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Conjugative plasmids disseminating ctx-m-15 among enterobacteriaceae from human, animals and the environment in Mwanza Tanzania: a need to intensify one health approach

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CONJUGATIVE PLASMIDS DISSEMINATING CTX-M-15 AMONG *ENTEROBACTERIACEAE* FROM HUMAN, ANIMALS AND THE ENVIRONMENT IN MWANZA TANZANIA: A NEED TO INTENSIFY ONE HEALTH APPROACH

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A Dissertation Submitted in Partial Fulfillment of the Requirement for the Degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology

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ABSTRACT

Globally, *bla*$_{\text{CTX-M-15}}$ beta-lactamases are the most popular extended spectrum beta-lactamase alleles that are widely distributed due its mobilization by mobile genetic elements in several compartments. We aimed to determine the conjugation frequencies and replicon types associated with plasmids carrying *bla*$_{\text{CTX-M-15}}$ gene from Extended Spectrum Beta-lactamase producing isolates in order to understand the dissemination of resistance genes in different compartments. A total of 51 archived isolates carrying *bla*$_{\text{CTX-M-15}}$ beta-lactamases were used as donors in this study. Antibiotic susceptibility tests were performed as previously described for both donors and transconjugants. Conjugation experiment was performed by a modified protocol of the plate mating experiment, and plasmid replicon types were screened among donor and transconjugant isolates by multiplex Polymerase Chain Reaction in a set of three primer panels. *Escherichia coli* was recovered from majority of isolates. The conjugation efficiency of plasmids carrying *bla*$_{\text{CTX-M-15}}$ was 88.2% (45/51) with conjugation frequencies in the order of 10^{-1} to 10^{-9} and a 100% transfer efficiency observed among *E. coli* of animal origin. Majority of donors (n = 21) and transconjugants (n = 14) plasmids were typed as either Inc FIA or Inc FIB. Resistance to non-beta-lactam antibiotics was transferrable in 34/45 (75.6%) of events. Ciprofloxacin, tetracycline and sulphamethoxazole-trimethoprim resistance was co-transferred in 29/34 (85.3%) such events. Gentamicin resistance was transferred in 17/34 (50%) of events. Majority of plasmids carrying *bla*$_{\text{CTX-M-15}}$ were conjugatively transferred by IncF plasmids along with non-beta lactam resistance. There is a need for more research on plasmids to understand how plasmids, especially multi replicon plasmids interact and the effect of such interaction on conjugation. One Health approach is to be intensified to address antimicrobial resistance which is a public health threat.

Keywords: *Conjugation; CTX-M-15; replicon; plasmid; non-beta lactam antibiotics; One-health*

DECLARATION

I, Caroline Anold Minja, do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor concurrently submitted for a degree or similar award in any other institution.

Caroline Anold Minja

Candidate Name	**Signature**	**Date**

The above declaration is confirmed

Prof. Gabriel Shirima

Supervisor (1)	**Signature**	**Date**

Prof. Stephen E. Mshana

Supervisor (2)	**Signature**	**Date**

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CERTIFICATION

The undersigned certify that they have read the dissertation titled: "*Conjugative Plasmids Disseminating CTX-M-15 among Enterobacteriaceae Recovered from Human, Animals and the Environment of Mwanza Tanzania*: *A Need to Intensify One Health Approach"* and recommended for examination in fulfilment of the requirements for the degree of Master's in Life Sciences of the Nelson Mandela African Institution of Science and Technology.

Prof. Gabriel Shirima

Supervisor (1)	**Signature**	**Date**

Prof. Stephen E. Mshana

Supervisor (2)	**Signature**	**Date**

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DEDICATION

This work is dedicated to my sons-Reynold and Samwel. Always work with integrity and be an inspiration.

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LIST OF ABBREVIATIONS AND SYMBOLS

AMR	Antimicrobial Resistance
CLSI	Clinical and Laboratory Standards Institute
CREATES	Centre for Research, Agricultural Advancement, Teaching Excellence and Sustainability in Food and Nutritional Security
CUHAS	Catholic University of Health and Allied Sciences
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetra-Acetic Acid
ESBL	Extended Spectrum Beta-Lactamase
HGT	Horizontal Gene Transfer
Inc	Incompatibility Group
IS	Insertion Sequences
MGE	Mobile Genetic Element
NAP	National Antimicrobial Resistance Action Plan
OriT	Origin of Transfer
PBRT	PCR based Replicon Typing
PCR	Polymerase Chain Reaction
SHV	Sulfhydryl Variable
ST	Sequence Type
T4SS	Type IV Secretion System
TEM	Temoneira
Tn	Transposon

CHAPTER ONE

INTRODUCTION

1.1 Background of the problem

Third generation cephalosporins and aztreonam antibiotics are responsible for the resistance exhibited by Extended Spectrum Beta Lactamase (ESBL) producing bacteria with exception to cephamycins, carbapenems and beta-lactamase inhibitors such as Clavulanic acid (Bush *et al*., 1995; Paterson & Bonomo, 2005). The third generation class of antibiotics were identified after penicillins, but ESBL enzymes against these drugs were not recognized until the first medicinal application of penicillins. The spread of these enzymes continue to be a challenge to the activity of these antibiotics which provided solution to the treatment of *enterobacteriaceae* and other gram-negative bacterial infections (Paterson & Bonomo, 2005). Extended Spectrum Beta Lactamases; are named for the extended action of the enzyme against a wide variety of antibiotics including ceftazidime, ceftriaxone, and cefotaxime which are also substrates for these enzymes. It is the single nucleotide mutations of one or more amino acid near the active site of the previous broad spectrum TEM-1, TEM-2, or SHV-1 beta-lactamases that evolved ESBL (Paterson & Bonomo, 2005). In natural environments, ESBL enzymes are chromosomally mediated by the selection pressure induced by beta-lactamase producing soil organisms or the irrational use of third generation antibiotics (Paterson & Bonomo, 2005). However, as reviewed by Cantón *et al*. (2012), the evolution of plasmid-mediated ESBL is attributed to the incorporation of resistance determinants by chromosome mobilizing elements such as insertion sequences or transposons that evolve plasmid mediated ESBL after causing mutation on such genetic environments.

The CTX-M beta-lactamases, are the most important class of beta-lactamases named after their strong hydrolytic activity against cefotaxime than other extended spectrum cephalosporins (Blair *et al*., 2015; Rossolini *et al*., 2008). There are over 100 CTX-M beta-lactamase alleles in five distinct phylogenetic groups which were originally thought to be predominant in S. America, E. Europe, and the Far East, but currently are the most popular ESBL worldwide (Blair *et al*., 2015).

Beta-lactamases of CTX-M may be chromosomally (Hirai *et al*., 2013) or plasmid-encoded, this accounts for their clonal and horizontal spread among diverse hosts harbouring these enzymes especially *Escherichia coli.* Specifically, the precursors of plasmid-mediated CTX-

M-15 are environmental *Kluyvera* spp whose chromosomal CTX-M clusters incorporated to the chromosome of host bacteria by mobilizing elements like *ISEcp1* or *ISCR1* (Cantón *et al*., 2012). The location of *ISEcp1* upstream $bla_{\text{CTX-M}}$ genes together with multiple inverted repeats downstream the gene facilitates the expression and ongoing transposition of bla_{KLU} genes to various CTX-M enzymes that include plasmid-mediated CTX-M-15 (Cantón *et al*., 2012; Naseer & Sundsfjord, 2011). The mobilization potential of *ISEcp1* for chromosome-linked multi-resistant determinants in other members of *enterobacteriaceae* increases with the additional possession of *ISCR1*; another mobile genetic element (MGE) embedded in a Class 1 integron that mobilizes unrelated CTX-M groups from similar or different species. This describes how bla_{KLU} mobilization by insertion sequences or transposons increases the expression and spread of mobilized CTX-M genes and steers the multidrug resistance effect (Cantón *et al*., 2012).

In Tanzania, $bla_{\text{CTX-M-15}}$ gene is the predominant ESBL allele with highest proportions in animals (90.9%) and the environment (92.3%) compared to that in human (72.5%) (Seni *et al*., 2018). It is also attributed to infections occurring in hospital and community settings (Blomberg *et al*., 2005; Mshana *et al*., 2011). Along with the gene's role in causing human infections, it is also isolated from companion and wild animals (Moremi *et al*., 2016; Seni *et al*., 2016). The successive spread of the gene in multiple ecological niches raises the burden of ESBL infections and threatens the failure of current successful antibiotics. Moreover, existence of similar CTX-M-15 clones circulating in human, animal and environmental interfaces (Seni *et al*., 2018) suggests a continuous flow of antimicrobial resistant determinants that threaten ongoing infection prevention and control strategies and increase antimicrobial selection pressure in these settings. With Tanzania in the midst of implementing her National Action Plan on AMR 2017-2022 (Ministry of Health [MoH], 2017), the strategic objective number two within this plan is on "*Strengthening the Knowledge and Evidence Based through Surveillance and Research*". The goal of this study fits in this objective that aimed to determine conjugation frequencies of plasmids carrying $bla_{\text{CTX-M-15}}$ gene between human, animal and environmental ESBL isolates and characterize plasmid replicon types associated with the transferred gene in order to understand factors that select the gene in either setting.

1.2 Statement of the problem

The prevalence of bacteria producing ESBL varies from 25% to 50% percent in Tanzania (Mshana *et al*., 2013; Mshana *et al*., 2009), and specifically the overall prevalence of *bla*$_{\text{CTX-M-15}}$ in human, animal and environment sources is 22.6% (Seni *et al*., 2018), with *E. coli* as the predominant strain. Plasmids carrying *bla*$_{\text{CTX-M-15}}$ genes circulate in both community (Mshana *et al*., 2016) and hospital settings (Mshana *et al*., 2011) in combination with quinolone and aminoglycoside resistance genes (Seni *et al*., 2016). Furthermore, it was observed that majority of *E. coli* found in the environment and from fish carries *bla*$_{\text{CTX-M-15}}$ (Moremi *et al*., 2016; Mshana *et al*., 2016). The successive adaptation of these dominant Antimicrobial resistant (AMR) vectors in diverse niches exceedingly limit therapeutic options especially in developing countries like Tanzania where proposed One Health *bla*$_{\text{CTX-M-15}}$ surveillance have unfavorably focused on either human, animal or environment interface (Frumence *et al*., 2021). Therefore, there is a possibility of an extensive variation in the epidemiology of *bla*$_{\text{CTX-M-15}}$ allele from human, animals and environmental isolates in Mwanza Tanzania.

1.3 Rationale of the study

Multiple clones of CTX-M-15 producing *E. coli* circulate between human, animals, and the environment as previously summarized (Seni *et al*., 2018). Mobile genetic elements (MGE) carrying the allele are exchanged between bacteria in these settings (Mshana *et al*., 2013). With the increasing persistence of *bla*$_{\text{CTX-M-15}}$ gene in Tanzania and limited information on the presence of the gene's alleles in any compartment, even non-conjugative bacteria may acquire and transmit the gene evolving new resistant strains. This study has therefore added to the understanding of the importance of IncF plasmids in the dissemination of multidrug-resistant determinants in human, animal and environment settings and is an evidence for the importance of One Health based interventions and research to address AMR.

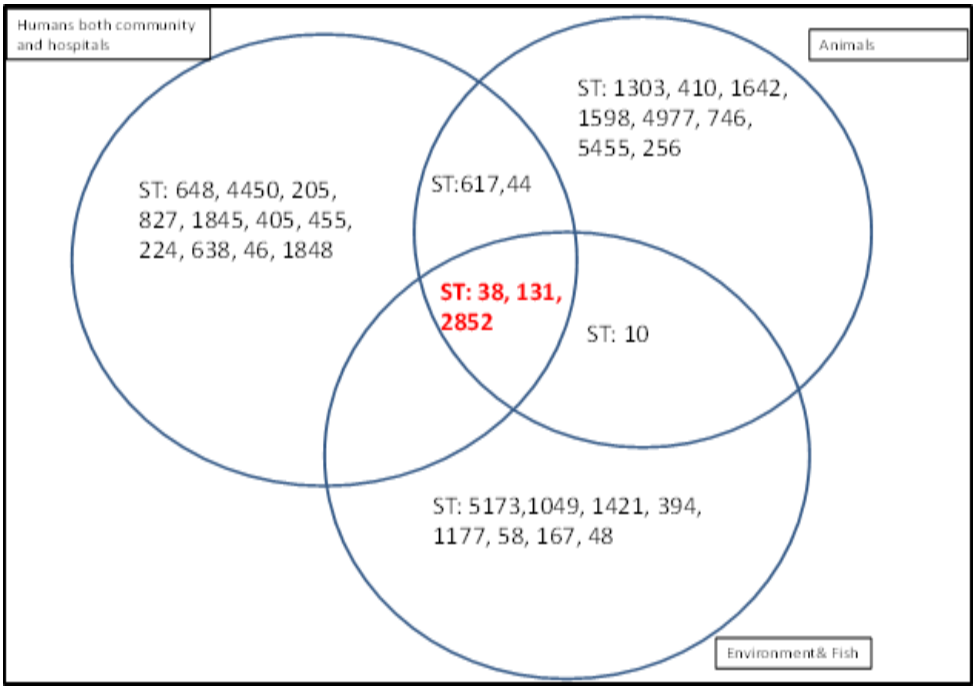

Figure 1: *Escherichia coli* genotypes detected in various compartments with ST 38, 131 and 2852 found in all sources

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1.4 Research objectives

1.4.1 General objective

To establish conjugation frequencies of *bla*$_{\text{CTX-M-15}}$ and plasmid replicon types from human, animal and environmental ESBL isolates in order to understand the gene's flow rate and implement better antibiotic stewardship approaches.

1.4.2 Specific objectives

(i) To determine conjugation frequencies of *bla*$_{\text{CTX-M-15}}$ from human, animals and environmental ESBL isolates of Mwanza Tanzania.

(ii) To determine plasmid replicon types spreading *bla*$_{\text{CTX-M-15}}$ gene among *enterobacteriaceae* donors and transconjugants from human, animals and the environment of Mwanza Tanzania.

1.5 Study hypothesis

(i) Null hypothesis: There is no significant difference between *bla*$_{\text{CTX-M-15}}$ carrying plasmids circulating in human, animal and environment ESBL isolates in Mwanza Tanzania.

(ii) Alternate hypothesis: There is a significant difference between *bla*$_{\text{CTX-M-15}}$ carrying plasmids circulating in human, animal and environment ESBL isolates in Mwanza Tanzania.

1.6 Significance of the study

This study has emphasized the importance of conjugation in disseminating multidrug-resistant bacteria in human, animal and the environment of Mwanza Tanzania and further calls for more intensive One Health approaches to address the threat.

1.7 Delineation of the study

This study focused on One Health Surveillance of antimicrobial resistance in human animal and environmental interfaces. Results obtained supports the continuing nation-wide AMR mitigation strategies and calls for improvements that must engage all arms focusing on human animal and environment health management.

CHAPTER TWO

LITERATURE REVIEW

2.1 An overview of the conjugation process in prokaryotes and eukaryotes

Conjugation, the process of genetic exchange is a unique source of genetic variation in both prokaryotes and eukaryotes as reflected by genotypically diverse elements involved in the process. Conjugation can not only occur between closely related organisms within a genus but also between distantly associated organisms across genera and kingdoms (Duy *et al*., 2019; Matsuo *et al*., 2010; The *et al*., 2016) with the involvement of functional genes in the latter organism (s). Conjugation involves nicking of a conjugative element at the origin of transfer (oriT) by relaxase enzymes that form a relaxosome in the cytoplasm. The relaxosome prepares the ssDNA of a conjugative element for transfer and releases the relaxase. Next, the ssDNA is transferred to the recipient's type IV secretory system (T4SS) by a coupling protein. Finally, the transferred ssDNA is replicated to required copy numbers in the recipient host (Zechner *et al*., 2012).

Mechanisms used by MGEs to facilitate conjugation process varies depending on the type of element, transfer or integration mechanism involved and the type of target sequence involved (Wozniak & Waldor, 2010). Terms like conjugative elements, mobilizing elements, site specific homologous integration, and site-independent non-homologous integration reflect the complexity of the process in both donors and recipients. Conjugation studies have grouped the ones known different but now conjugatively related; viruses, plasmids and transposons (Reanney, 1976) which possess related mechanisms of transfer or integration as previously established from similar conserved sequences obtained from conjugative proteins and the type IV secretion systems in these organisms (Juhas *et al*., 2008).

2.2 Mobile genetic elements: How they disseminate and evolve AMR genes

Among prokaryotes, the existence of a compressed arrangement of their genome in a cascade of functional genes is core to the successful creation of novel mosaic regions that through recombination and HGT increases diversity by the addition, deletion or capture of genes (Burrus & Waldor, 2004). Reported by Norman *et al*. (2009), the super genome concept illustrates how the private and communal gene pools of bacterial chromosomes or MGE facilitate the exchange of small DNA fragments respectively. While the communal gene pool

possesses extra-chromosomal elements including translocative elements (rearranges chromosomal genes) operative elements (exchanges between extra-chromosomal backbones but can be integrated into the host's private gene pool) and dispersive elements like conjugative plasmids (the permanent vector of HGT that transfers operative elements following their incorporation by translocative elements); the private gene pool have genomic islands (Juhas *et al*., 2008; Norman *et al*., 2009); which together form a diverse source of gene exchange among bacteria hosts.

Plasmids are extra chromosomal genomic elements in bacteria that can autonomously replicate in suitable hosts and are vectors of HGT, a well-adapted means of spreading antimicrobial resistance genes between organisms (Carattoli, 2013). Plasmids are the most common MGE classified as self-transmissible like conjugative plasmids or mobilizable like non-conjugative plasmids whose mobilization is via conjugative plasmids or conjugative genetic elements like transposons, insertion sequences and others. It is by utilizing available transfer systems in the former or following transfer activation by conjugative elements in the latter that conjugation is successful (Han *et al*., 2018). Moreover, another form of HGT among small plasmids is through trans-mobilization by conjugative elements (Moran & Hall, 2019; Ramsay & Firth, 2017; Salyers & Shoemaker, 1994).

In non-conjugative plasmids, mobilization and mutation events acting on the plasmid's genome may account for the rapid evolution of resistant plasmids to co-integrate plasmids when multi-replicon plasmids are involved (San Millan *et al*., 2014). Complexity increases if the co-integrate plasmid comprises of multiple resistant genes and involves a broad range of hosts that through mobilization may disseminate such genes to other plasmids. Transposition is the driving force for macro mutations caused by MGE exchange of resistance genes from different plasmids or acquisition of such genes from other replicons (Sýkora, 1992), these mutations evolve and adapt bacteria in their environments. Example, the global dissemination of $bla_{\text{CTX-M-15}}$ is by diverse and mosaic plasmids from bacteria isolates of human, animal and the environment where mutation events exacerbate the mobilization of the gene through insertion, deletion or DNA sequence rearrangements around the gene. Vounba *et al*. (2019), reported the faecal carriage of $bla_{CTX\text{-}M\text{-}15}$ gene in chicken by plasmid types I1, FIB, R and HI1 with proof of an epidemiological variation of these plasmids among *enterobacteriaceae*. In the study, the reported multidrug resistance was due to the co-transfer and selection for multi-replicon $bla_{CTX\text{-}M\text{-}15}$ plasmids through mutation.

Other common mobile genetic elements include transposons and insertion sequences. Transposons can transfer or facilitate the transfer of intact DNA sequences into other chromosomal sites in the same cell or to other cells. Illustratively, transposition events are facilitated by long transposon (Tn) sequences or short insertion sequences (IS) after the excision and integration of such sequences into targets.

The effects of transposition differ with the type of target, target sequence or integration mechanism; transposition target may be a specific gene sequence or plasmid co-integrate, it is site specific or site independent integration mechanisms that control the homologous or non-homologous recombination on the target. For example, when the transposon target is a gene associated with a resistance phenotype, transposition events may result to an overexpression, inactivation or mobilization of the phenotype when the Tn or IS is located upstream, in between or when two such Tn flanks the gene respectively (Hawkey, 2017) .

Conjugative transposons and conjugative plasmids mobilizes genes associated with transposons by facilitating their transfer conjugatively or self-transfer respectively. Different from other conjugative elements, conjugative transposons do not transfer associated genes when transferred to recipient cells because they are not part of the transfer machine; instead, they mobilize non-conjugative MGE to transfer such phenotypes through insertion sequence integration of homologous DNA in those elements. However, a self-initiated transfer of Tn associated genes is by homologous site-specific integration of conjugative plasmids carrying the gene. In both aspects, IS first serve as independent elements of transpositional gene mobilization through a homologous site specific integration of a self-transmissible mobile genetic element (conjugative plasmids or conjugative transposon) or chromosomal integration of the gene's target sequence within a conjugative transposon, secondly the IS facilitates the simultaneous expression and transfer of genes associated with the mobile element promoting its dissemination to other recipients (Janatova *et al*., 2014). For example, transposon Tn2 possess a transposition region having $bla_{\text{CTX-M-15}}$ associated with *ISEcp1* insertion sequence received from a multidrug-resistant self-transmissible plasmid (Monárrez *et al*., 2019), an arrangement well known to disseminate the gene around the globe (Coque *et al*., 2008). The gene's genetic environment can, therefore explain an increased frequency of transfer.

2.3 Plasmids disseminating CTX-M ESBLs

Globally, CTX-M beta-lactamases are diverse and keep evolving in various epidemic plasmids. For example, CTX-M-3 beta-lactamases sporadically disseminate by IncL/M plasmids in Poland and later throughout Europe unlike CTX-M-15 which are mainly transmitted by IncFII plasmids (Peirano & Pitout, 2010), CTX-M-1 reported in the food chain are disseminated by IncI1, IncN, and HIIB from bacteria isolates of both health and diseased human (Duy *et al*., 2019), animals of food origin (Kaldhone, 2017), and the aquatic environment (Zurfluh *et al*., 2014).

The narrow host range IncF family of plasmids successfully adapt *enterobacteriaceae* in several environments because it is a self-transmissible, low copy number plasmid whose replication and regulation is host-dependent, these plasmids keep disseminating to be maintained in respective hosts. Second, the TraD coupling protein in IncF plasmids is a virulence factor that activates virulence genes, including AMR genes existing in any host harbouring IncF plasmids (Szczepanowski *et al*., 2005). Another aspect of concern is Inc F plasmid's fertility inhibition systems which inhibit the transfer and regulation of other plasmids in the same strain; this system is inactive in F like plasmids because IncF possesses a self-regulatory promoter that overpowers such systems permitting their epidemic spread. Lastly, the co-residency of IncF multi-replicons (FA, FII and FB) with other replicons, drive the selective divergence of replicon FII that compensate Inc F fitness costs and evolve F like plasmids as mosaic and compatible broad-host-range plasmids (Villa *et al*., 2010). These reasons explain why IncF plasmids are successful over other conjugative plasmids like Inc P or IncW in both natural and selective bacteria communities.

Alternatively, the broad host range, high copy number plasmids like IncP, IncN and IncW that are host independent for replication control and regulation are reported to predominate the soil (Yahia *et al*., 2018), and polluted environments (Amos *et al*., 2014); transferring or mobilizing selective genes like antibiotic resistance genes, pesticide tolerance, catabolic degradative genes and metal tolerance genes (Anjum *et al*., 2011). In these environments, digestives and organic fertilizers from human or the food chain (Klümper *et al*., 2015; Wang & Yu, 2012) are reported reservoirs of $bla_{\text{CTX-M-15}}$ positive bacteria in the soil and in such polluted environments.

In Tanzania, IncF plasmids carrying *bla*$_{\text{CTX-M-15}}$ are the main plasmids reported to disseminate this gene in bacteria isolated from human, animal and environment (Moremi *et al*., 2016), the highest proportion is reported in animals (90.9%) and the environment (92.3%) compared to that in human (72.5%) (Seni *et al*., 2018). As Tanzania is implementing her National Antimicrobial resistance Action plan of 2017-2022, (MoH, 2017), studies focusing on dominant AMR resistant genes like *bla*$_{\text{CTX-M-15}}$ gene are important as they implement the objective of providing Evidence Based Research and Surveillance of AMR.

2.4 Environmental changes disseminate resistance determinants

In non-selective environments, bacteria exist as natural populations, but a change in environmental factors can modify, evolve or replace such niches. Environmental exposures like antimicrobial resistance genes can affect the abundance, distribution and transfer rates of bacteria residing in these natural environments. In a study where ESBL producing *E. coli* existed naturally in a river with few dominant plasmid genotypes, replacement with an increased number of the same but genetically diverse bacteria occurred when wastewater effluents were introduced at sites downstream the river (Dinatale, 2017). It was further reported that evolved plasmids in such bacteria contained alternative plasmid genes and addictive systems that selectively favored their adaptation. Another epidemiologically different but related study reported wastewater effluent disseminating CTX-M-15 in non-selective natural environments (Amos *et al*., 2014). In both studies, IncF plasmids dominated such effluent exposed sites and competitively replaced pre-existing plasmids along with the spread of CTX-M-15 in higher conjugation frequencies.

2.5 Factors affecting transfer efficiency and persistence of mobile genetic elements

The presence of one plasmid in a bacteria may enhance or prevent the stable transfer of another through the process of facilitation (Sagai *et al*., 1977). This property was reported later Gama *et al*. (2017a, 2017b, 2017c), whereby co-residing plasmids existing in the same host cell with varying transfer efficiencies, the most efficient facilitated the transfer of other plasmids. It is possible that the close interaction between plasmids in the cell triggered the formation of a mating pair to transfer the plasmids.

When co-resident plasmids are in different host cells, surface exclusion can prevent the transfer of other plasmids, or conjugation efficiencies may decrease when the transfer of one gene is independent of another (Mitra *et al*., 2019). Alternatively, for small plasmids which

were previously retro transferred from cells (Moran & Hall, 2019), the presence of more than one copy of oriT sequence activates their mobilization by conjugative plasmids. Among conjugative elements like conjugative plasmids; the higher the transfer rate, the higher the chance or probability that such plasmids have compensated for plasmid costs like plasmid loss or growth disadvantages similar to any co-carried gene in such element's gene pool that will be maintained in the respective bacteria population (Gama *et al*., 2018).

Conjugative relaxes which resolves co-integrates in recipient cells can also affect transfer efficiencies because recombination between plasmids of different incompatibility groups can result to a co-integrate expressing both incompatibility groups and which are reflected in each cell after resolution, the newly formed co-integrate replicon can either be maintained stably in the same cell, or if co-integrates in recipient cells are not resolved, conjugation rates are decreased (Wang *et al*., 2013). However, in such cases divergent mutation on one incompatibility group may evolve a newly compatible replicon to the once incompatible replicon and selectively transfer both plasmids (Sýkora, 1992). Alternatively, recombination events occurring between plasmids of the same incompatibility group in a single cell (Levin, 1994) and especially Inc F possessing cells (Coque *et al*., 2008) increases plasmid diversity while decreasing transfer efficiencies in these plasmids.

2.6 Fitness costs following conjugation transfer among enterobacteriaceae

Several plasmids and host factors that include host type, genetic environment and transferred elements involved contribute to physiological and energetic costs that follow transfer events as reviewed by Baltrus (2013). The reviewer suggested of HGT costs due to the replacement or insertions of DNA sequences in chromosomes that affect gene expression. The former alters functional proteins, while the latter imposes metabolic changes that depend on the plasmid size. In such alterations, unless compensatory mutations act on the DNA, fitness costs to select for HGT associated phenotypes remains very high (Harrison *et al*., 2015).

Genotype silencing of plasmid associated genes is a fitness advantage for plasmids harbouring such mutations, especially in non-selective environments (Humphrey *et al*., 2012). When such genes are antimicrobial resistance determinants, the existing environment becomes a reservoir for AMR genes that silently disseminate through mobile genetic elements in very low, close to undetectable transfer efficiencies.

In plasmids, low conjugation rates impose selection and adaptation costs to genes carried on the plasmid because resulting metabolic and physiological costs pose instability to plasmids and affect the expression of plasmid associated genes (Harrison & Brockhurst, 2012).

2.7 Methods of plasmid replicon typing

Typing plasmid replicons is essential for their correct classification and source tracing (Novick, 1987). In 2005, a PCR based replicon typing (PBRT) scheme classified plasmids based on common circulating replicons using 18 pairs of primers in 5 multiplex and three simplex reactions (Carattoli *et al*., 2005). However, due to hidden mutations that kept evolving unknown and novel replicons together with the reported difficulty of typing multi replicon plasmids (Villa *et al*., 2010); a continuous update of the scheme was expected. In response to the challenge, replicon target sequencing devised new PCRs for undetected replicons using PBRT kit as reported by Carattoli (2013) which detected IncFIA, FIB and FIC as subtypes of IncF in addition to two other new replicons that were devised to detect *qnr* genes in Salmonella (Villa *et al*., 2010). In the same year, new IncFII replicons (IncFII, Y, FIIK and FIIS) were proposed for *Yersinia, Klebsiella* and *Salmonella* respectively. To date, PBRT remains the gold standard for plasmid replicon typing among *enterobacteriaceae*.

2.8 Circulating sequence types disseminating CTX-M-15

Allelic variations that disseminate resistance genes differs among hosts and environments. Such variations could be a resultant of single nucleotide selection associated with HGT events that occur between bacteria clones within species harbouring the gene (Li *et al*., 2019). In the same study, multiple antimicrobial resistance genotypes co-exist within species of the gut microbiota before antibiotic use, and later some clones become distinctly selected in association with beneficial alleles where gene's abundance and HGT potential increases during antibiotic selection pressure. Similarly, the $bla_{\text{CTX-M-15}}$ is successfully disseminated by a clonal complex of sequence types circulating among enterobacteriaceae strains. Predominantly, the global ST131 of Inc F with other epidemiologically adapted sequences like ST 39 and ST4 in hospitals and the community (Mansour *et al*., 2015), ST 69 among human and animals in the community (Ewers *et al*., 2014) as well as ST10 (Said *et al*., 2015) and ST 2695 (Inwezerua *et al*., 2014) in soil and aquatic environments. In all studies, *Klebsiella spp and Escherichia coli* were reported as hotspots for such allelic differentiation. Therefore, the predominance of any clonal variant in a host is possibly a result of the

characteristic mobile genetic element that determine the persistence of the gene in either human, animal or the environment setting.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study isolates

All isolates used as donors in this study were obtained from the Catholic University of Health and Allied Sciences (CUHAS) in Mwanza Tanzania. A total of 51 *bla*$_{\text{CTX-M-15}}$ positive isolates were purposively selected and activated overnight in Luria Bertani (LB) broth at 37 $^{\circ}$C ready for use in conjugation and PBRT techniques.

Among the 51 isolates, twenty-two *bla*$_{\text{CTX-M-15}}$ positive isolates were obtained from a study that reported the magnitude of fecal carriage and diversity of ESBL genotypes among human residing in rural communities of Mwanza Tanzania (Mshana *et al*., 2016), 12 other *bla*$_{\text{CTX-M-15}}$ positive isolates were from a study that reported the fecal carriage of ESBL among companion and domestic farm animals that included pigs, chicken, dogs and goats (Seni *et al*., 2016). The remaining 17 environmental isolates were obtained from a study that investigated the presence of *bla*$_{\text{CTX-M-15}}$ from muddy soils and gut contents of freshwater fish from Lake Victoria in Mwanza Tanzania (Moremi *et al*., 2016).

3.2 Antibiotic susceptibility testing

Susceptibility testing of all donor isolates and the resulting transconjugants was performed by the disk diffusion method on Mueller Hinton agar (MHA) as recommended by the Clinical and Laboratory Standard Institute (CLSI), (CLSI, 2018). Antibiotic susceptibility was tested against tetracycline (30 µg), gentamycin (30 µg), ciprofloxacin (5 µg) and sulphamethoxazole-trimethoprim ((1.25/23.75 µg) (Hi-media, India), (Fig. 2).

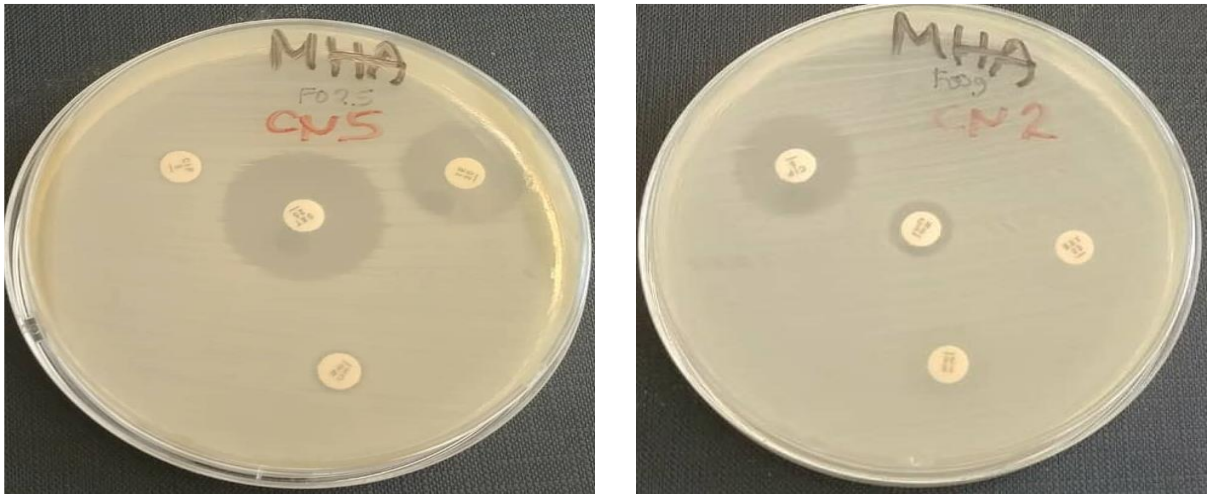


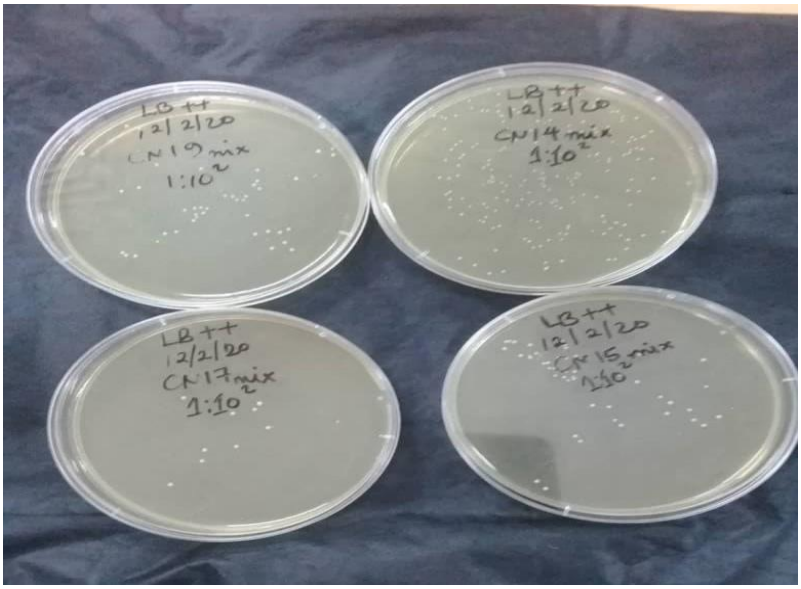
Plate1 Plate 2

Figure 2: Plates displaying antibiotic susceptibility results for donor strains on MHA

3.3 Conjugation experiment

A total of 51 known *bla*$_{CTX\text{-}M\text{-}15}$ positive isolates and *Escherichia coli* J53 ((F^-, *met, pro,* Az^r) – a mutant strain of *E. coli* (Plate 3, Fig. 3) (Jacoby & Han, 1996) obtained from the Institute of Medical Microbiology, Giessen, Germany, were used as donors and recipient strain, respectively. As previously described by Mshana *et al*. (2009) conjugation experiments were performed with some modifications. Shortly, the recipient strain was prepared by streaking *Escherichia coli* J53 in Luria Bertani (LB) plates supplemented with 100 µg/mL NaN_3 (LB++) while donor strains were selected in LB plates supplemented with 2 µg/mL cefotaxime only (LB+). From these, fresh overnight donor and recipient strains were prepared by picking single colonies emulsified in 10 mL LB broth and incubated overnight at 37 ˚C in a 150 rpm shaking incubator. After exactly 12 hours, equal volumes (500 µl) of donor and recipient strains were immediately mixed in 1.5 mL eppendorf tubes previously labeled transconjugant (Tc) while 1000 µL of donor strain were added in fresh tubes of similar volume–to be separately selected on LB+ and LB++ plates as respective controls. All tubes were incubated at 37 ˚C for 15 min, vortexed briefly, centrifuged at 12 000 g for 2 min and the pellet re-suspended in fresh 1000 µL LB broth. Finally, 0.1 mL of 10^{-1} to 10^{-4} transconjugant cultures were double selected on LB plates supplemented with 100 µg/mL NaN_3 and 2 µg/mL cefotaxime (Plates 4-7, Fig. 3). Conjugation efficiency (colony forming units/mL of donors divide by colony forming units/mL of transconjugants) was reported as transconjugants per donor cells, with the denominator obtained from an initial volume of 100 µl.

Plate 3: The recipient strain grown in LB media supplemented with Sodium Azide (100 µg/mL)



Plates 4-7: Transconjugants doubly selected on LB media supplemented with Sodium azide (100 µg/L) and Cefotaxime (2 µg/mL)

Figure 3: LB plates with the recipient and transconjugant strains

3.4 Genomic extraction of donor and transconjugants Deoxyribonucleic acid

Donor and transconjugant genomic DNA was extracted using a previously described chelex protocol with slight modifications (Casquet *et al*., 2012). First, 5 µL of proteinase K (10 mg/mL) were added into tubes containing 100 µL fresh LB emulsified colonies. In the same tubes, 300 µL of chelex buffer (Qiagen GmbH, Hilden, Germany) was added consecutively. The mixture was incubated for 3 hr at 55 $^{\circ}$C before adding 85 µL of 5 M NaCl and vortexed for 15 seconds to precipitate proteins. The supernatant was centrifuged at 13 000 g for 10 min followed by the addition of 300 µL of 100% cold ethanol and a 5 min centrifugation at 13

000 g that precipitated and pelleted the DNA. Lastly, the pellet was rinsed by pouring off the remaining fluid, adding 500 µL of 70% ethanol, centrifuging at 13 000 g for 5 min and leaving the pellet to air dry at 55 ˚C for 10 min. The DNA was then re-suspended in 50 µL nuclease-free water. Nanodrop (Thermo Scientific, Wilmington, DE) was used to check the quantity of the DNA, while the quality was confirmed by electrophoresis in 1.5% (w/v) agarose gel using TAE buffer. The obtained DNA samples were used in typing plasmid replicons or stored at –20 ˚C.

3.5 Polymerase chain reaction based replicon typing

After checking the quality and quantity of the DNA, targeted genes were amplified by a simplified version of the previously described PBRT technique (Johnson *et al*., 2007). Shortly, the eight Polymerase Chain Reaction (PCR) panels illustrated by Caratolli and colleagues (Carattoli *et al*., 2005), were reduced to three (Johnson *et al*., 2007), (Table 1). Using a readily reconstituted master mix, PCR was performed according to manufacturer's instructions (New England BioLabs, Inc. Beverly, MA) under the following conditions; 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 90 s at 72 °C; then a final extension of 5min at 72 °C. Amplicons were visualized on 1.5% tris-acetate EDTA agarose gels alongside a 100 bp DNA ladder (New England BioLabs, Inc. Beverly, MA). The sample was considered positive for replicon gene (s) if an amplicon of the expected band size was observed.

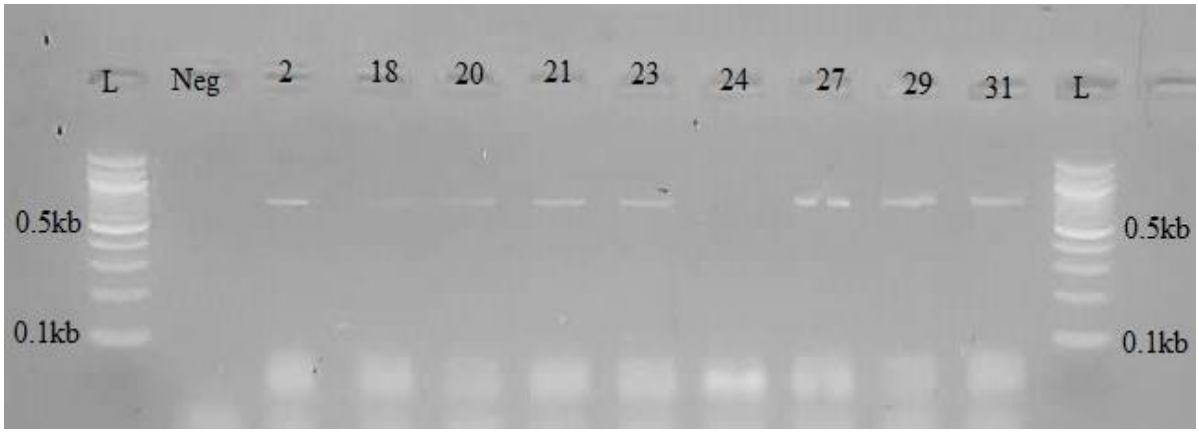


Figure 4: Gel image of 9 transconjugants with replicon type FIB, 702 bp

Table 1: Primers used in PCR based replicon typing of donor and transconjugant isolates

Replicon	Direction	Sequence 5' to 3'	Annealing Temp. (^{o} c)	Amplicon size (bp)
Panel 1				
B/O	F	5'-gcggtccggaaagccagaaaac-3'	60	159
	R	5'-tctgcgttccgccaagttcga-3'		
FIC	F	5'-gtgaactggcagatgaggaagg-3'	60	262
	R	5'-ttctcctcgtcgccaaactagat-3'		
A/C	F	5'-gagaaccaaagacaaagacctgga-3'	60	465
	R	5'-acgacaaacctgaattgcctcctt-3'		
P	F	5'-ctatggccctgcaaacgcgccagaaa-3'	60	534
	R	5'-tcacgcgccagggcgcagcc-3'		
T	F	5'-ttggcctgtttgtgcctaaaccat-3'	60	750
	R	5'-cgttgattacacttagctttggac-3'		
Panel 2				
K/B	F	5'-gcggtccggaaagccagaaaac-3'	60	160
	R	5'-tctttcacgagcccgccaaa-3		
W	F	5'-cctaagaacaacaaagccccg-3'	60	242
	R	5'-ggtgcgcggcatagaaccgt-3'		
FII_S	F	5'-ctgtcgtaagctgatggc-3'	60	462
	R	5'-ctctgccacaaacttcagc-3'		
FIB	F	5'-ggagttctgacacacgattttctg-3'	60	702
	R	5'-ctcccgtcgcttcagggcatt-3'		
Y	F	5'-aattcaaacaacactgtgcagcctg-3'	60	765
	R	5'-gcgagaatggacgattacaaaacttt-3'		
Panel 3				
I1	F	5'-cgaaagccggacggcagaa-3'	60	139
	R	5'-tcgtcgttccgccaagttcgt-3'		
F_{repB}	F	5'-tgatcgtttaaggaattttg-3'	60	270
	R	5'-gaagatcagtcacaccatcc-3'		
X	F	5'-aaccttagaggctatttaagttgctgat-3'	60	376
	R	5'-tgagagtcaatttttatctcatgttttagc-3'		
HI1	F	5'-ggagcgatggattacttcagtac-3'	60	471
	R	5'-tgccgtttcacctcgtgagta-3'		
N	F	5'-gtctaacgagcttaccgaag-3'	60	559
	R	5'-gtttcaactctgccaagttc-3'		
HI2	F	5'-tttctcctgagtcacctgttaacac-3'	60	644
	R	5'-ggctcactaccgttgtcatcct-3'		
L/M	F	5'-ggatgaaaactatcagcatctgaag-3'	60	785
	R	5'-ctgcaggggcgattctttagg-3'		

3.6 Data presentation and analysis

Data was analyzed using Excel version 2013 (Microsoft Inc, Washington DC, USA) where categorical variables were summarized as proportions or percentage.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Isolates Characteristics

A total of 51 CTX-M-15 positive donor isolates were used in this study, whereby 22 (43.14%) came from human, 12 (23. 52 %) from animals and 17(33.32%) were from the environment that included soil (6/17) and fish (11/17). The distribution of bacteria species among the isolates is presented in Table 2. *Escherichia coli* was the only species isolated from both human and animals whereas the environment that included isolates from fish and soil was comprised of *E. coli, K. pneumoniae, C. braakii and E. cloacae.*

Table 2: Bacteria species distributed among donor isolates of human, animal and environment

Sample origin	Isolate origin	Frequency n %	Species	Species n (%)	Total n (%)
Human	Human	22 (43.14)	*E. coli*	22 (43.1)	22 (43.14)
Animal	Goat	1 (1.96)	*E. coli*	1 (1.96)	12 (23.52)
	Pig	3 (5.88)	*E. coli*	3 (5.88)	
	Dog	6 (11.76)	*E. coli*	6 (11.76)	
	Chicken	2 (3.92)	*E. coli*	2 (3.92)	
Environment	Soil	6 (11.76)	*E. coli*	6 (11.76)	17 (33.32)
			E. coli	2 (3.92)	
	Fish	11 (21.57)	*K. pneumoniae*	3 (5.88)	
			C. braakii	2 (3.92)	
			E. cloacae	4 (7.84)	
Total (n)		**51 (100)**			**51 (100)**

The reason for the observed high level of *E. coli* dominance is due to successful colonization of *E. coli* species in human and animal gastrointestinal tract (GIT) (Hosuru *et al*., 2020). The GIT can serve as exchange hotspots and reservoirs of antimicrobial resistance genes. Likewise, *Escherichia coli* and *Klebsiella pneumoniae* are frequently isolated in infections associated with CTX-M-15 in hospitals (Mshana *et al*., 2009) and the community including household (Obeng-Nkrumah *et al*., 2019), aquatic environment (Lyimo *et al*., 2016) and the soil (Gekenidis *et al*., 2020). It is, therefore, possible that the food chain as previously explained by Irrgang *et al*. (2017) is the reservoir of $bla_{\text{CTX-M-15}}$ gene among animals passing it to human and the environment.

4.2 Conjugation efficiency of *bla*$_{\text{CTX-M-15}}$ gene among isolates of human, animals and the environment

Among 51 CTX-M-15 positive donor isolates, 45 (88.2%) transferred plasmids by conjugation with a transfer rate (transconjugants per donor cells) ranging from 4.8×10^{-1} to 1.5×10^{-9} observed in a human and environment isolates respectively (Table 3).

Table 3: Conjugation efficiency of human, animal and environment donor isolates

Sample ID	Source	Species	Conjugation efficiency
CN1	Fish	*E. cloacae*	8.2×10^{-5}
CN2	Fish	*E. cloacae*	2.3×10^{-4}
CN3	Fish	*E. cloacae*	5.2×10^{-5}
CN4	Fish	*E. cloacae*	NIL
CN5	Fish	*C. braakii*	7.5×10^{-6}
CN6	Fish	*E. coli*	7.6×10^{-3}
CN7	Fish	*E. coli*	NIL
CN8	Fish	*K. pneumoniae*	2.0×10^{-5}
CN9	Fish	*K. pneumoniae*	4.2×10^{-4}
CN10	Fish	*K. pneumoniae*	3.3×10^{-5}
CN11	Fish	*C. braakii*	9.4×10^{-4}
CN12	Pig	*E. coli*	4.7×10^{-5}
CN13	Pig	*E. coli*	2.6×10^{-6}
CN14	Pig	*E. coli*	9.8×10^{-5}
CN15	Local chicken	*E. coli*	4.7×10^{-5}
CN16	Local chicken	*E. coli*	8.4×10^{-7}
CN17	Goat	*E. coli*	4.1×10^{-6}
CN18	Dog	*E. coli*	2.1×10^{-5}
CN19	Dog	*E. coli*	1.2×10^{-7}
CN20	Dog	*E. coli*	5.0×10^{-5}
CN21	Dog	*E. coli*	1.1×10^{-6}
CN22	Dog	*E. coli*	6.0×10^{-4}
CN23	Dog	*E. coli*	9.6×10^{-6}
CN24	Environment	*E. coli*	1.5×10^{-9}
CN25	Environment	*E. coli*	2.6×10^{-7}
CN26	Environment	*E. coli*	3.5×10^{-6}
CN27	Environment	*E. coli*	2.9×10^{-7}
CN28	Environment	*E. coli*	6.1×10^{-6}
CN29	Environment	*E. coli*	7.2×10^{-3}
CN30	Human	*E. coli*	$1.0\text{x}10^{-3}$
CN31	Human	*E. coli*	$4.7\text{x}10^{-4}$
CN32	Human	*E. coli*	$2.1\text{x}10^{-4}$
CN33	Human	*E. coli*	$4.0\text{x}10^{-5}$
CN34	Human	*E. coli*	$5.4\text{x}10^{-5}$
CN35	Human	*E. coli*	$4.8\text{x}10^{-1}$
CN36	Human	*E. coli*	$1.7\text{x}10^{-4}$
CN37	Human	*E. coli*	$3.5\text{x}10^{-7}$
CN38	Human	*E. coli*	$8.1\text{x}10^{-5}$

Sample ID	Source	Species	Conjugation efficiency
CN39	Human	*E. coli*	$1.2x10^{-5}$
CN40	Human	*E. coli*	$2.7x10^{-5}$
CN41	Human	*E. coli*	$2.4x10^{-7}$
CN42	Human	*E. coli*	Nil
CN43	Human	*E. coli*	$5.5x10^{-6}$
CN44	Human	*E. coli*	$4.4x10^{-6}$
CN45	Human	*E. coli*	$2.9x10^{-6}$
CN46	Human	*E. coli*	NIL
CN47	Human	*E. coli*	$2.1x10^{-5}$
CN48	Human	*E. coli*	$1.2x10^{-4}$
CN49	Human	*E. coli*	$1.1x10^{-7}$
CN50	Human	*E. coli*	NIL
CN51	Human	*E. coli*	NIL

Nil: no conjugation

The transfer rates reported in this study are high and variable in the order 10^{-1} to 10^{-9}. High conjugation rates are enough to establish a long term persistence of plasmids in multiple hosts (Dionisio *et al*., 2005; Levin & Rozen, 2006; Millan *et al*., 2014) even in the absence of selection pressure (Dahlberg & Chao, 2003; Lopatkin *et al*., 2017). The transfer efficiency of plasmids carrying $bla_{\text{CTX-M-15}}$ observed for donor isolates was high (88%) despite the isolates varying frequencies of transfer, this justifies the persistence and dissemination potential of plasmids carrying $bla_{\text{CTX-M-15}}$ in a broader range of environments. In the remaining few isolates, CN4, CN7, CN42, CN46, CN50 and CN51 there was no conjugation, this might be due to the chromosomal integration of the gene (Ragupathi *et al*., 2019) or transposition events which can prevent plasmid mobility.

4.3 Transferable resistance of non-beta-lactam phenotype among isolates of human, animal and the environment

A summary of non-beta lactam resistance phenotypes transferred by plasmids carrying $bla_{\text{CTX-M-15}}$ gene is presented in Table 4. A total of 45 plasmids successful transferred the gene to transconjugants. Non-beta-lactam resistance phenotypes were observed in 34/45(75.6%) transconjugants. Donor resistance to ciprofloxacin (CIP), tetracycline (TE) and trimethoprim-sulphamethoxazole (SXT) was observed in 46/51 (90.2%), 47/51 (92.2%) and 48/51 (94.1%) of events, respectively, and was co-transferred in 29/34 (85.3%) of such events. Gentamicin was the least transferred with a frequency of 17/34 (50.0%).

Table 4: Antibiotic resistance phenotypes of donors and transconjugants of human animals and the environment

Sample no.	Source	Species	Donor's non-B-lactam resistance phenotype
CN1	Fish	*E. cloacae*	SXT*, CIP* ,CN*,TE*
CN2	Fish	*E. cloacae*	CIP, SXT, CN, TE
CN3	Fish	*E. cloacae*	CIP*, SXT*, TE*, CN*
CN4	Fish	*E. cloacae*	CIP, CN, TE, SXT
CN5	Fish	*C. braakii*	CIP*, SXT*, CN*, TE*
CN6	Fish	*E. coli*	CIP, SXT, CN, TE
CN7	Fish	*E. coli*	CIP, TE
CN8	Fish	*K. pneumoniae*	CIP*, SXT*, CN*, TE*
CN9	Fish	*K. pneumoniae*	CIP*, SXT*, CN*, TE*
CN10	Fish	*K. pneumoniae*	CIP, SXT, CN, TE
CN11	Fish	*C. braakii*	CIP, SXT, CN, TE*
CN12	Pig	*E. coli*	CIP*, SXT*, TE*
CN13	Pig	*E. coli*	TE, CIP, CN
CN14	Pig	*E. coli*	CIP*, SXT*, TE*, CN*
CN15	Local chicken	*E. coli*	CIP, SXT, CN, TE
CN16	Local chicken	*E. coli*	CIP, SXT, CN, TE
CN17	Goat	*E. coli*	SXT, TE*, CN, CIP*
CN18	Dog	*E. coli*	SXT
CN19	Dog	*E. coli*	SXT*, CIP*, TE, CN
CN20	Dog	*E. coli*	CIP*, SXT*, TE*
CN21	Dog	*E. coli*	CIP*, SXT*, TE*, CN*
CN22	Dog	*E. coli*	CIP*, CN*, TE*, SXT*
CN23	Dog	*E. coli*	SXT, TE, CN, CIP
CN24	Environment	*E. coli*	SXT*, CIP*, TE*
CN25	Environment	*E. coli*	SXT, TE, CIP
CN26	Environment	*E. coli*	CIP*, SXT*, TE*
CN27	Environment	*E. coli*	CIP*
CN28	Environment	*E. coli*	CIP*, SXT*, CN*, TE*
CN29	Environment	*E. coli*	CN, CIP*, SXT*, TE*
CN30	Human	*E. coli*	TE*, CIP*, CN, SXT*
CN31	Human	*E. coli*	CIP*, SXT*
CN32	Human	*E. coli*	SXT*, CIP*
CN33	Human	*E. coli*	TE*, CN*, CIP*, SXT*
CN34	Human	*E. coli*	SXT*, TE*, CN*, CIP
CN35	Human	*E. coli*	CIP*, CN*, SXT*, TE*
CN36	Human	*E. coli*	CIP*, CN*, SXT*, TE*
CN37	Human	*E. coli*	CIP*, CN*, SXT*, TE*
CN38	Human	*E. coli*	SXT*, TE*, CIP*, CN*
CN39	Human	*E. coli*	SXT, TE, CIP*, CN*
CN40	Human	*E. coli*	SXT*, TE*
CN41	Human	*E. coli*	SXT, TE*, CIP*, CN
CN42	Human	*E. coli*	SXT, CIP, CN, TE
CN43	Human	*E. coli*	CN*, CIP*, SXT*, TE*
CN44	Human	*E. coli*	SXT, TE, CIP
CN45	Human	*E. coli*	SXT, TE, CIP, CN
CN46	Human	*E. coli*	TE, SXT
CN47	Human	*E. coli*	SXT*, TE*, CIP, CN
CN48	Human	*E. coli*	SXT*, TE*, CIP, CN
CN49	Human	*E. coli*	CIP*, CN*, SXT*, TE*
CN50	Human	*E. coli*	SXT, TE
CN51	Human	*E. coli*	CN, CIP, SXT, TE

*Transferable resistance; SXT: Trimethoprim-sulphamethoxazole, CIP: ciprofloxacin, TE: tetracycline, CN: Gentamicin

A multidrug resistance phenotype is observed for donor and transconjugant isolates following antibiotic susceptibility testing. The conjugative spread of *bla*$_{\text{CTX-M-15}}$ gene by IncF plasmids along with tetracycline, aminoglycoside and quinolones have been reported (Rozwandowicz *et al.,* 2018). These plasmids harbor several combinations of resistance determinants and transfer them to human, animals and environment isolates through the ecological interaction of bacteria in these settings. Moreover, the genetic environment of *bla*$_{\text{CTX-M-15}}$ is dominated by multiple antibiotic resistance genes such as *aac (6')-lb-cr, tet (A, B), qnrS, qnr* and *sul* genes (Kiiru *et al*., 2013; Rafaï *et al*., 2015; Yousfi *et al*., 2016) whose phenotypic expression denotes the existing selection pressure for these antibiotics. Such selection can increase their transfer rate and possibly account for the high co-transfer of non-beta lactam antibiotics observed in this study. These observations together, confirm the conjugative spread of *bla*$_{\textit{CTX-M-15}}$ carrying plasmids along with tetracycline, aminoglycoside and quinolones in multiple hosts thus, increasing the host range while disseminating multi-drug resistance.

Finally, despite the transferability of *bla*$_{\text{CTX-M-15}}$ and other resistance phenotypes not confirmed genotypically, possible chromosomal or plasmid mutations causing genotype-phenotype discrepancies as previously unravelled by sequencing techniques (Kumburu *et al*., 2019; Mbelle *et al*., 2019; Ragupathi *et al*., 2019) can explain the observed resistance differences among donors and resultant transconjugants.

4.4 Replicon types of plasmids carrying *bla*$_{\text{CTX-M-15}}$

Common replicon types were FIA (n = 11) and FIB (n = 27) and occurred as single replicons that were transferrable in 14 transconjugants. Inc A/C and Y replicons were minor, and each occurred once. Among 14 isolates with plasmid replicons, 8 had replicons that were transferrable to respective transconjugants while the remaining 6 were only detected in transconjugants. Also, 15/51 plasmid donors did not transfer replicons to respective transconjugants, while no replicons were detected in 16/51 donors and their resultant transconjugants.

Table 5: Replicon types of plasmids carrying *bla*$_{\text{CTX-M-15}}$ among donors and transconjugants

Sample source	Conjugation efficiency	Conjugation range	Donor's plasmid replicon	Transconjugant replicon type
Human	1.2×10^{-4}		FIB	FIA
Human	8.1×10^{-5}		FIA, FIB	FIB
Dog	5.0×10^{-5}		FIB	FIB
Human	5.4×10^{-5}	10^{-6}–10^{-3}	FIB	FIB
Human	2.1×10^{-4}		FIB	FIB
Environment	7.2×10^{-3}		FIB	FIB
Dog	1.1×10^{-6}		FIB	FIB
Human	1.7×10^{-4}		FIB	FIB
Dog	9.6×10^{-6}		no rep	FIB
Dog	2.1×10^{-5}		no rep	FIB
Human	1.2×10^{-5}	10^{-7}–10^{-4}	no rep	FIB
Human	4.7×10^{-4}		no rep	FIB
Environment	2.9×10^{-7}		no rep	FIB
Fish	2.3×10^{-4}		no rep	FIB
Fish	NIL		FIA, Y	NA
Human	NIL		no rep	NA
Human	NIL	0	no rep	NA
Human	NIL		no rep	NA
Human	NIL		no rep	NA
Fish	NIL		no rep	NA
Fish	4.2×10^{-4}		A/C, FIA	no rep
Pig	2.6×10^{-6}		FIA	no rep
Human	5.5×10^{-6}		FIA	no rep
Dog	6.0×10^{-4}		FIA	no rep
Pig	9.8×10^{-5}		FIA	no rep
Human	2.9×10^{-6}		FIA	no rep
Human	4.0×10^{-5}		FIA	no rep
Human	4.8×10^{-1}	10^{-9}–10^{-1}	FIA	no rep
Dog	1.2×10^{-7}		FIB	no rep
Human	3.5×10^{-7}		FIB	no rep
Environment	1.5×10^{-9}		FIB	no rep
Environment	2.6×10^{-7}		FIB	no rep
Human	4.4×10^{-6}		FIB	no rep
Environment	3.5×10^{-6}		FIB	no rep
Human	2.1×10^{-5}		FIB	no rep
Fish	7.5×10^{-6}		no rep	no rep
Fish	9.4×10^{-4}		no rep	no rep
Human	2.7×10^{-5}		no rep	no rep
Local chicken	4.7×10^{-5}		no rep	no rep
Pig	4.7×10^{-5}		no rep	no rep
Human	2.4×10^{-7}		no rep	no rep
Fish	3.3×10^{-5}		no rep	no rep
Fish	2.0×10^{-5}	10^{-7}–10^{-3}	no rep	no rep
Fish	7.6×10^{-3}		no rep	no rep
Human	1.1×10^{-7}		no rep	no rep
Fish	5.2×10^{-5}		no rep	no rep
Goat	4.1×10^{-6}		no rep	no rep
Environment	6.1×10^{-6}		no rep	no rep
Local chicken	8.4×10^{-7}		no rep	no rep
Human	1.0×10^{-3}		no rep	no rep
Fish	8.2×10^{-5}		no rep	no rep

Nil: no conjugation, NA: no transconjugant

As presented above, IncF plasmids were common vectors of *bla*$_{\text{CTXM-15}}$ with frequency rates as low as $10^{-7} – 10^{-3}$. Replicon typing of plasmids carrying antimicrobial resistance genes are important for the detection, tracing and monitoring of these genes, these observations are in line with multireplicon FIA and FIB plasmids reported as major vehicles for the gene (Zurfluh *et al*., 2015). Inc Y plasmids carrying *bla*$_{\text{CTX-M-15}}$ associated with quinolone and aminoglycoside genes have been reported in the same setting (Moremi *et al*., 2016) justifying the high resistance rates for these antibiotics reported in this study (Table 4). Inc A/C plasmids carrying *bla*$_{\text{CTX-M-15}}$ gene are also reported (Lee *et al*., 2011), ensuring a diversity of plasmids adapted to spread the gene. Future studies aiming to address AMR under the umbrella of One Health should consider surveillance of the role of Inc F plasmids as a core objective in AMR mitigation programs.

Some donor replicons were not detected in respective transconjugants while other replicons were detected among transconjugants but missed in respective donors regardless of donor or transconjugant origin (Table 5). The absence of donor replicons in respective transconjugants may possibly result from conjugation failure, chromosomal integration of transconjugant plasmids (Coque *et al*., 2008) which drive the evolution of new undetected or unstable replicons, multi-replicon plasmids among donors (undetected by the method used) that destabilizes and prevent the transfer of other replicons (Dionisio *et al*., 2019), and horizontal exchanges between the chromosome and plasmid that modify or cause functional losses among donors or transconjugants and obscures the detection of existing replicons (Dionisio *et al*., 2005; Dionisio *et al*., 2019). Finally and as a shortcoming, the PBRT technique used in detecting plasmid replicons can give false-negative results when replicon sequences go undetected by the primer sets used, target replicon sequences undergo mutation through transpositional alterations by mobile genetic elements, and the unknown existence of new replicons in such plasmid (Johnson *et al*., 2007).

4.5 Transfer success of *bla*$_{\text{CTX-M-15}}$ among *Escherichia coli* isolates

Table 6 shows the percentage transfer of *bla*$_{\text{CTX-M-15}}$ among *E. coli* donor isolates. A total of fourty two *E. coli* donors were detected, and 37 (88.1%) successfully transferred the gene to the recipient, accounting 82.1% of all transconjugants. All *E. coli* originating from animals transferred the gene successfully.

Table 6: Transfer success of *bla*$_{CTX-M-15}$ among *E. coli* from human, animals and the environment

Source	*E. coli* donors n (%)	*E. coli* Transconjugants n *(%)*
Human	22 (52.4)	18 (81.8)
Animal	12 (28.6)	12 (100.0)
Environment	8 (19.0)	7 (58.3)
Total	42 (100.0)	37

Human and animal originating *E. coli* are adapted to disseminate ESBL genes by IncF plasmids (Rozwandowicz *et al*., 2018). The colonization and infection of animals by *E. coli* maximizes microbial interactions between non-pathogenic and pathogenic commensal *E. coli* in either companion or food-producing animals and facilitate the exchange of materials between them through conjugation. In addition, the increasing use of antibiotics in animals could select and transfer resistant pathogenic bacteria from animals to human and the environment with huge cost implications. Since AMR is a public health threat, the highest transfer rate observed in animal originating *E. coli* calls for more intensive integrated efforts to address AMR with experts from veterinary, human and ecological fields.

4.6 Study limitation

In this study, the transferability of *bla*$_{CTX-M-15}$ was not confirmed genotypically especially through sequencing which could have provided comparable data for donors and respective transconjugants during interpretation of results.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Majority of plasmids carrying *bla*$_{\text{CTX-M-15}}$ were conjugatively transferred by IncF plasmids along with non-beta lactam resistance. The heterogeneous nature of these plasmids continuously maintains and reserves the *bla*$_{\text{CTX-M-15}}$ gene in these settings. The 100% transfer efficiency among *E. coli* of animal origin is of concern since the networked interaction of animals with human and their environment continuously exchange and reserve resistance determinants in this interface. Therefore, there is a need for more research to understand the interaction and spread of circulating mobile elements especially among animals. Animals may also serve as dual targets for studies focusing on the horizontal transfer and evolution of antimicrobial resistance.

5.2 Recommendations

Since AMR is a persistent public Health challenge, the proposed 2017/22 National Antimicrobial resistance Action Plan (NAP), (MoH, 2017) envisions to combat the threat with One Health engagement being central to this plan. However, until recently (Frumence *et al*., 2021), only human and animal sectors were fully involved in implementing this plan. This study recommends an equal involvement of all sectors for a fully achievement and sustainability of NAP goals and objectives.

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RESEARCH OUTPUTS

Journal paper

Minja, C. A., Shirima, G., & Mshana, S. E. (2021). Conjugative Plasmids Disseminating CTX-M-15 among Human, Animals and the Environment in Mwanza Tanzania: A Need to Intensify One Health Approach. *Antibiotics*, *10*(7), 1-14. https://doi.org/10.3390/antibiotics 10070836

Poster presentation

Conjugative Plasmids Disseminating CTX-M-15 among *Enterobacteriaceae* isolates from human, animal and environments In Mwanza Tanzania: A need to Intensify One Health Approach.