaceae (Arakawa et al., 1995; Correia et al., 2003; Poirel

et al., 2010), In3-3 and In3-4 from environmental Delfia (Xu et al., 2007), In3-5 from hospital effluent *E. cloacae* (Barraud et al., 2013), In3-6 from hospital effluent *Acinetobacter johnsonii*, In3-7 from sewage sludge *Aeromonas allosaccharophila,* and In3-9 from hospital effluent *Citrobacter freundii* (Simo Tchuinte et al., 2016).

The integrons' internal variable regions that house the GCs were not detected in some of the *intI1* positive isolates, which could be due to defects or mutations at the 3′ CS or the GCs belonging to an unusual or complex class 1 integrons (Lee et al., 2011). According to the analysis of the sequencing results, some GCs were categorized as empty or undetermined. It could be that the sequences of that region have not been previously reported. The detection of integrons with empty GCs has been reported, and it shows the readiness of such isolates to capture GCs readily and subsequently express the ARGs harboured on them.

The findings of this study revealed a high prevalence of MDR Enterobacteriaceae from the various environmental matrices and a repertoire of various GC arrays harboured on the integrons they possess. A high occurrence of integrons was detected across all the isolates studied. The *intI1* genes have the highest detection frequency due to their capacity to recombine with different *attC* sites on the GCs (Deng et al., 2015; Kaushik et al., 2018). Although the genus Citrobacter had the least number of isolates, surprisingly, it harboured the most diverse GC arrays reported in this study. The integrons detected can rapidly acquire and then express ARGs harboured on GCs due to the presence of the promoters inherent in an integron structure. Since integrons are mobile, the GCs can be easily exchanged among various bacterial species in the different environmental matrices. These results prove that environmental sources also serve as a potential repertoire of mobile genetic elements harbouring different antibiotic resistance GCs.

These antibiotic-resistant disease-causing organisms, such as those reported in this thesis, according to the World Health Organization (WHO), will kill 10 million people per year by 2050 unless aggressive efforts are made to prevent this looming worldwide antimicrobial resistance (AMR) catastrophe (WHO, 2019). Unfortunately, this projection is a critical concern in South Africa, given that a sizeable section of the population has impaired immune systems. Other serious factors include poor cleanliness and insufficient infrastructure, which may make people more susceptible to infectious diseases, increasing the chance of death from AMR and causing economic loss (DAFF, 2017). The spread of ARB has thus challenged the tremendous gains of the existing antibiotics and their efficacy in decreasing morbidity and mortality (Magiorakos et al., 2012). It is common knowledge that effective antibiotics are crucial in modern medicine; however, the rapid growth of antibiotic resistance coupled with the slow discovery rate of new antibiotics jeopardize future antibiotic treatment choices, thereby necessitating the hunt for new, practical remedies (Brennan-Krohn & Kirby, 2019; O'Neill, 2016; Spellberg, 2014). One of such is the exploration of synergy among existing antibiotics. Two medications combined have a higher impact, thereby allowing current antibiotics to be salvaged for use in treating MDR bacteria, even if the bacteria are resistant against one or both antibiotics separately.



This research examined the inhibitory effect of combining two drugs belonging to different antibiotic classes to obtain a possible potentiating effect against some of the Enterobacteriaceae isolates harbouring integrons. The checkerboard method was used to determine the combined drug's fractional inhibitory concentration indices (FICIs). A total of 26.3% (10/38) of the interactions were categorized as synergistic, while 73.7% (28/38) were indifferent using previously described breakpoints (Brennan-Krohn & Kirby, 2019; Odds, 2003). None of the combinations was antagonistic. The time-kill assays (TKAs) revealed all the synergistic interactions as bactericidal. The most used antibiotic in this study with

interpretable results was gentamicin. It belongs to the aminoglycoside class of antibiotics and is used in treating MDR bacteria. Although they have been used for several decades to treat infections caused by non-fastidious Gram-negative bacteria (Castanheira et al., 2018), the most prevalent bacterial resistance mechanisms in this antibiotic class are the enzymatic modification aminoglycoside antibiotics (Wachino et al., 2020). The enzymes belong to families such as aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside adenyl transferases (ANTs) (Sparo et al., 2018; Wang et al., 2022). These enzymes are often encoded on gene cassettes of integrons, also present in the isolates investigated in this study.

The combinations of gentamicin with ceftazidime studied yielded a synergistic interaction, and further investigation of the TKAs revealed the bactericidal and synergistic effects of the combination compared to the individual agents. It has been reported that β-lactams such as ceftazidime are known to break the bacterial cell wall in a non-fatal way, allowing aminoglycosides such as gentamicin to enter bacteria and increase their killing effectiveness (Davis, 1982; Wang et al., 2022). Aminoglycosides, due to their synergistic antibacterial properties, when combined with β-lactam antibiotics, broaden the scope of treatment, accelerate bacterial clearance and reduce antibiotic resistance (Le et al., 2011). Several similar studies have reported the combination of aminoglycosides with β-lactams for treating MDR bacteria species (Sick et al., 2014; Tamma et al., 2012; Wang et al., 2022).



In general, the outcomes of the *in-vitro* assays revealed the combinations of gentamicin with tetracycline, ciprofloxacin, and ceftazidime against MDR *K. pneumoniae*; tetracycline-ceftazidime combination against MDR *E. coli*, colistin combinations with ceftazidime and gentamicin, and tetracycline-gentamicin combinations against MDR *C. freundii* may be future therapeutic alternatives.

## 9.2 Conclusion

The antibiotic fingerprints of most of the isolates revealed that all the isolates exhibited resistance against multiple antibiotics in different classes and were considered multidrug-resistant. The occurrence of MDR bacterial species from diverse environmental niches such as rivers, animal droppings, and effluents of hospital and wastewater treatment plants is a concern as it further limits therapeutic options. The detection of gene cassettes harbouring various antibiotic resistance genes on integrons in Enterobacteriaceae underlines the importance of monitoring antibiotic-resistant bacteria and the role of integrons in the rapid dissemination of antibiotic-resistance genes harboured by bacterial species. The high prevalence of integrons and the detection of their associated gene cassettes in these environmental matrices reveal integrons as a possible antimicrobial resistance driver within the environment. The probability of treating MDR bacterial isolates via combined antibiotics was also elucidated with certain drug combinations yielding synergistic and bactericidal outcomes. Hence, the combination of antibiotics is a potential option for treating integron-harbouring MDR Enterobacteriaceae members from different environmental matrices.



## 9.3 Recommendations

The following are suggested for future studies:

1. Due to the lack of detection of the class 3 integrons, whole genomic studies could be conducted to increase the likelihood of detecting this class of integrons.
2. Amplified gene cassettes with sequences that have not been previously reported and thereby categorized as undetermined could be further studied to determine their content and functions.
3. The combined antibiotics with synergistic and bactericidal interactions confirmed from the time-kill studies *in vitro* should be explored using *in vivo* models for the pharmacokinetic and pharmacodynamic studies.

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# APPENDICES

**APPENDIX 1**

University of Fort Hare
*Together in Excellence*

**ETHICS CLEARANCE**
**REC-270710-028-RA Level 01**

| | |
|---|---|
| Project Number: | OKO011SFAD01 |
| Project title: | **Exploration of antibiotics combination therapy for the management of multidrug-resistance in Enterobacteriaceae isolates from environmental sources harbouring class 1, 2, and 3 integrons.** |
| Qualification: | PhD in Microbiology |
| Student name: | Folake Temitope Fadare |
| Registration number: | 201608584 |
| Supervisor: | Prof A.I Okoh |
| Department: | Microbiology |
| Co-supervisor: | N/A |

On behalf of the University of Fort Hare's Research Ethics Committee (UREC) I hereby grant ethics approval for OKO011SFAD01. This approval is valid for 12 months from the date of approval. Renewal of approval must be applied for BEFORE termination of this approval period. Renewal is subject to receipt of a satisfactory progress report. The approval covers the undertakings contained in the above-mentioned project and research instrument(s). The research may commence as from the 15/10/20, using the reference number indicated above.

Note that should any other instruments be required or amendments become necessary, these require separate authorisation.
Please note that UREC must be informed immediately of

- Any material changes in the conditions or undertakings mentioned in the document;
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The student must report to the UREC in the prescribed format, where applicable, annually, and at the end of the project, in respect of ethical compliance.

UREC retains the right to

- Withdraw or amend this approval if
  - Any unethical principal or practices are revealed or suspected;
  - Relevant information has been withheld or misrepresented;
  - Regulatory changes of whatsoever nature so require;
  - The conditions contained in the Certificate have not been adhered to.

- Request access to any information or data at any time during the course or after completion of the project.

Your compliance with Department of Health 2015 guidelines and any other applicable regulatory instruments and with UREC ethics requirements as contained in UREC policies and standard operating procedures, is implied.

UREC wishes you well in your research.

Yours sincerely

Vithal, Renuka
Digitally signed by Vithal, Renuka
Date: 2020.12.04 10:34:37 +02'00'

**Professor Renuka Vithal**
**Chairperson: University Research Ethics Committee**
25 November 2020

**Appendix 2**

Primer sequences of target strains and their thermocycling conditions.

| Target strain | Target gene | Primer Sequence (5′ →3′) | Amplicon size (bp) | Thermocycling conditions |
|---|---|---|---|---|
| Klebsiella genus | *gryA* | F: CGCGTACTATACGCCATGAAGTA<br>R: ACCGTTGATCACTTCGGTCAGG | 441 | 94 ℃ for 5 mins; 35 [94 ℃, 30 s; 55 ℃, 45 s; 72 ℃, 45s]; 72 ℃ for 7 min. |
| *Klebsiella pneumoniae* | *16S-23S ITS* | F: ATT TGA AGA GGT TGC AAA CGA T<br>R: TTC ACT CTG AAG TTT TCT TGT GTT C | 130 | 94 ℃ for 5 mins; 30 [94 ℃, 30 s; 55 ℃, 30 s; 72 ℃, 40 s]; 72 ℃ for 10 min. |
| *Klebsiella oxytoca* | *pehX* | F: GATACGGAGTATGCCTTTACGGTG<br>R: TAGCCTTTATCAAGCGGATACTGG | 343 | 94 ℃ for 5 mins; 30[94 ℃, 30 s; 55 ℃, 30 s; 72 ℃, 40 s]; 72 ℃ for 10 min. |
| *Enterobacter cloacae* | *hsp60* | F: GTAGAAGAAGGCGTGGTTGC R: ATGCATTCGGTGGTGATCATCAG | 341 | 94 ℃ for 5 mins; 30 [94 ℃, 30 s; 54 ℃, 30 s; 72 ℃, 1 min]; 72 ℃ for 5 min. |
| Citrobacter genus | *Citrobacter urease* | F: TGAAGCTGAACTACCCGGAATC R: TGTCCAGGCTCAAAACGTAC | 454 | 94 ℃ for 4 mins; 30 [94 ℃, 30 s; 55 ℃, 40 s; 72 ℃, 1 min]; 72 ℃ for 7 min. |
| *Escherichia coli* | uidA | F: AAAACGGCAAGAAAAAGCAG R: ACGCGTGGTTACAGTCTTGCG | 147 | 94 ℃ for 5 mins; 30[94 ℃, 30 s; 58 ℃, 1 min; 72 ℃, 1 min]; 72 ℃ for 8 min. |
| Class 1 integron | *intI1* | F: CAGTGGACATAAGCCTGTTC<br>R: CCCGAGGCATAGACTGTA | 164 | 94 ℃ for 5 mins; 35[94 ℃, 60 s; 55 ℃, 60 s, 72 ℃, 30 s] 72 ℃, 10 min |
| Class 2 integron | *intI2* | F: TTATTGCTGGGATTAGGC<br>R: ACGGCTACCCTCTGTTATC | 232 | 94 ℃ for 5 mins; 32[94 ℃, 60 s; 59 ℃, 60 s; 72 ℃, 2 mins]; 72 ℃ for10 mins. |

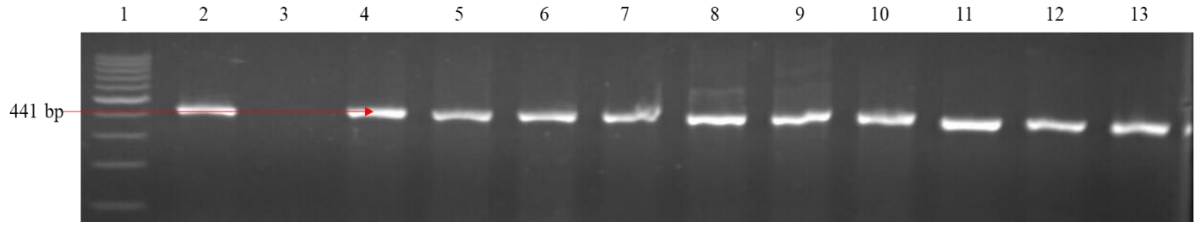


**Figure A1.** A gel showing representative positive isolates with *gryA* gene at 441 bp for the confirmation of genus Klebsiella. Lane 1: 100 bp molecular weight marker, Lane 2: Positive control (*K. pneumoniae* ATCC 35657$^{TM}$), Lane 3: Negative control (*E. coli* ATCC 8739), Lanes 4-13: Some *gryA* positive isolates.

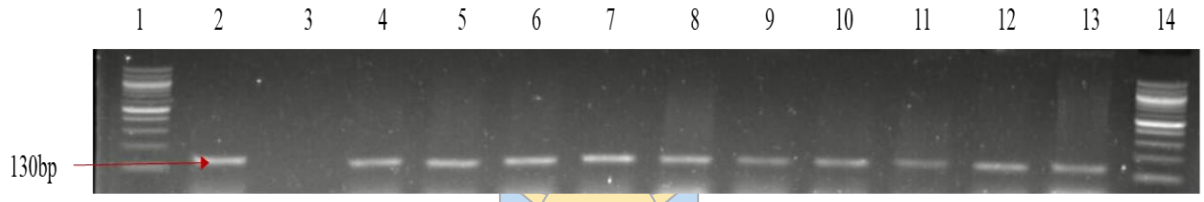


**Figure A2.** A gel showing representative positive isolates for *16S-23S ITS* gene at 130 bp for the confirmation of *Klebsiella pneumoniae*. Lanes 1 and 14: 100 bp molecular weight marker, Lane 2: Positive control (*K. pneumoniae* ATCC 35657$^{TM}$), Lane 3: Negative control (*E. coli* ATCC 8739), Lanes 4-13: Some isolates positive for *16S-23S ITS* gene.



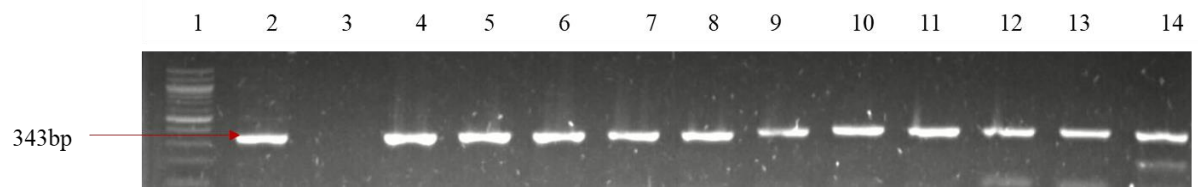


**Figure A3.** A gel showing isolates positive for *pehX* gene at 343 bp for *K. oxytoca* for the confirmation of *Klebsiella oxytoca*. Lane 1: 100 bp molecular weight marker, Lane 2: Positive control (*K. oxytoca* NCTC 11686), Lane 3: Negative control (*E. coli* ATCC 8739), Lanes 4-14: Some isolates positive for *pehX* gene.

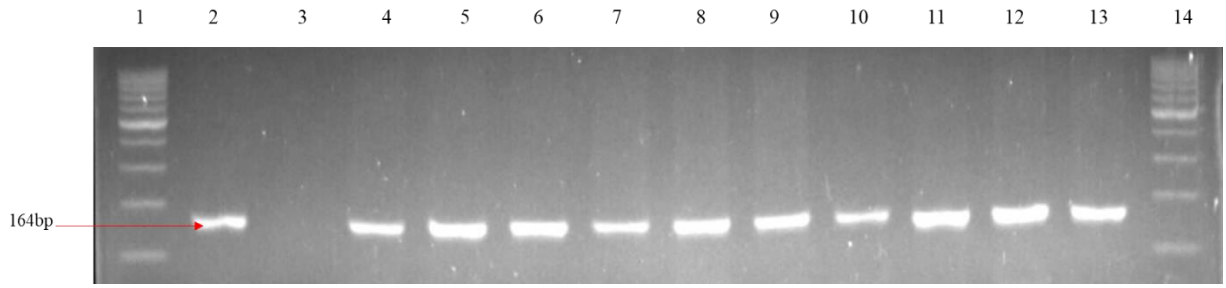


**Figure A4.** A gel showing the presence of the *IntI1* gene for class 1 integrons at 164 bp. Lanes 1 and 14: 100 bp molecular weight marker, Lane 2: Positive control (*Acinetobacter baumannii* ATCC 19606), Lane 3: Negative control, Lanes 4-13: Some isolates positive for *IntI1* gene.

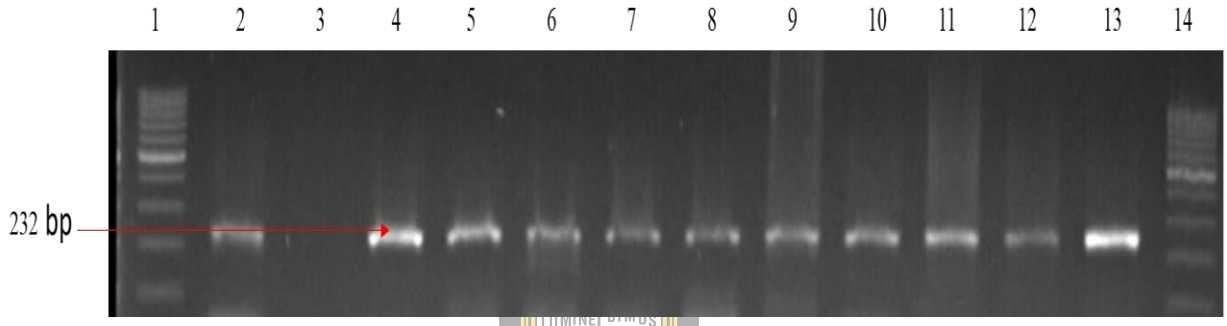


**Figure A5.** A gel showing isolates harbouring *IntI2* gene for class 2 integrons at 232 bp. Lanes 1 and 14: 100 bp molecular weight marker, Lane 2: Positive control, Lane 3: Negative control, Lanes 4-13: Some isolates positive for *IntI2* gene.



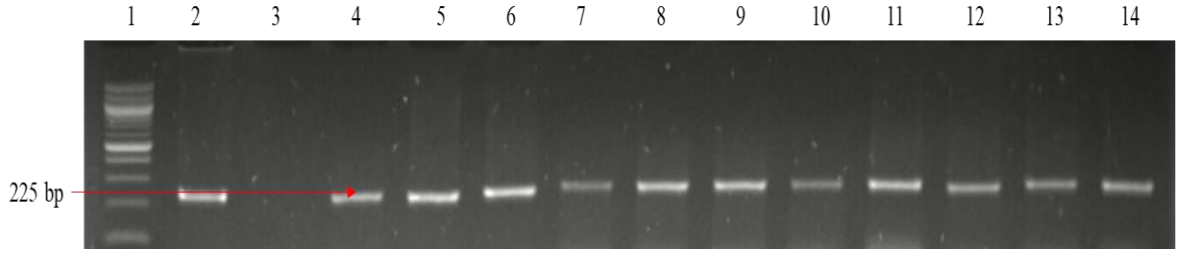


**Figure A6.** A representative gel showing isolates at 225 bp harbouring the *qacEΔ1* gene, a quaternary ammonium compound resistance gene. Lane: 100 bp molecular weight marker, Lane 2: Positive control (*Acinetobacter baumannii* ATCC 19606), Lane 3: Negative control, Lanes 4-14: Isolates positive for *qacEΔ1* gene.

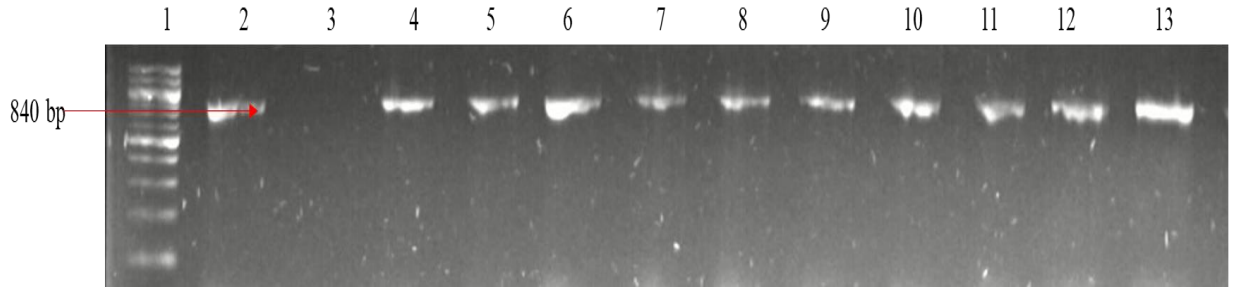


**Figure A7.** A representative gel showing isolates at 840 bp harbouring the *sul1* gene, a sulphonamide resistance gene. Lane 1: 100 bp molecular weight marker, Lane 2: Positive control (*Acinetobacter baumannii* ATCC 19606), Lane 3: Negative control, Lanes 4-14: Some isolates positive for *sul1* gene.

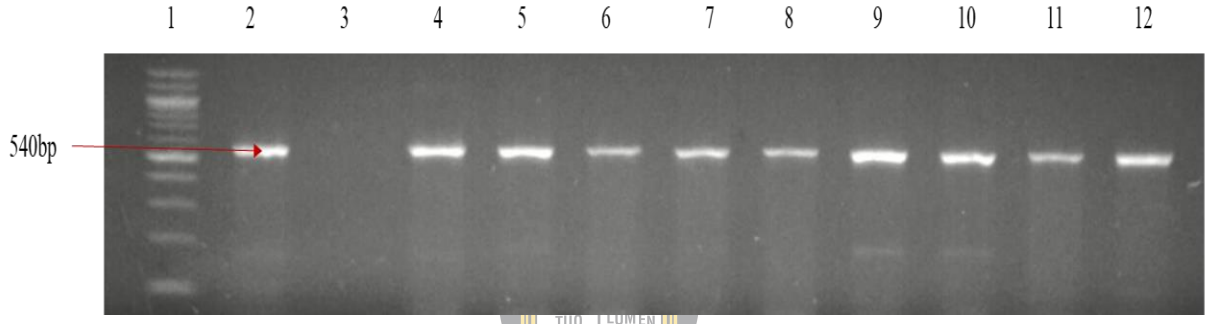


**Figure A8.** A representative gel showing isolates positive for the *P2R2* promoter gene at 540bp. Lane 1: 100 bp molecular weight marker, Lane 2: Positive control (*Acinetobacter baumannii* ATCC 19606), Lane 3: Negative control, Lanes 4-12: Isolates positive for *P2R2* promoter gene.



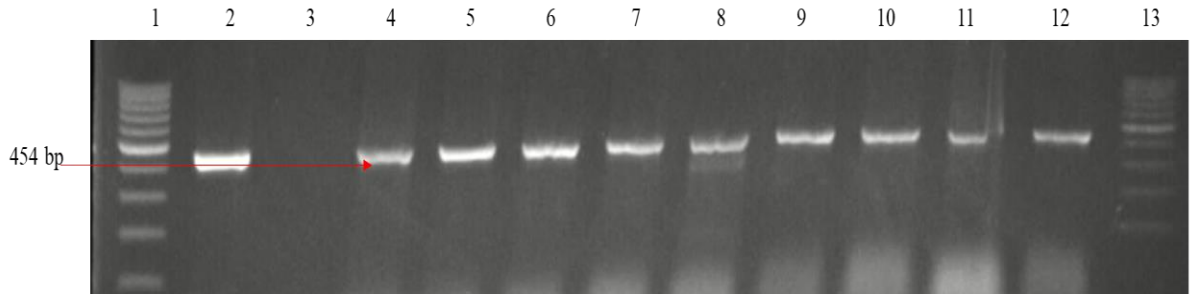


**Figure A9.** A representative gel showing isolates positive for the *Citrobacter* urease gene at 454 bp for the molecular confirmation of *Citrobacter* spp. Lanes 1 and 13: 100 bp molecular weight marker, Lane 3: Negative control, Lanes 4-12: Some isolates positive for *Citrourease* gene.

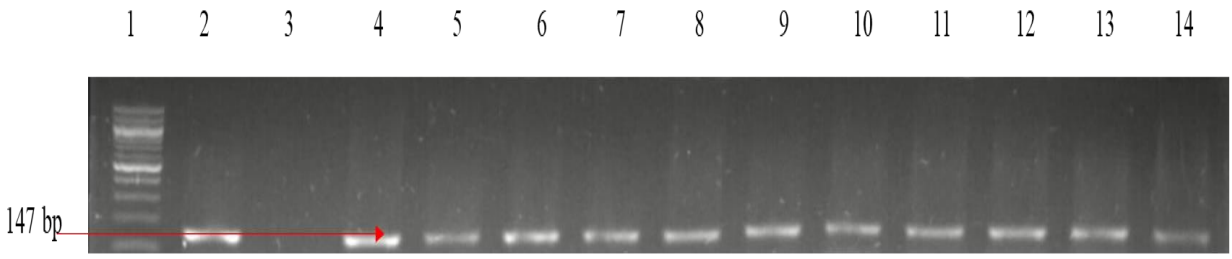


**Figure A10.** A representative gel showing isolates positive for the *uidA* gene at 147 bp for *E. coli*. Lane 1: 100 bp molecular weight marker, Lanes 2: Positive control (*E. coli* ATCC 8739), Lane 3: Negative control, Lanes 4-14: Some isolates positive for *uidA* gene.

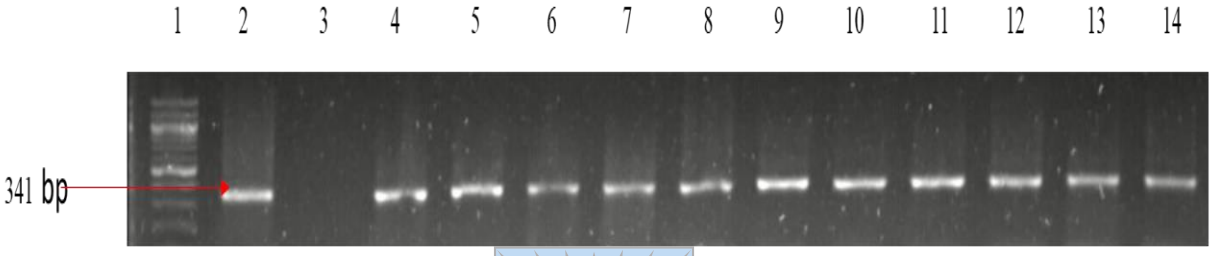


**Figure A11.** A representative gel showing isolates positive for the *hsp 60* gene at 341 bp. Lane 1: 100 bp molecular weight marker, Lanes 2: Positive control (*E. cloacae* ATCC BAA-2341$^{TM}$), Lane 3: Negative control, Lanes 4-14: Some isolates positive for *hsp 60* gene.



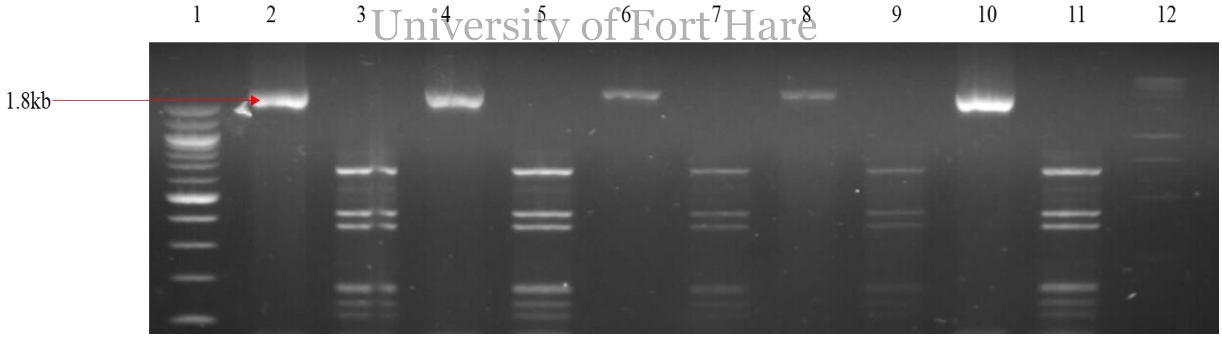


**Figure A12.** A representative gel of a restriction fragment length polymorphism showing undigested and digested of class 2 gene cassette region of using *aluI* enzyme. Lane 1: 100 bp molecular weight marker, Lanes 2-11: undigested amplicon and its respective digested RFLP amplicon, Lane 12: 1kb molecular weight marker.

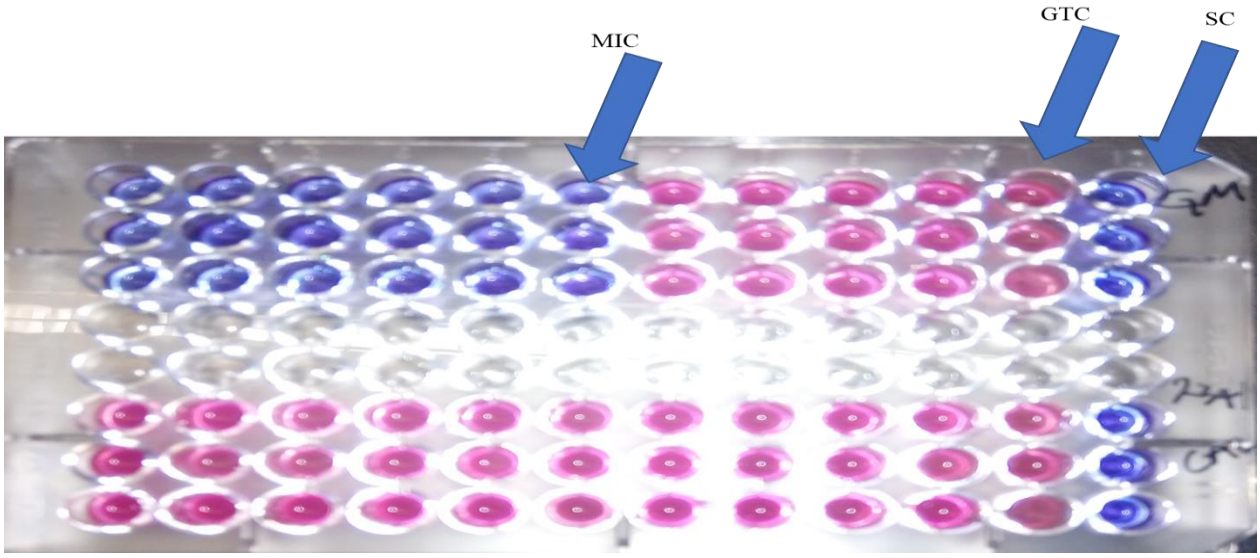


**Figure A13.** A microtiter plate showing the minimum inhibitory concentration of gentamicin and ceftazidime of a multidrug-resistant *K. pneumoniae* after the addition of 30 µL resazurin dye (*w*/*v*, 0.015%).

GTC: Growth control; SC: Sterility Control; MIC: Minimum inhibitory concentration; MIC gentamicin = 4µg/ml; and MIC Ceftazidime ≥128µg/ml.

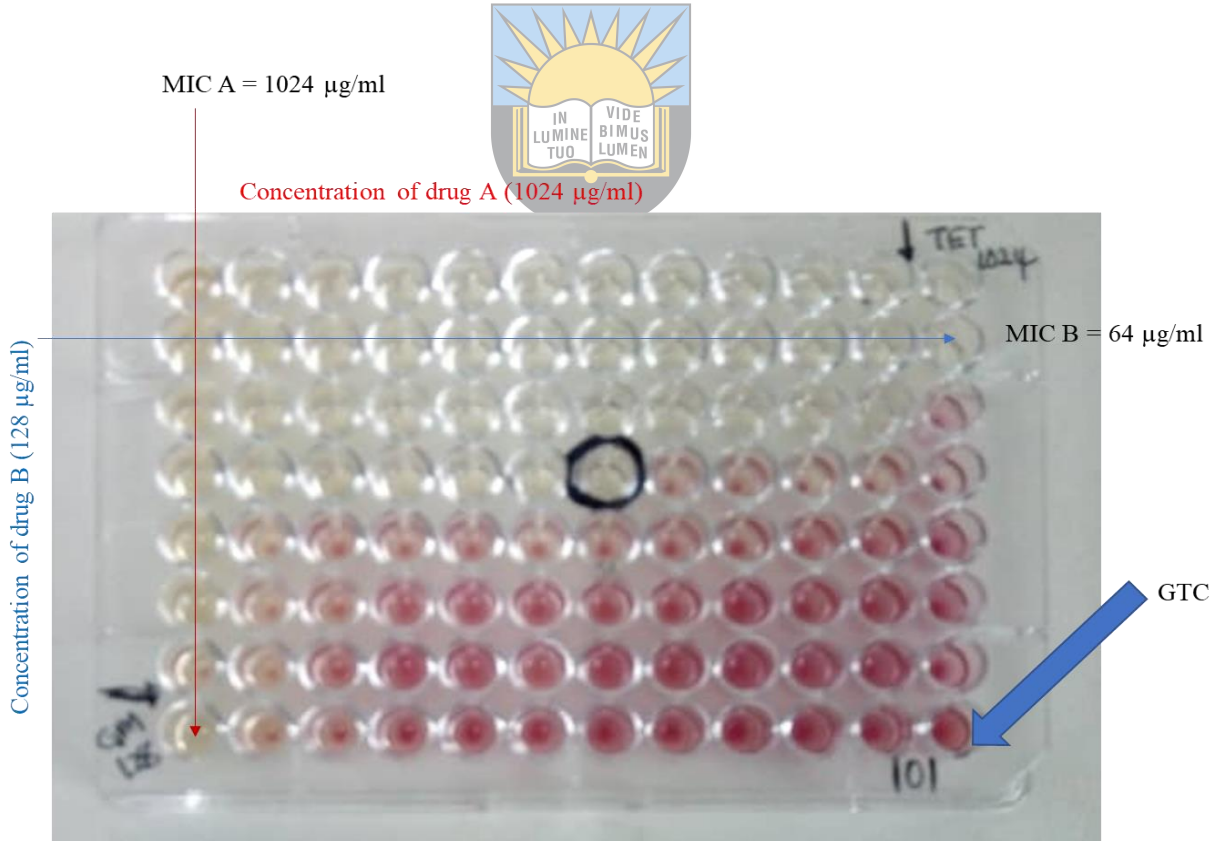


**Figure A14.** A microtiter plate showing the minimum inhibitory concentration of gentamicin and ceftazidime of a multidrug-resistant *E. coli* after the addition of 30 µl of the 2,3,5, triphenyl tetrazolium chloride dye.

GTC: Growth control MIC: Minimum inhibitory concentration. MIC A (tetracycline)= 1024µg/ml; MIC B (gentamicin )= 64µg/ml; well circled in black represents the MIC in combination.

**Sequences and accession numbers of some of the genes on the gene cassettes in the integrons detected in the study deposited in the NCBI gene bank were as follows:**

*dfrA21* OP380275

ATGAACCCGGAATCGGTCCGCATTTATCTGGTCGCTGCCATGGGTGCCAA

TCGGGTTATTGGCAATGGTCCCGATATCCCCTGGAAAATCCCAGGTGAGC

AGAAGATTTTTCGCAGGCTCACCGAGAGCAAAGTGGTCGTTATGGGCCGC

AAGACATTTGAGTCCATAGGCAAGCCCTTACCAAACCGCCACACAGTGGT

GCTCTCGCGCCAAGCTCGTTATAGCGCTCCTGGTTGTGCAGTTGTTTCAA

CGCTGTCACAGGCTATCGCCATCGCAGCCGAACACGGCAAAGAACTCTAC

GTAGCCGGCGGAGCCGAGGTATATGCGCTGGCGCTACCGCATGCCAACGG

CGTCTTTCTATCTGAGGTACATCAAACCTTTGAGGGTGACGCCTTCTTCC

CAGTGCTTAACGCAGCAGAATTCGAGGTTGTCTCATCCGAAACCATTCAA

GGCACAATCACGTACACGCACTCCGTCTATGCGCGTCGTAACGGCTAA



partial *dfrA7* OP380276

ATGGCCCTGATATCCCATGGTCAGCAAAAGGTGAGCAGTTACTCTTTAAA

GCGCTCACATATAATCAGTGGCTCCTTGTTGGAAGGAAAACATTTGACTC

TATGGGTGTTCTTCCAAATCGAAAATATGCAGTAG

*dfrA21* OP380277

ATGAACCCGGAATCGGTCCGCATTTATCTGGTCGCTGCCATGGGTGCCAA

TCGGGTTATTGGCAATGGTCCCGATATCCCCTGGAAAATCCCAGGTGAGC

AGAAGATTTTTCGCAGGCTCACCGAGAGCAAAGTGGTCGTTATGGGCCGC

AAGACATTTGAGTCCATAGGCAAGCCCTTACCAAACCGCCACACAGTGGT

GCTCTCGCGCCAAGCTCGTTATAGCGCTCCTGGTTGTGCAGTTGTTTCAA

CGCTGTCACAGGCTATCGCCATCGCAGCCGAACACGGCAAAGAACTCTAC

GTAGCCGGCGGAGCCGAGGTATATGCGCTGGCGCTACCGCATGCCAACGG

CGTCTTTCTATCTGAGGTACATCAAACCTTTGAGGGTGACGCCTTCTTCC

CAGTGCTTAACGCAGCAGAATTCGAGGTTGTCTCATCCGAAACCATTCAA

GGCACAATCACGTACACGCACTCCGTCTATGCGCGTCGTAACGGCTAA

*aadA1* OP380278

ATGAGGGAAGCGGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGT

TGGCGTCATCGAGCGCCATCTCGAACCGACGTTGCTGGCCGTACATTTGT

ACGGCTCCGCAGTGGATGGCGGCCTGAAGCCACACAGTGATATTGATTTG

CTGGTTACGGTGACCGTAAGGCTTGATGAAACAACGCGGCGAGCTTTGAT

CAACGACCTTTTGGAAACTTCGGCTTCCCCTGGAGAGAGCGAGATTCTCC

GCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGT

TATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACAT

TCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCT

TGCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCG

GAGGAACTCTTTGATCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAA

TGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGCGATGAGC

GAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGC

AGAATCGCGCCGAAGGATGTCGCTGCCGACTGGGCAATGGAGCGCCTGCC

GGCCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTTATCTTGGAC

AAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTT

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*aadA5* OP380279

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