DIFFERENCES IN ANTIBACTERIAL RESISTANCE PROFILES AND GENES BETWEEN *Escherichia coli* ISOLATES FROM HUMANS AND BLACK RHINOCEROS IN LAMBWE VALLEY, KENYA

BY

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DECLARATION

I declare that this thesis is my own original work and has not been presented in any other institution of higher learning for the award of a degree, diploma or certificate. This thesis or any part of it may not be reproduced without written permission from the author, supervisors or Maseno University.

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ABSTRACT

Antimicrobials have been in use for decades in disease management, but emergence of resistant pathogens such as *Escherichia coli* have rendered its future of concern to public health. Increased human-wildlife interaction is associated with emergence of devastating human diseases and resistance genes are increasingly being isolated. Of importance is the increased bacterial infection that requires prolonged treatment periods as seen in Lambwe Valley which points to antibacterial resistance among the community members of Lambwe Valley. However, major gaps exist on whether human and intensively managed wild animals such as black rhinoceros harbors bacterial strains with similar antibacterial resistance profiles. The present study aimed at investigating antimicrobial resistance patterns and genetic variation in *E. coli* isolates from human and black rhinoceros in Lambwe Valley. The specific objectives were to determine differences in antimicrobial resistance to seven commonly used antimicrobials and to characterize resistance genes in *E. coli* isolates from human and the black rhinoceros in Lambwe Valley. A cross-sectional study design was adopted in collecting 184 fecal samples from humans. The study employed a simple random technique in collecting human samples. In addition, 16 fecal samples from black rhinoceros were collected. Antimicrobial susceptibility was determined by disk diffusion method. Polymerase Chain Reaction was used for molecular characterization of antimicrobial resistance genes. *Escherichia coli* were isolated in all human samples and in 15 of the black rhinoceros samples. The prevalence of antimicrobial resistance in *E. coli* isolates from black rhinoceros to erythromycin was 86.7%, gentamicin was 80.0%, ampicillin was 73.3%, tetracycline was 40.0%, amoxicillin/clavulanic acid was 60.0%, cotrimoxazole was 33.3% and ceftriaxone was 13.3%, but 86.7% of the isolates were susceptible to chloramphenicol. The level of resistance of *E. coli* isolates from human to amoxicillin/clavulanic acid was 85.3%, cotrimoxazole was 83.1%, gentamicin was 28.3%, erythromycin was 76.1%, ampicillin was 75.0%, tetracycline was 64.7%, ceftriaxone was 58.3% and chloramphenicol was 29.9%. Human and black rhinoceros isolates showed significant similarity in resistance to ampicillin ($z=0.143$, $p=0.889$), tetracycline ($z=1.901$, $p=0.057$), chloramphenicol ($z=1.923$, $p=0.055$) and erythromycin ($z=0.935$, $p=0.3524$). However resistance to cotrimoxazole ($z=4.587$, $p=0.000$), ceftriaxone ($z=3.310$, $p=0.001$), amoxicillin/clavulanic ($z=0.935$, $p=0.352$) acid and gentamycin ($z=4.125$, $p=0.000$) was significantly different with gentamycin resistance being higher in black rhinoceros while cotrimoxazole, ceftriaxone and amoxicillin/clavulanic acid resistance was higher in human. Multi-drug resistance was 69.0% in humans and 43.4% in black rhinoceros with resistance phenotype being ampicillin, cotrimoxazole, chloramphenicol, tetracycline, amoxicillin/clavulanic acid and erythromycin. PCR analyses of selected samples indicated presence of bla_{TEM}, *tetA*, *tetB*, *dfrA1* and *sul1* genes in isolates from humans and black rhinoceros. The observed similarity in phenotypic and genotypic antimicrobial resistance profiles between human and black rhinoceros isolates suggests that antimicrobial resistance is no longer confined to humans, but is a wider environmental issue raising grave concern to public health. It also suggests that human and wildlife are experiencing high rate of resistance genes cross-transfer. Hence there is need for multi-sectorial coordinated action plan on surveillance of antimicrobial resistance by incorporating public health, livestock and wildlife sectors.

TABLE OF CONTENT

ABBREVIATIONS AND ACRONYMS

ADD: Agar Disk Diffusion

AIDS: Acquired Immunodeficiency Syndrome

AMR: Antimicrobial Resistance

ATCC: American Type Culture Collection

***blaTEM*:** Gene responsible for ampicillin resistance

BP: Base Pair

CDC: Center for Disease Control and Prevention

CLSI: Clinical and Laboratory Standards Institute

COOH: Carboxylic Acid

DNA: Deoxyneucleic Acid

E test: Epsilometer Strip Test

E. coli: *Escherichia coli*

EMB: Eosin Methyl Blue

ESBL: Extended spectrum beta lactamase

HIV: Human Immune virus

IMVIC: Indole, Methyl Red, Voges Proskauers, Citrate

IRT: Inhibitor Resistant

KOH: Potassium Hydroxide

MDR: Multi-Drug Resistance

MIC: Minimum Inhibition Concentration

MIL: Motility, Indole, Lysine Medium

PCR: Polymerase Chain Reaction

RTPCR: Real Time Polymerase Chain Reaction

sul1and ***dfrA1*****:** Genes responsible for cotrimoxazole resistance

tetA and ***tetB*****:** Genes responsible for tetracycline resistance

TSI: Triple Sugar Iron

WHO: World Health Organization

B-Lactam: Beta-lactam

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

CHAPTER ONE: INTRODUCTION

1.1. BACKGROUND

Although antimicrobial agents have been in use for decades, emergence of a wide range of antimicrobial resistant pathogens have threatened the future of antimicrobials and is a major concern to public health globally (WHO, 2015). Alexander Flemings and Howard Florey were the first to raise a red flag regarding the development of antimicrobial resistance (Fleming, 1945). Since then, pathogenic bacteria have shown increased resistance to a wide range of antimicrobial classes used in the management of both human and animal diseases (WHO, 2015; Mshana *et al.*, 2013). Some examples include the seven commonly used classes of antibiotics such as the penicillinase (ampicillin and penicillin), cephalosporins, fluoroquinolones (ciprofloxacin and norfloxacin), aminoglycosides (gentamicin and streptomycin), tetracyclines (tetracycline and doxycycline), sulfonamides (co-trimoxazole and trimethoprim), macrolides (erythromycin) and the chloramphenicol among others (Coates *et al.*, 2011). Currently, about 64% of those suffering from multi-drug resistant strains are likely to die compared to those suffering from non-resistant form of the same bacterial infection (WHO, 2015). In countries where data are available such as the USA, about 23,000 patients die yearly from infections caused by antimicrobial resistant bacteria (CDC, 2016).

Despite the scarcity of accurate antimicrobial resistance data in most African countries, available data suggest that Africa shares the global burden of antimicrobial resistance among pathogenic bacterial isolates among humans and livestock (Mshana *et al.*, 2013) which are normally under increased selective pressure due to misuse of antimicrobial agents (WHO, 2015). In Kenya, for instance, in addition to the emergence of pathogenic bacteria requiring prolonged treatment periods and resulting in increased cases of relapsing infections, studies from different regions of the country suggest an increase in the levels of multi-drug resistance

to commonly used antimicrobials (Wandili *et al.*,2015; Kipkorir *et al.*,2016; Sang *et al.*, 2012; Brooks *et al.*, 2006).

Besides the overuse of antimicrobial agents in hospitals, such as over-the-counter medication, and in livestock therapy (Zhang *et al.*, 2006; Singer *et al.*, 2003), their use in controlling bacterial infections in fruiting plants and aquaculture contributes to increased antimicrobial selective pressure (Thiele-Bruhn, 2003; Cabello, 2006).

Regardless of the point of use, antimicrobial agent selective pressure against microbes leads to accumulation of resistance genes that can be transferred within and between species, hence facilitates persistence of resistance genes (Davies *et al.*, 2010). Increased human modification of the environment increases opportunities for contact between humans and the wildlife (Mayer, 2000),which in turn is associated with emergence and spread of some of the most devastating human pathogens including HIV/AIDS (Hahn *et al.*, 2000) and influenza (Schoub, 2012). Contact between human and wildlife is fostered by anthropogenic habitat change brought about by the increase in human population (Radhouani *et al.*, 2014) and human activities such as poaching and conservation measures such as translocation and intensive management of endangered species such as black rhinoceros (Walpole *et al.*,2001; Emslie, 2012). It is foreseeable that contact between humans and wildlife may facilitate interspecies spillover of antimicrobial resistant genes. Indeed, it is clear from a number of studies that antimicrobial resistant bacteria is increasingly being reported from the wildlife (Silva *et al.*, 2010; Pesapane *et al.*, 2013; Botti *et al.*, 2013). However, it is yet to be established whether intensively managed black rhinoceros and humans carry *Escherichia coli* isolates with similar antimicrobial resistance profiles. Therefore, Therefore, there is need to establish whether intensively managed wild animals such as black rhinoceros may harbor antimicrobial resistance microbes which may complicate efforts on containment of antimicrobial resistance.

The declining efficacy of antimicrobial agents directly correlates with the spread of antimicrobial resistance genes (Kumarasamy *et al.,* 2010). Transmission of resistance is facilitated by mobile genetic elements such as plasmids, transposons, integrons and bacteriophage (Levy, 2002). Therefore, understanding interspecies cross-transmission of antimicrobial resistance genes and routes of its dissemination is the key to combating the threat of antimicrobial resistance. This will require an in-depth knowledge on the factors which facilitates environmental circulation of genetic elements conferring resistance and the spectrum of environmental reservoirs of antimicrobial resistance genes. The over-use of antimicrobial agents in human and livestock therapy (Singer *et al.,* 2003; Zhang *et al.,* 2006) and in agriculture and aquaculture (Thiele-Bruhn, 2003; Cabello, 2006) are some of the factors that not only contributes to the marked increase in antimicrobial resistance, but can lead to spill-over of antimicrobial agents to the wild. However, there is no empirical data on whether humans and intensively managed black rhinoceros harbor *E. coli* strains that carry similar antimicrobial resistant genes. Therefore, it would be important to establish the antimicrobial resistance profiles of human and black rhinoceros in Lambwe Valley in a bid to maintain the efficacy of available antimicrobial agents; and also to have therapeutic option to be used in management of the few, nineteen, black rhinoceros in Ruma national park, in case of a bacterial disease outbreak.

In-depth evaluation of the antimicrobial resistance genetic variation in different mammalian hosts is significant in understanding the epidemiology of interspecific gene transmission (Pesapane *et al.,* 2013). Due to the fact that *E. coli* is a coliform that inhabits gastrointestinal tract of all mammals (Stecher *et al.,* 2012), it makes a suitable model in demonstrating transmission of enterobacteriaceae between human and wildlife and in the studies involving contamination of the environment with fecal matter (Fahrenfeld*et al.,* 2013; Van den Bogaard *et al.,* 2000; Silva *et al.,* 2010). Although it is known that mammals contain distinct subtypes

of *E. coli* in their alimentary tract (Simpson, 2002), it has been shown by Pesapane *et al.* (2013) that banded mongoose foraging in human backyard gardens harbors *E. coli* strains having predominantly similar antimicrobial resistance profiles to humans isolates.Yet the distinctiveness of antimicrobial resistance in *E. coli* strains from human and intensively managed animals such as the black rhinoceros population, remains unknown.

The black rhinoceros, *Diceros micheli bicornis*, in African countries including Kenya, is under intensive conservation management. Due to the fact that black rhinoceros are critically endangered (IUCN, 2017; Emslie, 2012), with the small population being confined in small conservancy regions in Kenya (Emslie, 2012) for instance in Ruma National park found in Lambwe Valley. Since the conservation measures involved in recovery of black rhinos population which includes translocation coupled with tight security and continued monitoring of their movement in addition to human activities in Lambwe Valley which include poaching, use of a single water source with the wildlife and having a major road that passes through the park, bring the species under increased contact with humans; black rhinoceros makes an ideal candidate for investigating the spillover of antimicrobial resistance at the human-wildlife interface. Health reports from facilities in Ndhiwa Sub-County indicate that there has been an increase in patients presenting with enteric infections requiring prolonged treatment periods with antibiotics. Whether the observed prolonged treatment period is due to emergence of antimicrobial resistance is yet to be established. As such; this study endeavored to establish whether both human and black rhinoceros populations in Lambwe Valley harbor *E. coli* strains exhibiting similar antimicrobial resistance profiles.

1.2. Statement of Research Problem

In an effort to effectively combat antimicrobial resistance, the national development action plan on containment of antimicrobial resistance has placed a lot of emphasis in enhancing an

interlinked surveillance and sharing research data on antimicrobial resistance in human and livestock health. However, the significance of wildlife as sentinels for environmental reservoirs of antimicrobial resistance yet to be established. Thus it is foreseeable that increased human and wildlife closeness may facilitate cross-transfer and persistence of antimicrobial resistance genes in the environment. Moreover, studies from different regions points to increase in antimicrobial resistance among wildlife species. Such increase in antimicrobial resistance in wild animals brings a danger in the management of critically endangered wild animals such as black rhinoceros in case of an outbreak of a bacterial infection in the wild. Beside the observed increase in antimicrobial resistance in different regions in the country, health records from facilities in Lambwe Valley indicate prolonged periods in treatment of enteric bacterial infection using antimicrobial agents. Whether the observed prolonged period is contributed by emergence antimicrobial resistant pathogens is yet to be established. Also, it is not known whether both human and black rhinoceros populations in Lambwe Valley harbor *E. coli* strains exhibiting similar antimicrobial resistance profiles.

The global decline in antibiotic potency has been demonstrated to correlate with the spread of antimicrobial resistance genes in the environment. Although it has been established that antimicrobial resistance genes may act as a crucial marker for epidemiological surveillance of the spillover and interspecies cross-transfer of antimicrobial resistance genes, it is yet to be established whether humans and intensively managed wildlife species such as black rhinoceros harbors *E. coli* strains that carry similar antimicrobial resistant genes.

1.3. Justification of the Study

This study was necessitated by the fact that antimicrobial resistant cases are increasingly being reported both in the humans, environmental and in the wild animals' bacterial isolates

and this raises a grave concern to the future of the available antimicrobial agents. In addition, development of antimicrobial resistance has reduced therapeutic options for pathogenic bacteria leading to extended periods of hospital stay, increase in treatment cost and loss of life since new antimicrobial agents takes long to be produced and currently, there is no new antimicrobial agent in the pipeline for production. Also, in relation to conservation of black rhinoceros, human-wildlife interaction may lead to the spread of antimicrobial resistance genes in the environment which will complicate infection management among the critically endangered black rhinoceros in case of a bacterial disease outbreak.

Therefore, in an effort to effectively control antimicrobial resistance challenges, it is imperative to understand the significance of wildlife as sentinels of environmental reservoirs of antimicrobial resistance by profiling the antimicrobial resistance patterns in *E. coli* strains from wild animals and to determine the occurrence of such resistance in the human community living in the proximity to the wild animals. Understanding the potential of wildlife in maintaining and spreading antimicrobial resistance pathogens in the environment would be important in developing effective ways to maintain efficacy of available antibiotics, save money, man hours and human life as well as enhancing the conservation efforts of the critically endangered black rhinoceros. Hence findings on the burden of antimicrobial resistance are needed to inform clinical therapy decisions, to guide policy recommendations, and to assess the impact of antimicrobial resistance containment interventions.

1.4. Significance of the Study

According to World Health Organization, preservation of antibiotic potency through involvement of all stakeholders involved (public health and agriculture) is a top priority. Thus, the results of this study will inform policy makers on the need to consider wildlife as gate keepers in surveillance of environmental reservoirs of antimicrobial resistance in

addition to determining the spectrum of antimicrobial resistance reservoirs. The study finding will also inform public and veterinary health personnel on the potency of available antimicrobial agents and the need for judicious antimicrobial use through evidence based treatment. Also, the outcome of the study will go a long way in communicating to conservationists and wildlife management the risks posed by increased human-wildlife interaction in interspecies cross-transfer of antimicrobial resistance genes that would otherwise jeopardize the strides made in conservation measures incase these conserved animals requires treatment due to outbreak of a disease.

1.5. Main Objective

To investigate antimicrobial resistance patterns and genes in *E. coli* isolates from human and black rhinoceros.

1.6. Specific Objectives

1. To determine differences in antimicrobial resistance patterns of *E. coli* isolates from human and black rhinoceros to eight commonly used antimicrobials in Lambwe Valley.
2. To characterize antimicrobial resistance genes in *E. coli* isolates from humans and black rhinoceros in Lambwe Valley.

1.7. Null Hypotheses

1. *Escherichia coli* strains isolated from human and black rhinoceros show no difference in resistance to the eight commonly used antimicrobials.
2. There is no difference in genes that confer antimicrobial resistance in *Escherichia coli* strain from human and those from black rhinoceros.

CHAPTER TWO: LITERATURE REVIEW

2.1. Trends and Causes of Antimicrobial Resistance

Antimicrobials have been in use for decades in the treatment and prevention of bacterial infections, but with the emergence of resistant bacterial strains, their effectiveness have drastically declined (WHO, 2015). Although the decline in antimicrobial efficacy is not a new phenomenon, of concern is the unprecedented increase in the cases of antimicrobial resistant bacteria worldwide as compared to the slow turnover of new and effective antimicrobial agents (WHO, 2015). Pathogenic bacteria have demonstrated high resistance to a wide range of antimicrobial classes used in management of both human and animal diseases (Singer *et al.,* 2003; Mshana *et al.,* 2013). A point in case is the seven commonly used class of antibiotics such as; the penicillinase (ampicillin and penicillin), fluoroquinolones (ciprofloxacin and norfloxacin), aminoglycosides (gentamicin and streptomycin), tetracyclines (tetracycline and doxycycline), sulfonamides (cotrimoxazole), macrolides (erythromycin) and the chloramphenicol (Coates *et al.*,2011)**.** In some regions, hardly any of the available antimicrobial options are effective to common infections (WHO, 2015). The first red flag regarding antimicrobial resistance was raised by Alexander Flemings and Howard Florey (1945) following their discovery of the penicillin. Afterwards, antimicrobial resistance has been increasing rapidly all over the world (WHO, 2015). It is estimated that about 64% of people suffering from multi-drug resistant strains of bacterial infections are likely to die than those suffering from non-resistant strains of the same bacterial infections (WHO, 2015). In the United State of America alone, at least 2 million people are infected with multi-drug resistant strains of bacteria that results in 23,000 fatalities annually (CDC, 2016).

Despite the scarcity of accurate data on antimicrobial resistance in most African countries, the available data suggests that Africa shares the global trends of increasing antimicrobial resistance to commonly prescribed antimicrobials (Ndihokubwayo *et al.,* 2013). Elevated levels of resistance to ampicillin, cotrimoxazole, streptomycin, kanamycin, chloramphenicol, tetracycline, gentamycin and penicillin among others have been documented in Zimbabwe (Mbaga *et al.,* 2010), Mozambique (Mandomando *et al.,* 2010), Egypt (El Kholy *et al.,* 2003) and Kenya (Sang *et al.,* 2012; Kipkorir *et al.,*2016). Central Africa, Gabon (Alabi *et al.,* 2013) and Nigeria (Okesola *et al.,* 2009; Okonko *et al.,* 2009) have seen an increase in multi-drug resistant strains, with Nigeria recording elevated levels of resistance to Nalidixic acids (Okesola *et al.,* 2009; Mshana *et al.,*2013).

East African countries are also struggling with the antimicrobial resistance burden (Omulo *et al.,* 2015) which is further complicated by poor standards of hygiene, lack of clean drinking water which may lead to contamination of water bodies with microbes harboring antimicrobial resistance genes (Brooks *et al.,* 2006; Ndihokubwayo *et al.,* 2013). In addition, poor hospital practice ranging from limited laboratory diagnosis to monitor antimicrobial susceptibility, misuse of antimicrobials agents (Ndihokubwayo *et al.,* 2013; Tadesse, 2014) for example over-the-counter prescription and failure to complete prescribed antimicrobial agents. Several cases of multi-drug resistance to commonly prescribed antimicrobials agents against the gastrointestinal bacterial infections have been reported in East Africa, for instance, studies in Ethiopia (Tadesse, 2014; Beyene *et al*., 2011) and Uganda (Kitara *et al.,* 2011) have documented decline in antimicrobial efficacy due to emergence of antimicrobial resistant pathogenic bacteria. In Kenya, there are reports of declining efficacy of antimicrobial agents in managing pathogenic microbes (Wandili *et al.,* 2015; Kipkorir *et al.,*2016; Sang *et al.,* 2012). These phenomena point to a rapid development of antimicrobial

resistant pathogens in the human population. However, there is limited data on whether the antimicrobial resistance pathogens in human can spill to the wildlife ecosystem.

A number of factors have been shown to lead to antibiotic resistance. First, antimicrobial resistance is believed to arise from the misuse of antimicrobial agents in therapeutic and prophylactic management of diseases in both human and livestock (Singer *et al.,* 2003). Secondly, the increasing use of antimicrobials in the control of bacterial infections in fruiting plants and in aquaculture (Thiele-Bruhn, 2003; Cabello, 2006). Antimicrobial misuse leads to an increase in selection pressure in the environment which facilitates the transfer of resistance genes within the intra and interspecific communities. Cross-transfer of resistance genes in the microbial communities leads to the buildup of resistance gene pool in the environment (Davies *et al.,* 2010). Buildup of resistance genes will foster epidemic spread of antimicrobial resistance genes from one region to another that will lead to a global threat to antimicrobial efficacy (Laxminarayan *et al.,* 2013). However, due to the interplay emanating from between human-animals interaction in facilitating spread and persistence of antimicrobial resistance in the environment, there is scanty information on contribution of human-wildlife interaction to spillover of antimicrobial resistance between humans and wildlife species. As such, there is need to establish whether wild animals harbor antimicrobial resistant microbes hence act as environmental reservoirs of antimicrobial resistance.

2.2. Risk Factors Associated With Human-Wildlife Interaction

Human-wildlife interaction is thought to be the primary cause of emergence and spread of most devastating human diseases (Jones *et al.,* 2008) including HIV/AIDS (Hahn *et al.,* 2000) and influenza (Schoub, 2012). The increase in human-wildlife interaction is fostered by anthropogenic habitat change (Radhouani *et al.,* 2014). Human population pressure has resulted in encroachment into hitherto wild environments (Griffin *et al.,* 2003; Cilimburg *et*

al., 2000). Possible sources of interactions or points of contact between human and wildlife include sharing of common watering point and pasture (Martinez, 2009; Mayer, 2000; Deem *et al.,* 2001), hunting and poaching activities, and also wildlife handling common in intensive management of endangered species of the African black rhinoceros (Walpole *et al.,* 2001; Emslie, 2012). Whatever the form of interaction, contact between humans and wildlife may affect pathogen transmission dynamics, in ways that will complicate clinical and veterinary management of infections caused by such pathogens (Smolinski *et al.,* 2003).

Poaching and loss of wildlife habitat is the basis of the global conservation strategies of threatened species (Walpole *et al.,* 2001; Emslie, 2012). The population of the black rhinoceros, has declined by more than 95% in the last five decades due to poaching and habitat loss (Walpole *et al.,* 2001; Emslie, 2012). Currently, the small population estimated at 760 individuals in the wild is found only in Kenya and Tanzania (IUCN, 2017; Emslie, 2012). For example, a number of black rhinoceros were translocated from a private ranch and reintroduced to Ruma National Park between December 2011 and January 2012 (KWS, 2012). Thus intervention measures such as translocation coupled with tight security has been put in place to allow for the recovery of black rhinoceros population. Such intense conservation measures and other human activities have brought these animals under increased contact with humans. Yet it remains unknown if the increased contact between human and intensively managed species of wildlife such as the Eastern black rhinoceros might lead to cross-transmission and maintenance of antimicrobial resistance in the environment.

A number of studies have linked the increasing spillover of antimicrobial resistance genes to wildlife, which is thought to be free from antimicrobial contamination, to proximity of wildlife to human dominated ecosystems; the first case of antimicrobial resistance in migratory birds was reported in Japan (Sato, Oka, Asagi, & Ishiguro, 1978). Presence of

multi-drug resistance (Botti *et al.,* 2013) and methicillin resistant bacteria whose origin is traced to humans and livestock (Porrero *et al.,* 2013) was seen in several wild animals in Europe. Also, a multi-drug resistant *E. coli* isolated from wild rabbits in Portugal (Silva *et al.,* 2010) and those exhibiting high degree of genetic similarity with isolates from humans was seen from mongoose in Botswana (Pesapane *et al.,* 2013); whereas, a multi-drug resistant *E. coli* strain from rat was reported in Kenya (Gakuya *et al.,* 2001). From the literature, it is clear that interaction between human and wild animals can enhance the spread and cross-transmission of antimicrobial resistant pathogens. Of concern, is that it is not known whether wild animals such as black rhinoceros may harbor microbes with similar antimicrobial resistance profiles to those of humans. Therefore, in absence of interaction between human and black rhinoceros in the wild, it can be predicted that *E. coli* isolates from human would have higher levels of antimicrobial resistance compared to isolates from black rhinoceros.

In addition, harsh environmental conditions may subjects bacteria to stressful factors that will lead to controlled adaptive response. The adaptive measures employed by bacteria include cellular changes that leads to reduced growth and metabolic activities (Poole, 2012). For example, high temperature, high osmolarity, alkaline pH and high salt, will trigger expression of a highly regulated sigma factor (*RpoE (sE), EnvZ/ OmpR* and *CpxRA*) in *E. coli* bacterium, this will not only make them overcome the prevailing stressful condition, but also leads to up-regulation of resistance genes such as: β-lactammase, aminoglycosides and multi-drug efflux genes (Hirakawa *et al.,* 2003; Nishino *et al.,* 2010). In addition, activation of *ompC* pathway will lead to suppression of *ompF*, yet *ompF* is a major portal for entry of β-lactam antimicrobial agents (ampicillin, penicillin, cefalothin, cefuroxime), while *ompC* forms a smaller channels hence leading to inability of β-lactams to penetrate the bacterial membrane (Hirakawa *et al.,* 2003). Therefore, when bacterial adaptive measures kicks in they will be able to survive in unfavorable environment and at the same time expressing other genes

responsible for antimicrobial resistance in absence of antimicrobial selection pressure (Hirakawa *et al.,* 2003; Nishino *et al.,* 2010). As such, increase in up-regulation of antimicrobial resistance genes can be caused by intrinsic response to environmental stressors. Thus, the impact of up-regulation of multi-drug resistance genes in *E. coli* strains to human health rests in the possibility that once the resistance genes have been mobilized to commensal or pathogenic bacteria in the wildlife, the gene will naturally amplify and be transmitted afterwards between bacterial species of human importance. This will then find its way to humans through contact points such as contaminated watering points.

2.3. Genetic Characterization of Antimicrobial Resistance

Decline in antimicrobial potency worldwide correlates with the environmental spread of antimicrobial resistance genes (Kumarasamy *et al.,* 2010). Therefore, understanding the dynamics of resistance genes transfer in a bacterial population, its reservoirs and their spread in the environment is important in combating the threats caused by antimicrobial resistance. Such understanding requires empirical data on the spectrum of their environmental reservoirs

Beside overuse of antimicrobial agents in hospitals, agriculture and in the community (Zhang *et al.*, 2006), the use of sub-lethal antimicrobial doses in farm animals also contributes to persistence of resistance genes in the environment. Mobilization of antimicrobial resistance genes from one host to another is facilitated by mobile genetic elements such as plasmids, transposons, integrons and bacteriophages (Middleton *et al.,* 2005; Levy, 2002). Several studies have documented the progressive increase of antimicrobial resistance overtime; in early 1990 *E. coli* strain resistant to ampicillin, cefaltin, tetracycline, chloramphenicol, sulfisomidine, trimethoprim and gentamycin was reported in Ethiopia (Ringertz *et al.*, 1990). Towards the end of 20th Century and early 21st Century, *E. coli* strain resistant to amoxillin/clauvalate, erythromycin (Sang *et al.*, 1997) and kanamycin (Kikuvi *et al.*, 2006)

was reported in Kenya. Currently, resistance to ampicillin and trimethoprim/sulphamethoxazole is about 95% and to tetracycline is about 81% (Kipkorir *et al.*,2016; Sang *et al.,* 2012).

Antimicrobial resistance genes are spread through contamination of the environment with resistant strains and ingestion of contaminated food and water (Brooks *et al.,* 2006). Physical factors created by wind, human movement and watersheds are important factors in the spread of resistant genes from one geographical region to another and from one community to another (Martinez, 2009; Levy, 2002). Indeed the global distribution of *Beta*-lactamase in *E. coli* defines the evolution and spread of bacterial resistance (Davies *et al.,* 2010). About a thousand β-lactam resistant genes have been identified (Davies *et al.,* 2010). The high rate of extended spectrum β-lactamase producing *E. coli* has restricted the use of β-lactam group of antibiotics (Viswanathan *et al.,* 2012; Pfeila *et al.,* 2010). In fact, the rate of *E. coli* adaptive mutation is in the order of 10^{-5} per genome per generation with a mean selective advantage of 1% that has implication in the evolution of antimicrobial resistance (Perfeito *et al.,* 2007). So far, extended spectrum β-lactamase producing *E. coli* has been isolated in vegetables (Reuland *et al.,* 2014), water (Fahrenfeld *et al.,* 2013), farm animals (Smet *et al.,* 2008) and dust (Diaz-Mejia *et al.,* 2008). In addition, *E. coli* strains expressing a wide array of ***TEM*** resistance genes; ***tetA*** and ***tetB; sul1*** and ***dfrA1*** genes have also been isolated from black-headed gulls (Dolejska *et al.,* 2007), wild rabbits (Silva *et al.,* 2010) and banded mongoose (Pesapane *et al.,* 2013). Moreover, Pesapane *et al.* (2013) demonstrated that *E. coli* isolates from banded mongoose foraging in human backyard gardens had a high degree of genetic similarity. In addition, a number of studies on angulates demonstrate a high degree of *E. coli* cross-transmission, as seen in domestic and wild animals with overlapping pasture and watering points (Vander-Waal *et al.,* 2014b), among societies of giraffes (Vander-Waal *et al.,* 2014a) humans, livestock and the mountain gorillas in Uganda (Rwego *et al.,* 2008).

However, the distinctiveness of the genes that confer antimicrobial resistance in bacterial isolates from intensively managed animals such as the black rhinoceros population remains unknown.

In studying interspecies cross-transfer of antimicrobial resistance, *Escherichia coli* have been demonstrated as a suitable model in investigating enterobacteriaceae transmission between humans and wildlife (Farnleitner *et al.,* 2010; Szekely *et al.,* 2010), since they exist in pathogenic and commensal forms in different mammalian hosts (Stecher *et al.,* 2012). The alimentary tract of mammals is inhabited by distinct subtype of *E. coli* strains (Simpson, 2002), yet Pesapane *et al.,* (2013) using *E. coli* isolates from banded mongoose foraging in human backyard gardens demonstrated that only those isolates having similar antimicrobial resistant patterns with the isolates from human had a high degree of genetic similarity. In sum, it can be reasoned that mammalian hosts presenting genetically similar *E. coli* strains are either exposed to a common *E. coli* strain, or are experiencing high rate of genetic cross-transfer. However, major gaps in knowledge exist on whether the antimicrobial resistant *E. coli* strains from humans and those from wildlife harbor similar genes that confer resistance to antimicrobial agents. Therefore, it is predicted that in absence of cross-transfer of antimicrobial resistance genes at the interface between human and wildlife due to contamination of either watering or feeding point, *E. coli* isolates from human would not have antimicrobial resistance gene profile similar to those present in isolates from black rhinoceros in the wild.

In elucidating the presence of antimicrobial resistant genes among bacterial population, molecular testing methods are invaluable methods which help. In molecular analysis, the techniques used include Real time polymerase chain reaction (RTPCR) which quantifies and expresses antimicrobial resistance genes in real time. The conventional polymerase chain reaction (PCR) is used to detect the presence of and to characterize genes responsible for

antimicrobial resistance. Molecular methods use specific antimicrobial resistance gene primers to validate the existence of these genes in the suspected microorganism (Ehlers *et al.*, 2009). These methods have proven to be reliable in testing antimicrobial resistance patterns (Louw *et al.*, 2012) and may reduce the risk of increasing bacterial resistance by preventing use of unnecessary antibiotics in patients who may not benefit from them.

2.4. Antimicrobial Susceptibility Testing for Pathogenic *E. coli*

Drug susceptibility testing is often used to select the effective antimicrobial drug to use in treatment and control of infectious diseases by pathogens that are resistant to drugs which is essential as a guideline for clinical management (CLSI 2016). Agar disc diffusion (ADD), Minimum inhibition concentration (MIC) and Epsilometer strip test (E test) are some of the antimicrobial testing methods used.

Agar disc diffusion is a paper discs impregnated with antimicrobials at varied concentration to be used to test for drug antimicrobial activities *in vitro*. These discs are carefully placed on agar plates where the drugs will diffuse into the agar extending its antibacterial activities; therefore, the activity of the drug against the bacterial correlates with the zone of inhibition around the disc (Morello *et al.*, 2003; CLSI 2016).

Minimum inhibition concentration (MIC) is used for direct measurements of minimum zone of inhibitory concentration (MIC) of an organism with an antimicrobial dilution test (Morello *et al.*, 2003; CLSI 2016). The test is performed by inoculating the wells of a plate with the bacterial culture and dilutions of antibiotics are arranged across the rows. The MIC can directly be determined by observing the exact concentration required to inhibit bacterial growth (CLSI 2016). If the tested bacteria have a MIC equal to or below the susceptible breakpoint, the organism is susceptible. An MIC equal to or above the 'resistant' breakpoint indicates that the organism is resistant regardless of dose administered on location of the

infection. Minimum inhibition concentration in the intermediate range means that the organism is resistant to the drug unless dosing modification is used. MIC tests are more qualitative than an ADD test, but must be performed according to strict guidelines (CLSI 2016).

Epsilometer Strip Test is an expansion of the disk diffusion method since the inoculation protocol is the same. E-test provides a direct qualification of antimicrobial susceptibility of microorganisms (Morello *et al.*, 2003; CLSI 2016). The test uses an epsilometer test (E-test) device for direct quantification of antimicrobial susceptibility of microorganisms. The method combines the ease of disk diffusion and accuracy of the MIC broth dilution techniques. The E-test utilizes a rectangular plastic strip device that contains predefined, continuous exponential gradient of antibiotic concentration that corresponds to MIC dilutions. The antibiotic impregnated plastic strips are placed on the surface of the solid agar. After 24 hours of incubation, an elliptical zone of inhibition of bacterial growth is seen around the test strip. The zone edge intersects the plastic strip at a specific level corresponding to the inhibitory concentration of the drug that inhibits the microorganism (Morello *et al.*, 2003; CLSI 2016). The inhibitory concentration is a direct measure of the susceptibility of the organism to the particular test drug.

CHAPTER THREE: MATERIALS AND METHODS

3.1. Study Site

This study was conducted in Lambwe Valley, western Kenya (Appendix I). Lambwe Valley lies within latitudes 0^0 30' and 0^0 45' South and longitudes 34^0 10' and 34^0 20' East (Allsopp *et al.*, 1972). It has a total area of 324 km^2 of which 120 km^2 at the valley bottom is occupied by Ruma National Park. The area has a warm humid climate, with a mean annual temperature of 22^0C and a daily mean minimum and maximum ranging from 17^0C to 30^0C (Njoka *et al.*, 2003). The area receives mean annual precipitation of 1346mm and has a bimodal rainfall pattern between March to May and September to November (Allsopp *et al.*, 1972; Njoka *et al.*, 2003). Ruma National Park is served by the Lambwe River, a seasonal river that meanders through the park and drains into Lake Victoria. According to 2016 health reports from Homa Bay County Health facilities, a total 32,112 cases of enteric infection was reported in 2015 within the region with 64.5% being cases of non-specific diarrhea while 35.5% being cases of typhoid fever.

3.2. Study Population

The study focused on the population of black rhinoceros in Ruma National Park that were translocated from private ranches in a bid to conserve them. Black rhinoceros are critically endangered. There are about 21 black rhinoceros in Ruma National Park. Thus, it is an ideal candidate to test the hypothesis that *Escherichia coli* strains isolated from human and black rhinoceros show no difference in susceptibility to eight commonly used antimicrobials and that they harbor different genes that confer resistance to commonly used antimicrobials. With the increased interaction between human and the critically endangered black rhinoceros in

Ruma National Park due to conservation measures, poaching, tourism activities, sharing of a single water source between human communities and the wildlife and also having a public road passing through the park, it was imperative to ascertain the impact of such interaction to spillover of antimicrobial resistance at the interface between human and black rhinoceros.

3.3. Inclusion Criteria

With the small population of black rhinoceros in Ruma National Park, they were sampled to saturation. Whereas, human participants from the communities surrounding Ruma National Park were recruited to this stud after they gave informed written consent of participation to the study (Appendix II).

3.4. Exclusion Criteria

With human participants, the following groups were excluded from the study: children under the age of 18 years without available parent or legal guardian to give informed consent and the very aged who would not understand the scope of the study and could not give informed written consent.

3.5. Study Design

This was a cross-sectional study involving *E. coli* strains isolated from black rhinoceros in Ruma National Park, and from human communities surrounding Ruma National Park.

3.6. Sample Size Determination

Sample size was determined according to the formula by Kothari, (2004)

$$\mathbf{n = [z^2\, p\, q] / e^2}$$

Where: **n** is the desired sample size, **z** is the statistics for a level of confidence; **p** is the estimated proportion of an attribute that is present in the population (expected prevalence); **q** is **1-p**; **e** is the acceptable margin of error for proportion being estimated (precision).

In this study, **z** value (at 5% type 1 error (P<0.05) of 1.96; **p** value of 0.50 was assumed, according to Kothari, (2004). Since *E. coli* is a commensal enterobacteriaceae inhabiting gastrointestinal tract of all mammals, the sample size had an alpha level a priori at 0.08 chosen as a good compromise between an appropriate precision, the high prevalence of the commensal *E. coli* in gastrointestinal tract of mammals and the resources available for this study.

Therefore: $\mathbf{[1.96^2 * 0.5\ (1\text{-}0.5)]/\ (0.08^2) = 150}$

Thus, in this study, the sample size of human participants was 150. To buffer for sampling errors and errors arising from sample handling since samples were collected and stored in field conditions, 22% of the calculated sample size was added, thus, bringing the final sample size to 184.

3.7. Sampling Procedure and Sample Collection

Fecal specimens were collected following the protocol by Carroll *et al.*,(2015). Fecal samples from humans were collected following simple randomized sampling design (Kothari, 2004) with modification, where the after selecting the first household, every third household was selected for sampling. A swap of fresh fecal samples was taken aseptically and inoculated in Carry Blair media (Himedia pvt Ltd. Mumbai, India) in a screw capped universal bottle. The samples were then transferred in ice cold box at 8^0C from the field to the central storage facility and stored for 3-4 days at 4^0C awaiting transportation to Maseno University microbiology laboratory. The isolates from black rhinoceros fecal samples (n=16) and those

from human fecal samples were then transferred in ice cool box at 8^0C to Microbiology laboratory for processing and microbiological analysis.

Sweeps of *E. coli* cultures from both human and black rhinoceros samples showing resistance were first sub-cultured on nutrient agar plates and incubated for 24 hours at 37^0C then preserved at 4^0C in Soya bean Casein Digest Medium (Himedia pvt Ltd. Mumbai, India) with 15% glycerol, awaiting DNA extraction and subsequent molecular analysis. All the samples collected were sterilized by autoclaving at a temperature of 121^0C for 15 minutes (Lauer *et al.,* 1982) and finally incinerated.

3.8. Identification of *E. coli* in Stool Samples

Isolation and identification of *E. coli* was performed according to protocol by Morello *et al.*(2003) where the transportation media was gently agitated and aliquot sub-cultured to Tryptone phosphate broth (Himedia pvt Ltd. Mumbai, India), an enrichment media, and incubated at 37^0C for 20 hours. After 20 hours, the enrichment broth was gently agitated and an aliquot streaked on Eosin Methyl Blue (EMB) agar (Himedia pvt Ltd. Mumbai, India) and incubated at 37^0C for 24 hours. Colony color and morphology was then determined by visualization using a hand lens. The typical *E. coli* which appeared as brilliant green colonies in eosin methyl blue (EMB) agar were sub-cultured into Triple Sugar Iron (TSI) media (Himediapvt Ltd. Mumbai, India) in caped tubes and incubated at 37^0C for 48 hours. The isolates from TSI showing both acidic slant and butt were sub-cultured to Motility, Indole, Lysine medium (MIL) (Himedia pvt Ltd. Mumbai, India) and to indole, methyl red and vogesproskauer (IMVIC) (Himedia pvt Ltd. Mumbai, India) for biochemical differentiation and identification of *E. coli.* In motile-lysine medium, *E. coli* colonies were able to propel themselves progressively in one direction moving away from the point of inoculation. This directional propulsion of colonies was an indicative of enterobacteriaceae that possess

flagella and the presence of *Escherichia coli.* In addition, lysine was broken down by decarboxylase enzymes possessed by some bacteria giving a positive test of deep purple colour. Further test in IMVIC broth was carried to differentiate *E. coli* from other *enterobacteriaceae*. In this study indole was produced when 0.2ml- 0.3ml Kovac's reagent was added to 5ml of 24 hours tryptophan broth culture and a red coloured ring observed at interface, indicating presence of *E. coli* strains. Methyl red test was positive indicated by formation of bright red color throughout medium due to fermentation of glucose; *E. coli* strains produce acid from fermentation of glucose, mannitol and sorbitol hence low pH indicated by bright red color in the medium. Vogesproskauer test depends on the production of acetylemethylcarbinol or butylenes glycol from dextrose in medium. This is oxidized to diacetyl by adding alkali (KOH), which shows pink color for positive results (Morello *et al.,* 2003). Vogesproskauer (VP) test was negative when 0.6ml α -naphthol and 0.2ml of 40% KOH solution were added respectively to 5ml of 48 hours VP culture and vortexed. Thus with the observed phenotypic and biochemical tests characteristic, *E. coli* was isolated.

3.9. *Escherichia coli* Antimicrobial Susceptibility Test

Escherichia coli isolates were tested for susceptibility against eight commonly used antimicrobial agents (ampicillin (10mcg), gentamicin (10mc), tetracycline (30mcg), cotrimoxazole (25mcg), chloramphenicol (30mcg), ceftriaxone (30mcg), amoxicillin/clavulanic acid (30mcg) and erythromycin (15mcg) (Himedia pvt Ltd. Mumbai, India)).

The choice of antimicrobials for the susceptibility test used in this study was informed by two factors: first, guidance by Clinical and Laboratory Standard Institute (CLSI. 2016), where antimicrobial agents are placed into 6 different groups. The group A antimicrobial agents (ampicillin and gentamicin) are those that are considered appropriate for inclusion in primary

testing panels; group B agents (amoxicillin/clavulanic acid, ceftriaxone and cotrimoxazole) are those that may warrant primary testing and are reported selectively when organisms are resistant to agents of the same antimicrobial class in group A; while group C include supplemental antimicrobial agents (chloramphenicol and tetracycline) that may be tested in regions harboring endemic strains resistant to several primary antimicrobial agents for instance those resistance to β-lactams (CLSI 2016). Secondly, selection was narrowed down to those antimicrobial agents that are easily accessed as over-the-counter medication and those that are commonly used in treatment of both human and veterinary infections. These include all the above mentioned antimicrobial agents, with exception of amoxicillin/clavulanic acid and ceftriaxone which are used in treating human conditions. The addition of amoxicillin/clavulanic and ceftriaxone was to determine if wild animals may harbor resistance to antibiotics not open for veterinary use. The selected antimicrobial agents are also a representative of the seven main classes of antibiotics that are commonly in use in the management of both human and animal diseases, they include: ampicillin (penicillins) and amoxicillin/clavulanic acid (β-lactamase inhibitor combination), gentamicin (aminoglycosides), tetracycline (tetracyclines), cotrimoxazole (folate pathway inhibitors), chloramphenicol (phenicols), ceftriaxone (cephems) and erythromycin (macrolides) (CLSI 2016).

Antibiotic susceptibility test was performed following the protocol by Kirby-Bauer disk diffusion method (Bauer *et al.,* 1966). Antimicrobial susceptibility breakpoints were interpreted according to the recommended standards by the Clinical and Laboratory Standard Institute (CLSI 2016).

Escherichia coli cultures were swabbed onto a Muller-Hinton agar (Himedia pvt Ltd. Mum-bai, India). Eight different antibiotic discs (ampicillin (10mcg), gentamicin (10mc), tetracycline (30mcg), cotrimoxazole (25mcg), chloramphenicol (30mcg), ceftriaxone

(30mcg), amoxicillin/clavulanic acid (30mcg) and erythromycin (15mcg)) were applied using sterile forceps onto the culture plates. The culture plates were dried on working bench for 5min, after which, they were incubated at 37°C for 24 hours (Bauer *et al.,* 1966). *E. coli* ATCC 25922 was used as a control for potency of antimicrobial agent discs. Zones of inhibition were measured to the nearest millimeter and isolates classified as sensitive (S), intermediate (I) and resistant (R) (CLSI 2012).

Escherichia coli cultures showing resistance was sub-cultured in nutrient agar (Himedia pvt Ltd. Mumbai, India) and incubated for 24 hours at 37^0C to facilitate recovery of stressed cells. Sweeps of the bacterial growth on nutrient agar plates were then preserved at 4^0C in Soya bean Casein Digest Medium (Himedia pvt Ltd. Mumbai, India) with 15% glycerol, pending DNA extraction and subsequent molecular analysis.

3.10. DNA Extraction

Escherichia coli isolates preserved at 4^0C in Soya bean Casein Digest Medium (Himedia pvt Ltd. Mumbai, India) with 15% glycerol were brought to room temperature then DNA extraction was carried out using DNeasy purification kit (QIAGEN), following the manufacturer's instructions (Appendix III). DNA concentration and purity was assessed spectrophotometrically using Nano-drop spectrometer (QIAGEN).

3.11. Detection of Resistance Genes

Selected isolates were subjected to molecular analysis to determine presence of antimicrobial resistance genes in six *E. coli* isolates from human and four isolates from black rhinoceros. The isolates which showed high level of antimicrobial resistance were purposively selected as potential candidates of genes that confer resistance. The selected isolates were analyzed for presence of $\boldsymbol{bla_{TEM}}$ gene that codes for ampicillin resistance, ***tetA*** and ***tetB*** genes which codes

for tetracycline resistance and ***Sul1*** and ***dfrA1*** genes which codes for cotrimoxazole resistance. The selection of these three antimicrobial agents ampicillin, tetracycline and cotrimoxazole, were selected based on the protocol by Clinical and Laboratory Standards Institute (CLSI 2016) with antimicrobial agents in group A and group B selected.

Polymerase chain reaction was used to detect the presence of resistance genes in a final reaction volume of 50µl (Table 3.1). Amplification was carried out using Rotor-Gene PCR cycler (QIAGEN) using published primers (Table 3.2). In $\boldsymbol{bla_{TEM}}$ gene amplification, the following conditions were used, initial denaturing at 94^0C for 5 min followed by 30 cycles consisting of 94^0C for 1 min, annealing at 58^0C for 45 sec, elongation at 72^0C for 2 min and final extension 72^0C for 10 min then 4^0C until visualization. In amplification of ***tetA*** gene, the following condition was used, initial denaturing at 94^0C for 1 min followed by 30 cycles consisting of 94^0C for 1 min, annealing at 57^0C for 30 sec, elongation at 72^0C for 2 min and final extension 72^0C for 10 min then 4^0C until visualization. In amplification of ***tetB*** gene, the following condition was used, initial denaturing at 94^0C for 1 min followed by 30 cycles consisting of 94^0C for 1 min, annealing at 55^0C for 1 min, elongation at 72^0C for 2 min and final extension 72^0C for 10 min then 4^0C until visualization. In amplification of ***Sul1*** gene, the following condition was used, initial denaturing at 94^0C for 5 min followed by 36 cycles consisting of 94^0C for 15 sec, annealing at 63^0C for 30 sec, elongation at 72^0C for 1 min and final extension 72^0C for 7 min then 4^0C until visualization. While in amplification of ***dfrA1*** gene, the following condition was used, initial denaturing at 94^0C for 5 min followed by 35 cycles consisting of 94^0C for 30 sec, annealing at 60^0C for 40 sec, elongation at 72^0C for 1 min and final extension 72^0C for 7 min then 4^0C until visualization.

Table 3.1: PCR master mix for DNA amplification

Component	Quantity for 1 reaction	Concentration
2X Tag Mix	25.0µl	1X
Forward Primer	2.0µl	400nM
Reverse Primer	2.0µl	400nM
DNA Template	5.0µl	<1000ng
PCR grade dH_2O	16.0µl	
Total Volume	**50.0µl**	

Table3.2: Primer sequences used for PCR amplification

Antimicrobial Agent	Gene	Sequence
Beta-lactams	***blaTEM***	**(F)** CATTTCCGTGTCGCCCTTAT
		(R) TCCATAGTTGCCTGACTCCC
Sulfonamide	***Sul1***	**(F)**TTCGGCATTCTGAATCTCAC
		(R)ATGATCTAACCCTCGGTCTC
Trimethoprim	***dfrA1***	**(F)**AAGAATGGAGTTATCGGGAATG
		(R)GGTAAAAACTGGCCTAAAATTG
Tetracycline	***Tet(A)***	**(F)** GGTTCACTCGAACGACGTCA
		(R) CTGTCCGACAAGTTGCATGA
	tet(B)	**(F)** CCTCAGCTTCTCAACGCGTG
		(R) GCACCTTGCTGATGACTCTT

Note: the table shows genetic sequences of the primers that targets genes responsible for different antimicrobial resistance

3.12. DNA Visualization in Gel Electrophoresis

PCR amplicons for ***tetA*** and ***tetB*** gene were loaded onto 1.5% agarose gel stained with ethidium bromide and electrophoresed at 100 volts for 35min. Amplicons for ***blaTEM*** gene were loaded onto 1% agarose gel stained with ethidium bromide and electrophoresed at 100 volts for 35min. Also, PCR products for detection of ***sul1*** gene product were loaded onto 2.2% agarose gel stained with ethidium bromide and electrophoresed at 90 volts for 180 min. While those of ***dfrA1*** were loaded onto 2% agarose gel stained with ethidium bromide and electrophoresed at 100 volts for 35 min. In running the amplicons, 10x Tris-Borate-EDTA (TBE) buffer was used. All the electrophoresed aamplicons were visualized through UV trans-illuminator (QIAGEN) and gel pictures taken. Molecular weight marker with 100bp increment (100bp, DNA ladder, Invitrogen) was used as a size standard.

3.13. Data Analysis

The resistant pattern was measured and presented as a percentage using descriptive statistics. The Mann-Whitney test was used to compare proportion of *E. coli* isolates that showed resistance across the eight antibiotics between human and black rhinoceros samples (formula; Appendix V). In addition the Z-test for difference in proportion of two samples was used to compare proportions of resistance *E. coli* isolates for each antibiotic (formula; Appendix V). In all the analysis, the cut off for statistical significance was at 95% confidence level.

3.14. Ethical Issues

This study was cleared by the School of Graduate Studies, Maseno University. Ethical approval for the study was obtained from Maseno University Ethical Research Committee; the ethical clearance certificate reference number is MSU/DRPI/MUERC/00420/17 (Appendix IV). Research permit for fieldwork component of the study was obtained from the

Kenya Wildlife Services; reference number KWS/BRM/5001. Study permission for collection of human stool samples was sought from Ndhiwa Sub-County Hospital in Homa-Bay County and informed written consent was obtained from human study participants prior to sample collection (Appendix II).

The information collected from human participant was kept as confidential as stipulated according to ethical requirements; where, the samples were coded by the use of numbers that did not identify the study participants. In addition, these codes were not indicated on the signed consent forms. All soft copies data in spreadsheet were encrypted and stored in a password protected folder. The data were only accessed by the principal investigator and the supervisors.

3.15. Limitations of the study

For one to gain more insight on the spill-over of antimicrobial resistance genes at the human-wildlife interface, it would have been more meaningful to analyses antimicrobial susceptibility across all pathogenic bacterial strains. Secondly, to have a full picture of the cross-transfer of genetic elements at the human-wildlife interface, it would have been prudent to do DNA fingerprint of *E. coli* strain from human and black rhinoceros by analyzing the variation in their house keeping repetitive extragenic palindromic genes. However, the similarity of antimicrobial resistance genes among different bacterial species may act as a crucial marker for the spillover and mixing of genetic elements in bacterial population at the interface between human and the wildlife.

CHAPTER FOUR: RESULTS

4.1. RESULT

In this study, a total of 184 and 16 faecal samples were collected from human and black rhinoceros during the study period (June 2017 to November 2017). Out of these, 93.8% (n=15) of isolates from black rhinoceros and 100% (n=184) of human samples tested positive of *E. coli* based on their morphological appearance and biochemical reaction on various bacteriological (differential and selective) culture media.

4.1.1. Antibacterial Resistance in *E. coli* Isolates from Black Rhinoceros and Human in Lambwe Valley

Escherichia coli isolates from human and black rhinoceros showed varied degree of susceptibility with amoxicillin/clavulanic acid (85.3% and 60%), cotrimoxazole (83.1% and 33.3%), gentamicin (28.3% and 80%), erythromycin (76.1% and 86.7%) and ampicillin (75.0% and 73.3%), tetracycline (64.7% and 40.0%), ceftriaxone (58.3% and 13.3%) and chloramphenicol (29.9% and 6.7%) respectively, Table 4.1.

Table 4.1: Antimicrobial susceptibility profiles of *Escherichia coli* isolates from humans and black rhinoceros in Lambwe Valley

Antimicrobial Agent	% Susceptibility in Humans (n=184)			% Susceptibility in Black Rhinoceros (n=15)		
	R	I	S	R	I	S
Ampicillin	75.0	11.4	13.6	73.3	26.7	0.0
Gentamicin	28.3	23.4	48.3	80.0	6.7	13.3
Tetracycline	64.7	13.0	22.3	40.0	6.7	53.3
Cotrimoxazole	83.1	4.4	12.5	33.3	0.0	66.7

Chloramphenicol	**29.9**	**24.5**	**45.6**	**6.6**	**6.7**	**86.7**
Ceftriaxone	**58.3**	**20.3**	**21.4**	**13.3**	**0.0**	**86.7**
Amoxycillin/ Clavulanic acid	**85.3**	**12.5**	**2.2**	**60.0**	**13.3**	**26.7**
Erythromycin	**76.1**	**19.0**	**4.9**	**86.7**	**13.3**	**0.0**

Legend: Ampicillin, tetracycline, amoxicillin/clavulanic acid and erythromycin resistance were higher both in human and black rhinoceros isolates. Isolates from black rhinoceros were highly susceptible to chloramphenicol. R= resistance, I= intermediate and S= sensitive.

The observed antimicrobial resistance pattern in six out of the eight antimicrobial agents tested was higher in *E. coli* strains isolated from human than in those from black rhinoceros (Figure 4.1). Interestingly, two of the eight antimicrobial agents tested (erythromycin and gentamycin) gave an unexpected results where proportion of antimicrobial resistance was higher in *E. coli* strains isolated from black rhinoceros than those from human (Figure 4.1). When the overall results of all the eight antimicrobial agents were subjected to Mann-Whitney test, it was found that there was no statistical difference in overall antimicrobial resistance between *E. coli* strains from human and those from black rhinoceros (U=25.000, P=0.462). Further statistical interrogation of individual antimicrobial agent by comparing proportions of resistance in *E. coli* isolates from human and those from black rhinoceros to each antimicrobial agent using Z-test showed that there was no difference in antimicrobial resistance patterns in *E. coli* isolates from human and black rhinoceros in 50% of the antimicrobial agents (ampicillin Z=0.143, p=0.889; tetracycline Z=1.901, p=0.057; chloramphenicol Z=1.923, p=0.055 and erythromycin Z=0.935, p=0.3524); whereas, the remaining 50% showed difference in their antimicrobial resistance profiles. Among those which exhibited difference in resistance patterns (cotrimoxazole, ceftriaxone and

amoxicillin/clavulanic acid and gentamycin); 37.5% of these antimicrobial agents (cotrimoxazole, ceftriaxone and amoxicillin/clavulanic acid) pointed to higher antimicrobial resistance pattern in isolates from humans than in black rhinoceros, whereas, gentamycin resistance was higher in isolates from black rhinoceros than those from humans.

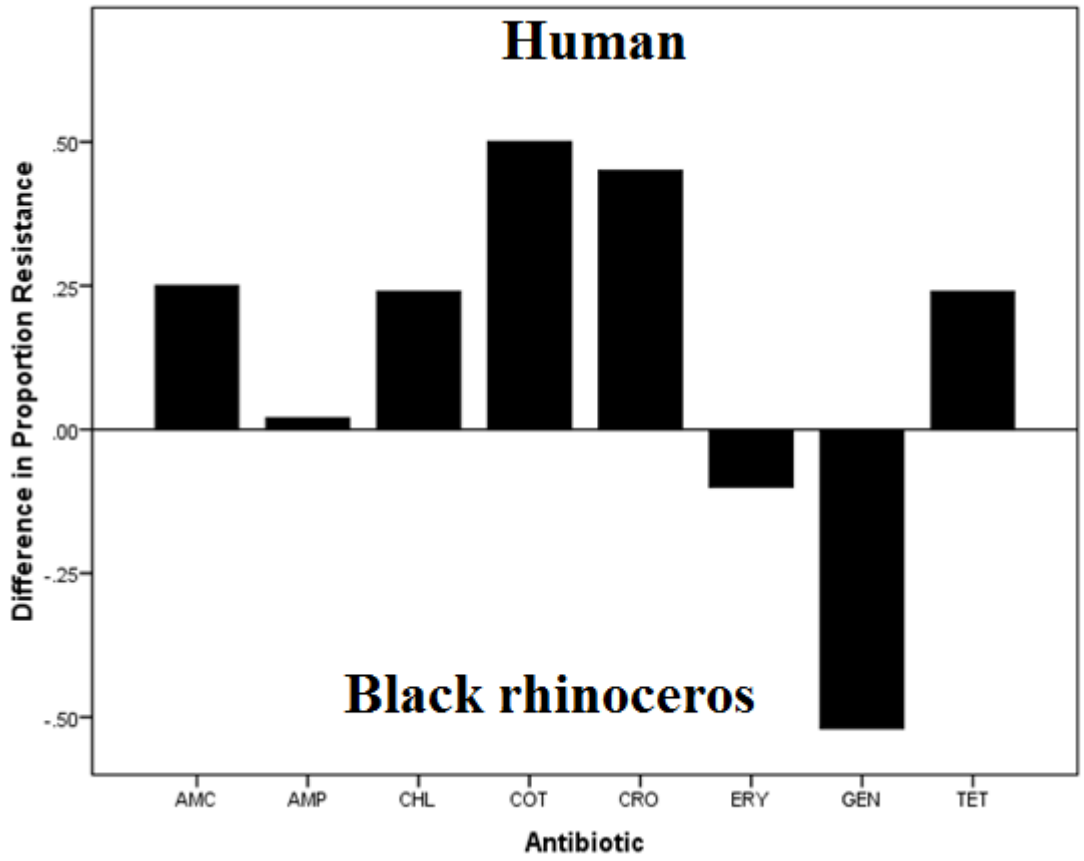

Figure 4.1: Difference in proportion of antimicrobial resistance in *E. coli* isolates from human and black rhinoceros in Lambwe Valley.

Note: Difference in proportion was obtained by subtracting proportions of isolates that showed resistance in black rhinoceros from those of humans. A positive value means resistance was higher in humans while negative value means resistance was higher in black rhinoceros. AMC= amoxicillin/clavulanic acid, AMP= ampicillin, CHL= chloramphenicol, COT= cotrimoxazole, CRO= ceftriaxone, ERY= erythromycin, GEN= gentamycin, and TET= tetracycline.

In addition to resistance profiles to individual antibiotics, *E. coli* strains resistant to more than two antimicrobial agents were identified and represented as a case of multi-drug resistance (MDR). The proportion of MDR phenotype of *E. coli* isolated from black rhinoceros was 43.4%, while that from human isolates was 69%. The isolates were mostly resistant to ampicillin, gentamycin, tetracycline, ceftriaxone, amoxicillin/clavulanic acid and erythromycin.

4.1.2. Antimicrobial Resistance Genes in *E. coli* Isolates from Human and Black Rhinoceros

All the 6 selected isolates resistant to ampicillin from human and 3 of 4 from black rhinoceros gave positive amplicons for $\boldsymbol{bla_{TEM}}$ genes (Plate 4.1). In tetracycline resistance, 4 of 6 *E. coli* isolates from human and 3 of 4 *E. coli* isolates from black rhinoceros harbored ***tetA*** genes (Plate 4.2), while one *E. coli* isolate each from human and black rhinoceros were positive for ***tetB*** genes (Plate 4.3). In addition, 2 of 6 *E. coli* isolates from human and 1 of 4 isolates from black rhinoceros harbored ***dfrA1*** genes for trimethoprim resistance (Plate 4.4), while 3 of 6 isolates from human and 1 of 4 isolates from black rhinoceros expressed ***sul1*** genes (Plate 4.5) responsible for sulfonamide resistance.

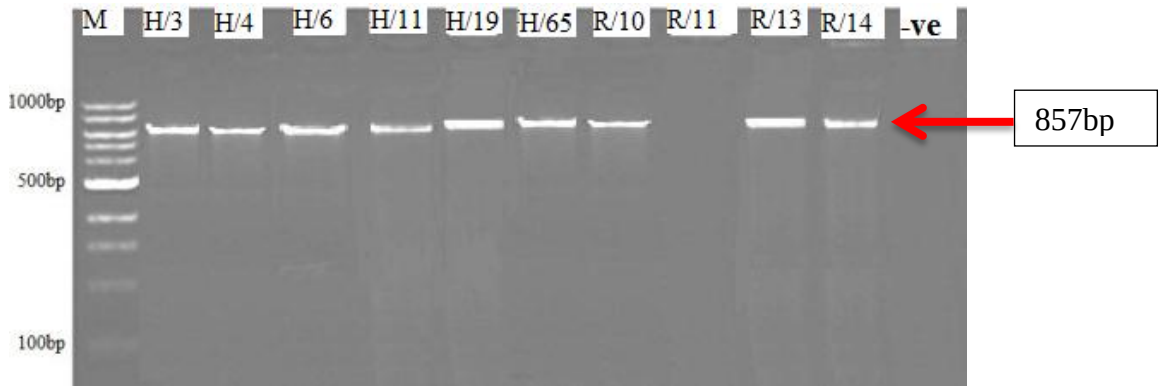

Plate 4.1: Micrograph showing presence of *blaTEM* gene (857bp) in *Escherichia coli* isolated from human and black rhinoceros.

Note: M= DNA ladder, H/3, H/4, H/6, H/11, H/19 and H/65 represent human samples and R/10, R/11, R/13 and R/14 represent black rhinoceros samples; **-Ve**= negative control. All the 6 samples from humans and 3 samples from black rhinoceros showed presence of ***blaTEM*** **gene** respectively.

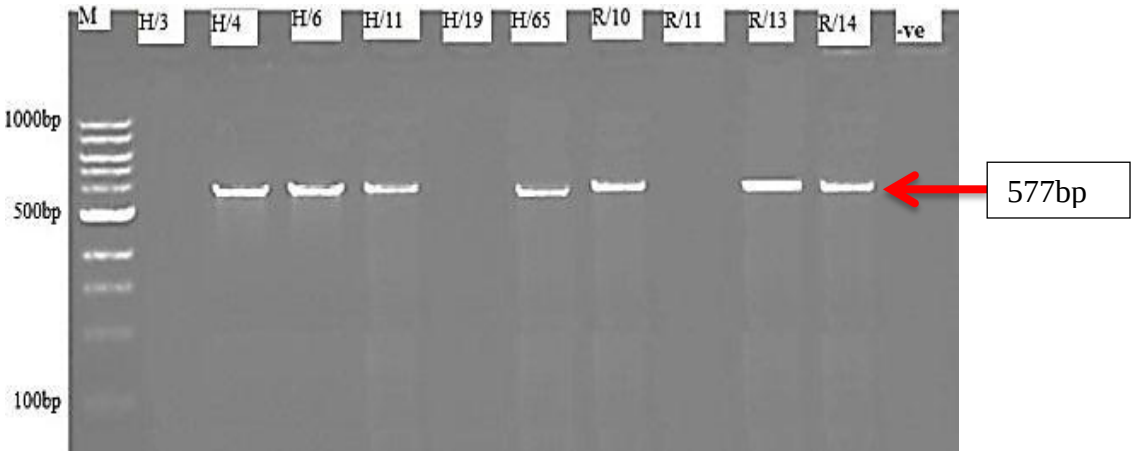

Plate 4.2: Micrograph showing presence of *TetA* gene (577bp) in *Escherichia coli* isolated from human and black rhinoceros

Note: M= DNA ladder, H/3, H/4, H/6, H/11 and H/65 represent human samples and R/10, R/11, R/13 and R/14 represent black rhinoceros samples; **-Ve**= negative control. Four samples from humans and 2 samples from black rhinoceros showed presence of ***TetA*** **gene** respectively.

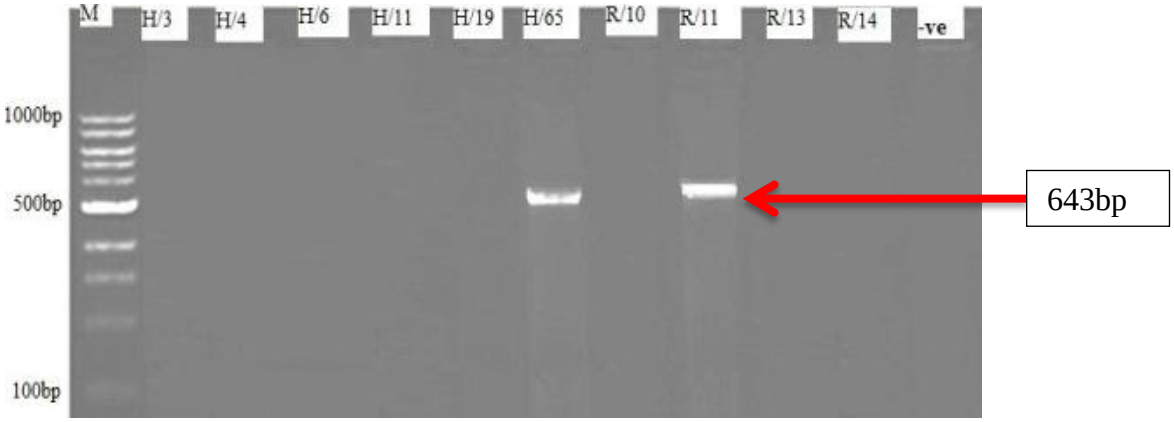

Plate 4.3: Micrograph showing presence of *TetB* gene (634bp) in *Escherichia coli* isolated from human and black rhinoceros

Note: M= DNA ladder, H/3, H/4, H/6, H/11 and H/65 represent human samples and R/10, R/11, R/13 and R/14 represent black rhinoceros samples; **-Ve**= negative control. One sample from humans and black rhinoceros showed presence of ***TetB* gene** respectively.

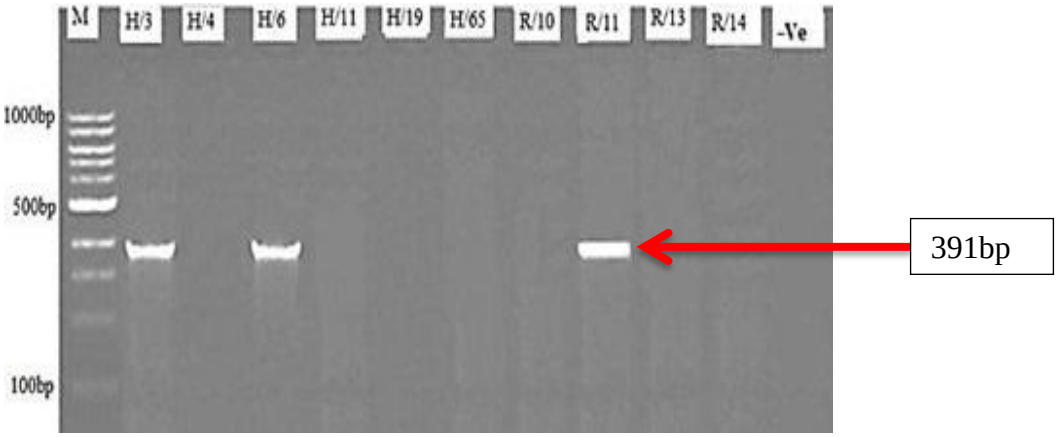

Plate 4.4: Micrograph showing presence of *dfrA1* gene (391bp) in *Escherichia coli* isolated from human and black rhinoceros

Note: M= DNA ladder, H/3, H/4, H/6, H/11 and H/65 represent human samples and R/10, R/11, R/13 and R/14 represent black rhinoceros samples; **-Ve**= negative control. Two samples from humans and one sample from black rhinoceros showed presence of ***dfrA1* gene** respectively.

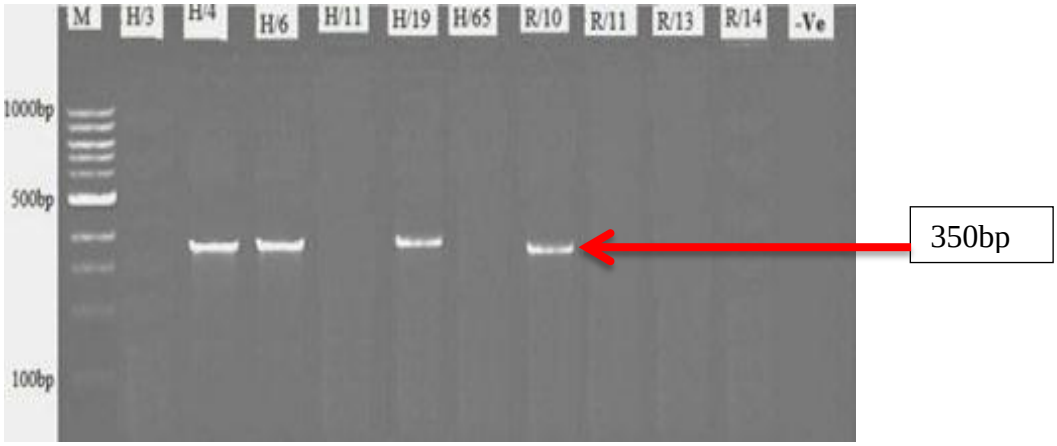

Plate 4.5: Micrograph showing presence of *sul1* gene (350bp) in *Escherichia coli* isolated from human and black rhinoceros

Note: M= DNA ladder, H/3, H/4, H/6, H/11 and H/65 represent human samples and R/10, R/11, R/13 and R/14 represent black rhinoceros samples; **-Ve**= negative control. Three samples from humans and one sample from black rhinoceros showed presence of ***sul1*gene** respectively.

CHAPTER FIVE: DISCUSSION

5.1. Antibacterial Resistance in *E. coli* Isolates from Black Rhinoceros and human

The results of this study showed that there is homogeneity in antimicrobial resistance in *E. coli* strains isolated from human and those from black rhinoceros in Lambwe Valley, as 50% of the *E. coli* isolates from both humans and black rhinoceros showed no difference in antimicrobial resistance. In other words, black rhinoceros in Ruma National Park harbor *E. coli* strains having antimicrobial resistance patterns similar to those from *E. coli* isolates from human population living around the park. This is a cause of alarm because it is a pointer that bacterial isolates from human and those from the black rhinoceros in Lambwe Valley are subjected to the same antimicrobial selective pressure against ampicillin, tetracycline, chloramphenicol and erythromycin. The current finding contradicts previous studies which demonstrated that *E. coli* isolates from human showered high levels of antimicrobial resistance than in wild animals isolate (Pesapane *et al.,* 2013; Goldberg *et al.,* 2007). For example: in Botswana isolates from human and banded mongoose showered resistance to ampicillin (79% and 33%) and tetracycline (35% and 10%) repsectively (Pesapane *et al.,* 2013), while in Uganda isolates from human and chimpanzee showered resistance to ampicillin (55% and 5%), tetracycline (49% and 6%) and trimethoprim (72% and 4%) respectively (Goldberg *et al.,* 2007).

The cause of the homogeneity in antimicrobial resistance between *E. coli* isolates from human and black rhinoceros to ampicillin, tetracycline, chloramphenicol and erythromycin can be hypothesized to be two factors related to contamination of the environment by anthropogenic factors. First, the park is found on the floor of a valley surrounded by hills inhabited by human population. Fecal matter from human and livestock may contaminate the environment which will then be washed downstream by runoff water into the only river

(Lambwe River). The river flows from human populated habitat, meanders through the park then enters human settlement as it flows to Lake Victoria. Since black rhinoceros in Ruma National Park are not subjected to antimicrobial therapy, the resistance microbes could be present in human and livestock populations because of continuous use and misuse of antimicrobial agents (Singer *et al.,* 2003). Wildlife could then be exposed through human and livestock fecal waste contamination of the environment or due to ecotourism activities in the region. It is known that anthropogenic perturbations influence interspecies transmission among microbes (Goldberg *et al.,* 2008). Secondly, the peri-wild baboons and vervet monkeys that forage on human backyards may also act as conduits that transfer antimicrobial resistance microbes from human habitats into the wild. Previous studies have demonstrated that organisms in direct contact with humans may act as shuttles of antimicrobial resistance genes from human to the wild (Goldberg *et al.,* 2007; Eze *et al.,* 2015; Pesapane *et al.,* 2013). Thus, this study demonstrates that antimicrobial resistance is no longer an issue confined to humans and livestock, but instead it is a wider environmental issue of public health concern.

An interesting result from the current study was the unexpected high levels of gentamicin resistance in *E. coli* isolates from black rhinoceros. The observed gentamicin resistance might be due to a high selective pressure in the wild since gentamycin is an antibiotic produced naturally by *Micromonospora purpureochromogenes* which is widely present in the environment (water and soil) as an important saprotrophic bacterium (Kumar *et al.,* 2008). The ubiquitous distribution of the bacterium *Micromonospora purpureochromogenes* might explain the high selective pressure exhibited by *E. coli* isolates from black rhinoceros in the wild. Therefore due to the presence of these antibiotics in the environment, the commensal *E. coli* will acquire antimicrobial resistance through interspecies cross-transfer of resistance

properties (Davies *et al.*, 2010) and thus be able to survive and compete for resources in such an environment.

From the study it was observed that there was a high level of multi-drug resistance among the *E. coli* strains from human and black rhinoceros. This is surprising since the acquisition of resistance genes by microbes makes them incur serious energy cost hence unstable (Andersson, 2006). Therefore in absence of selective pressure, for instance overuse of antimicrobial agents, these multi-drug resistance strains observed both in human and black rhinoceros populations should be short-lived. Intrinsically, the impact of high levels of multidrug resistance by commensal *E. coli* strains in the wildlife on human health rests in the possibility that the resistant strains of commensal or pathogenic bacteria will naturally be transmitted between intraspecific or interspecific bacterial species of human importance.

5.2. Antibacterial Resistance Genes in *E. coli* isolates from Human and Black Rhinoceros

In this study, it was observed that *E. coli* isolates from humans and black rhinoceros harbors ***bla$_{TEM}$***, ***tetA***, ***tetB***, ***dfrA1*** and ***sul1*** genes that are known to confer resistance to ampicillin (Briñas *et al.*, 2002), tetracycline (Bryan *et al.*, 2004) and cotrimoxazole (Coelho *et al.*, 2017) respectively. Thus, the observation points to the possibility that the antimicrobial resistant *E. coli* from both humans and black rhinoceros harboring similar antimicrobial resistance gene profiles are being exposed to a common antimicrobial resistant *E. coli* strain or are experiencing high rate of bacterial cross-transfer. As a word of caution, it should be understood that since a small sample size for molecular characterization of resistance genes were purposively selected, the presence of antimicrobial resistance genes in *E. coli* isolates from human and black rhinoceros should not be taken to correspond to the burden of antimicrobial resistance genes in the two population but as a pointer that the phenotypic antimicrobial resistance observed in both humans and black rhinoceros has a genetic basis.

Since development of antimicrobial resistance genes in humans correlates with the overuse and misuse of antimicrobial agents in hospitals and in the community (Zhang *et al.,* 2006; Ndihokubwayo *et al.,* 2013; Tadesse, 2014), the homogeneity in antimicrobial resistance genes in *E. coli* isolates from black rhinoceros and human can be due to contamination of the environment (Ndihokubwayo *et al.,* 2013); sharing of common contaminated watering point (Martinez, 2009); wildlife handling in intensive management (Emslie, 2012) and presence of peri-wild animals foraging in human backyards which picks antimicrobial resistance genes and shuttles them to other wild animals not in direct contact with human being (Goldberg *et al.,* 2007; Eze *et al.,* 2015; Pesapane *et al.,* 2013).

It is known that expression of antimicrobial resistance genes by microbes makes them incur serious energy cost thus, they will be unstable and not survive for long in an environment free of selective pressure (Andersson, 2006). An alternative explanation to the high levels of antimicrobial resistance observed in the wild type *E. coli* strains from the study region may be due to the harsh environmental conditions experienced in Lambwe Valley. These environmental condition which include: high level of salinity in water, strong alkaline soils and the high environmental temperature (Allsopp, 1972) can subjects bacteria to myriad of stressful factors; apparently there is no current data on physico-chemical parameters in Lambwe Valley other than the work by Allsopp (1972) . Such stressors will make the bacteria to elicit highly controlled adaptive response that manifest cellular changes. For example, high temperature, high osmolarity, alkaline pH and high salt, will trigger expression of a highly regulated sigma factor (*RpoE (sE), EnvZ/ OmpR* and *CpxRA*) in *E. coli* bacterium, this will not only make them overcome the prevailing stressful condition, but also leads to up-regulation of resistance genes such as: β-lactammase, aminoglycosides and multi-drug efflux genes (Hirakawa *et al.,* 2003; Nishino *et al.,* 2010). In addition, activation of *ompC* pathway will lead to suppression of *ompF*, yet *ompF* is a major portal for entry of

β-lactam antimicrobial agents (ampicillin, penicillin, cefalothin, cefuroxime), while *ompC* forms a smaller channels hence leading to inability of β-lactams to penetrate the bacterial membrane (Hirakawa *et al.,* 2003; Nishino *et al.,* 2010). The cellular changes manifested will lead to reduced growth rate and metabolic activities (Poole, 2012) and since antimicrobial agents kill rapidly replicating bacteria (Eng *et al.,*1991), these isolates will be able to survive in this harsh environment.

The detection of $\boldsymbol{bla_{TEM}}$, ***tetA, tetB***, ***dfrA1***and ***sul1*** genes in the current study is in line with previous work. For instance, $\boldsymbol{bla_{TEM}}$, ***tetA, tetB***, ***dfrA1***and ***sul1*** genes have been isolated from wild rabbits in Northern Portugal (Silva *et al.,* 2010) and in black-headed sea gulls in Czech Republic (Dolejska *et al.,* 2007). In addition, $\boldsymbol{bla_{TEM}}$ gene was isolated from ampicillin resistant *E. coli* in healthy humans, animals (Briñas *et al.,* 2002) and in Iberian Lynx in Spain (Gonçalves *et al.,* 2013). Also, $\boldsymbol{bla_{TEM}}$ gene has also been isolated from clinical *E. coli* isolates resistant to ampicillin in; Trans- Nzoia County Hospital, Kenya (Kipkorir *et al.,* 2016), Polish hospital (Ojdana *et al.,* 2014) and in a South African hospital (Ehlers *et al.,* 2009). Mureithi *et al.,* (2015) demonstrated 56% $\boldsymbol{bla_{TEM}}$ genes in *E. coli* isolates from baboons in Kenya not subjected to antimicrobial therapy.

The observed activities against amoxicillin/clavulanic acid in the current study is worrying as it suggests production of inhibitor-resistant TEM (IRT) enzymes that plays an important part in rapid hydrolysis of clavulanic acid thus mediating amoxicillin-clavulanic acid resistance (Canton *et al.,* 2008). Therefore, due to the presence of TEM (IRT) in the *E. coli* strains in the environment and the swiftness of interspecies cross-transfer of antimicrobial resistance genes (Davies *et al.,* 2010), the few available B-lactam/clavulanic acid antimicrobial agents will be rendered ineffective within a short time hence complicating treatment of bacterial diseases.

It is crucial to note that, unlike in humans, $\boldsymbol{bla_{TEM}}$ gene was not detected in all the isolates from black rhinoceros that showed resistance to ampicillin. Absence of $\boldsymbol{bla_{TEM}}$ gene might be an indication that other β-lactam genes or TEM mutant genes (Briñas *et al.,* 2002) are responsible for the observed resistance to ampicillin in isolates from black rhinoceros in Lambwe Valley. Moreover, it might be a pointer that resistance mechanisms other than β-lamactamase encoding genes, for instance activation of *ompC* pathway which is triggered in case of high temperature, high osmolarity, alkaline pH and high salt, (Hirakawa *et al.,* 2003; Nishino *et al.,* 2010) are involved in conferring the observed resistance.

Isolation of ***tetA*** and ***tetB*** genes from tetracycline resistant in the current study is in line with other findings globally. For instance, ***tetA*** and ***tetB*** genes have also been demonstrated in *E. coli* isolates from wild birds (black head gulls) in Czech Republic (Dolejska *et al.*,2007) and in wild rabbits in Portugal (Silva *et al.* 2010) not subjected to antimicrobial agents. Thus, the findings of the current study supported by previous studies indicate that *E. coli* strains from wild animals harbor ***tetA*** and ***tetB*** genes that have the potential to be spread to human beings.

Since ***tetA*** and ***tetB*** genes were not detected in all isolates in the current study, it is an indication that other ***Tet*** genes that either code for active efflux pump or ribosomal protection (Tuckman *et al.,* 2007) may be responsible for the observed tetracycline resistance in the study region.

In the present study, ***sul***-genes, a determinant for sulphanomide resistance and ***dfrA***-genes, a determinant for trimethoprim resistance were detected. Although not much has been done on resistance genes associated with cotrimoxazole resistance in the wildlife, these results are in line with the work of Silva *et al.,* (2010) who found ***sul***-genes being expressed by *E. coli* isolates from wild angulates and wild birds in Portugal and the work of Dolejska *et al.,*

(2007) who reported presence of both ***dfrA1*** and ***sul1*** from *E. coli* isolates from black-headed gulls in Czech Republic. In addition, ***sul1*** gene (Arabi *et al.,* 2015) and ***dfrA1*** gene (Heidary *et al.,* 2014) were reported in *E. coli* isolates from clinical samples in Iran, and in African Republic (Frank *et al.,* 2007).

CHAPTER SIX: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary

This study contend itself with the observation that *Escherichia coli* isolated from human and black rhinoceros populations within Lambwe Valley showed homogeneity in antimicrobial resistance profiles to ampicillin, tetracycline, chloramphenicol and erythromycin. Isolates from black rhinoceros were more resistant to gentamycin compared to isolates from human being, while those from humans where more resistant to cotrimoxazole, ceftriaxone and amoxicillin/clavulanic acid. In addition, isolates from both human and black rhinoceros showed high levels of multi-drug resistance to ampicillin, gentamicin, tetracycline, ceftriaxone, amoxicillin/ clavulanic acid and Erythromycin.

Consistent with the study's observation is the fact that isolates from humans and black rhinoceros in Lambwe Valley harbored similar genes (***bla$_{TEM}$; tetA*** and ***tetB; dfrA1*** and ***sul1*** genes) that confer antimicrobial resistance to ampicillin, tetracycline and cotrimoxazole respectively. The observed patterns suggests that *E. coli* isolates from human and black rhinoceros may be experiencing high rate of *E. coli* cross-transfer with *E. coli* acting as a vehicle that shuttles antimicrobial resistance genes at human-wildlife interface. Thus, wildlife may act as environmental reservoirs to antimicrobial resistance genes.

6.2. Conclusions

Two conclusions emerged from this study and are as follows;

1. The high levels in antimicrobial resistance profiles of *E. coli* isolates from humans and black rhinoceros in Lambwe Valley to ampicillin, tetracycline, cotrimoxazole, amoxicillin/ clavulanic acid and erythromycin, suggests that there is reduced therapeutic options; such that for management of bacterial infections in human,

gentamicin will be the drug of choice while in case of a bacterial infection outbreak among the critically endangered black rhinoceros, ceftriaxone and chloramphenicol will be the most appropriate.

2. The homogeneity in antimicrobial resistance genes between *E. coli* isolated from human and black rhinoceros populations within Lambwe Valley that confer antimicrobial resistance to ampicillin, tetracycline and cotrimoxazole (***bla$_{TEM}$; tetA*** and ***tetB; dfrA1*** and ***sul1*** genes respectively), suggests that human and black rhinoceros are exposed to a common *E. coli* strain or are experiencing cross-transfer of antimicrobial resistant microbes.

6.3. Recommendations

1. With the high levels in antimicrobial resistance profiles of *E. coli* isolates from humans and black rhinoceros in Lambwe Valley leading to reduced therapeutic options for management of bacterial infections, there is need for evidence based antimicrobial therapy both in human health and in animal health to determine the best regiment of antimicrobial agent that will be effective.
2. With the current evidence of homogeneity in antimicrobial resistance profile between *E. coli* isolates from human and black rhinoceros, there is need for coordinated action plan on surveillance and monitoring of antimicrobial resistance, research and sharing of knowledge between public health, livestock sector and also the wildlife sector.

6.4. Suggestions for Future Studies

1. Due to homogeneity of antimicrobial resistance profiles between human and black rhinoceros, there is need to establish the pathways of interspecies spillover and cross-transfer of antimicrobial resistant genes at the human-wildlife interface.

2. There is need to establish factors that trigger the high level of gene plasticity observed in the wild type *E. coli* isolates from human and black rhinoceros population in Lambwe Valley.
3. To establish if whether human and black rhinoceros are exposed to similar *E. coli* isolates, there is need to do gene sequencing and to establish the phylogenetic relationship of *E. coli* isolates from human, environment and wild animals in Lambwe Valley. This will adequately explain mixing of genetic makeup among *E. coli* strain in different hosts and the environment.

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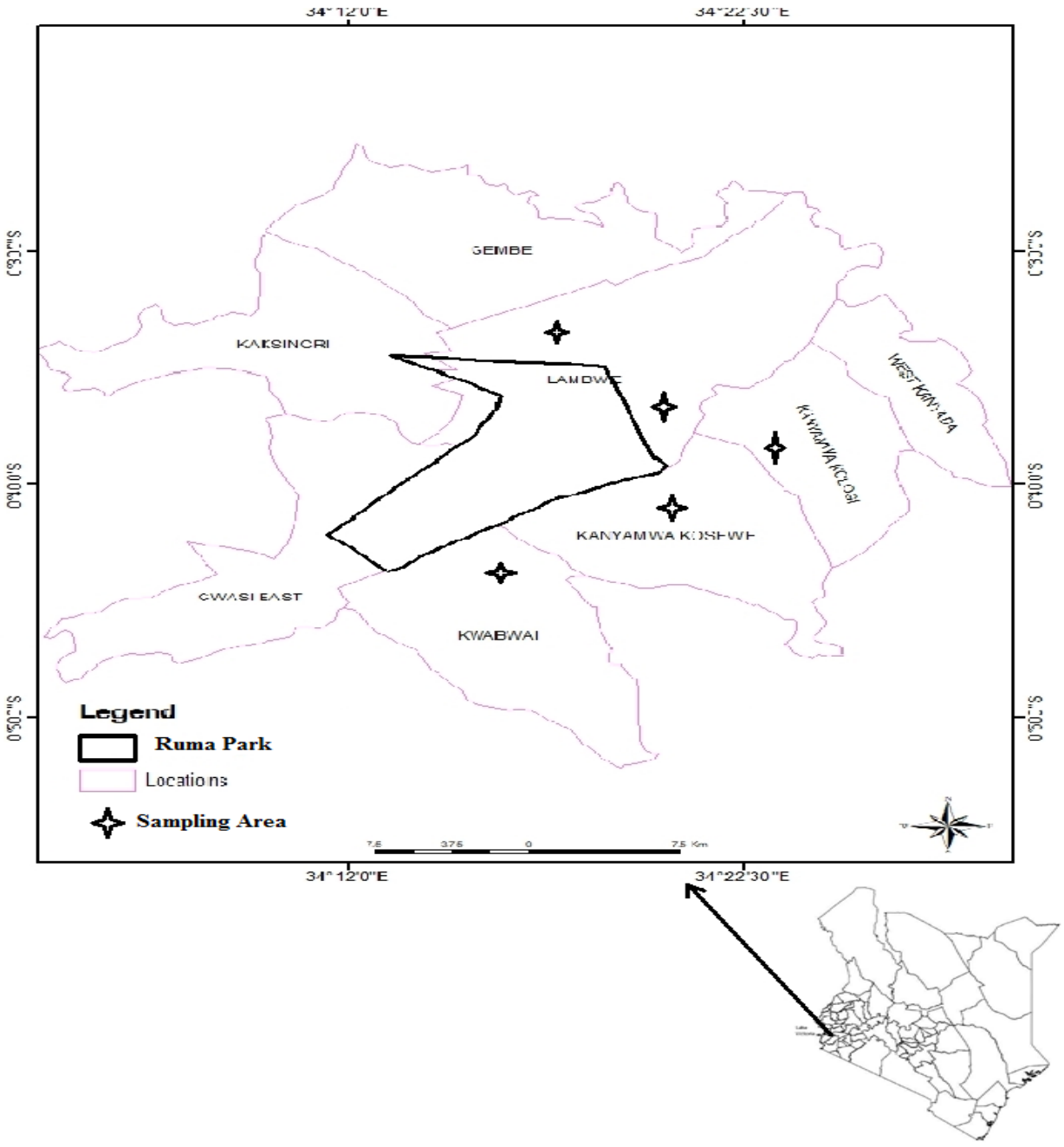
APPENDICES

APPENDIX I: Map of the Study Site



Map showing location of Ruma National Park in Lambwe Valley. Surrounding the park boundaries is a demarcation enclosing human settlement.

APPENDIX II: Consent Form
CONSENT FOR INVESTIGATIONAL STUDIES

MASENO UNIVERSITY

SCHOOL OF PHYSICAL AND BIOLOGICAL SCIENCES

Project Title: Difference in Antimicrobial Resistance Profiles and Genes between *Escherichia coli* Isolates from Humans and Black Rhinoceros in Lambwe Valley, Kenya

Investigator: Collins KipkorirKebenei

SUPERVISORS: Dr. Patrick O. Onyango; Dr. Paul O. Ang'ienda

This is a cross-sectional research study. Research studies include only people who choose to participate in it. Please take your time to decide if you want to or want your child to join this study. Discuss it with your doctor and other people providing your medical care about the study.

Mr. Collins Kipkorir Kebenei from Maseno University is inviting you to enroll in a research study to answer specific questions about persistence in antimicrobial resistance menace as one of the major problem that affect the potency of antimicrobials in the world. The information from this study is vital in formulating new strategies to preserve utility of available antimicrobials; in epidemiological study of resistance gene in the environment and shed light on the spectrum of resistance gene reservoirs and factors facilitating their dissemination and persistence in the environment.

You and/ or your child are being asked to take part in this study because you or your child may have been exposed to antimicrobial resistant bacteria that may cause increase in cases of disease relapse that requires prolonged treatment periods thus increasing the cost of treatment and loss in man hours.

WHY IS THE STUDY BEING DONE?

The purpose of this study is to investigate antimicrobial resistance pattern, characterize the antimicrobial resistance gene diversity and the epidemiological study of resistance gene in the environment within Lambwe Valley. This research is done to better understand how to safe guard the utility of available antibiotics.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

In the overall study, about 184 participants across all ages, who live around Ruma National Park will take part in the study upon their informed consent. This study will take place in Lambwe Valley only.

WHAT IS INVOLVED IN THE STUDY?

The study will be a community based where we will ask you or your child to give a fecal sample for subsequent laboratory diagnosis and determination of drug susceptibility.

HOW LONG WILL I OR MY CHILD BE IN THE STUDY?

You will be required to provide the study with required sample only once.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

There may be no direct benefit for being in this study. You will have access to additional modern diagnostic procedures like PCR diagnostic techniques to identify the level of drug resistance, thus your doctor will be furnished with information on the range of effective antimicrobials to be used in your treatment.

WHAT OTHER OPTIONS ARE THERE?

It is not mandatory for you or your child to be in this study. Taking part is strictly voluntary.

WHAT ABOUT CONFIDENTIALITY?

We will keep the information we collect about you or your child as confidential as stipulated by ethical requirement. The samples will be coded by use of numbers. Only the principle investigator and the study data managers will have access to this code. Any test results we obtained will be shared with the health care providers at the hospital.

WHAT ARE THE COSTS?

There are no costs to you or your child to participate in this study. You or your child will not receive any payment for taking part in this study.

STORAGE AND USE OF SAMPLES FOR FUTURE STUDIES

Samples taken for this study may be stored in a freezer at Post Graduates Molecular laboratory, Maseno University and may be used for future scientific studies related to gastrointestinal infection. However, these samples will only be used with approval from the principal investigator and you will not be conducted for additional consent. You or your child may still participate in this study if you do not consent to us using your sample or your child's samples for future scientific studies about gastrointestinal infections in your community. If you check "**No**" then your sample or your child sample will be stripped of their identification number in the database after completion of this study and such samples will be destroyed.

Consent for participating in the study (Please check the box below)

Yes ☐

☐ agree to allow my samples or my child samples to be used for future studies.

Assessment of Informed Consent		
	Yes	No
Do you understand the consent form?		
Do you have any question?		
Question(s):		
Do you or your child have to participate in this study		
Will we take your or your child's fecal sample during this study		

SIGNATURE

Signing below indicates that you have been informed about the research study in which you voluntarily agree to be part or let your child be part of the study; that you have asked questions about the study that you may have; and that the information given to you has permitted you to make a fully informed and free decision about your participation or your child's participation in the study. By signing this consent form, you do not waive any legal rights.

Name of the participant: Signature of the participant (if adult):…….Date:…../…/……
Name of Parent/ Legal Guardian: Signature: ……..Date:…/…./…...
If Legal Guardian, indicate relationship:
Name of person obtaining informed consent: Signature: ……..Date:…./…./…...
Name of Principal/ Investigator: Signature: ……Date: …/…./….
Name of Supervisor: **a.**Supervisor1:……Signature:…….Date:…./…./…. **b.**Supervisor 2:……Signature: …….Date:…./…./….

APPENDIX III: Qiagen DNeasy DNA Extraction Protocol for Bacterial Cultures

Adapted from QIAgen DNeasy handbook.

Procedure:

1. Appropriately label a 1.5 ml tube for each sample.
2. Add 1.75 ml of bacterial culture to a labeled 2 ml tube.
3. Spin tubes at 20,000 x g for 5 minutes in centrifuge. Decant liquid.
4. Add 180 ul of enzymatic lysis buffer to you tube and vortex 10-20 s.
5. Incubate at 37° C for 30 min.
6. Add 25 ul of proteinase K to the tube
7. Add 200 ul of Buffer AL to the tube.
8. Vortex the tube briefly.
9. Incubate at 56° C for 30 min. *Now is a good time to label all the tubes you need for the rest of the protocol.*
10. Add 200 ul of 100% ethanol to the tube
11. Vortex briefly.
12. Using a micropipette, transfer entire contents (~600 ul) of tube to labeled spin column.
13. Centrifuge column at 10,000 x g for 1 min.
14. Remove column from collection tube. Place column in new collection tube.
15. Add 500 ul of buffer AW1 to the column and centrifuge at 10,000 x g for 1 minute.
16. Remove column from collection tube. Place column in new collection tube.
17. Add 500 ul of buffer AW2 to the column and centrifuge at 20,000 x g for 3 minute.
18. Carefully remove tubes from centrifuge, do not let flow-through contact column. If this happens, spin tube again for 1 min at 20,000 x g.
19. Transfer the column to a 1.5 ml tube and add 200 ul of buffer AE to the column. Let column stand at room temperature for 1 minute.
20. Centrifuge at 10,000 x g for 1 minute. Discard the column and store the DNA appropriately (4° C for short term, -20° C for long term).

Materials required

Qiagen DNeasy Blood and Tissue kit

200 and 1000 ul pipette tips

1.5 ml microcentrifuge tubes

2.0 ml microcentrifuge tubes

Overnight bacterial cultures

Equipment required

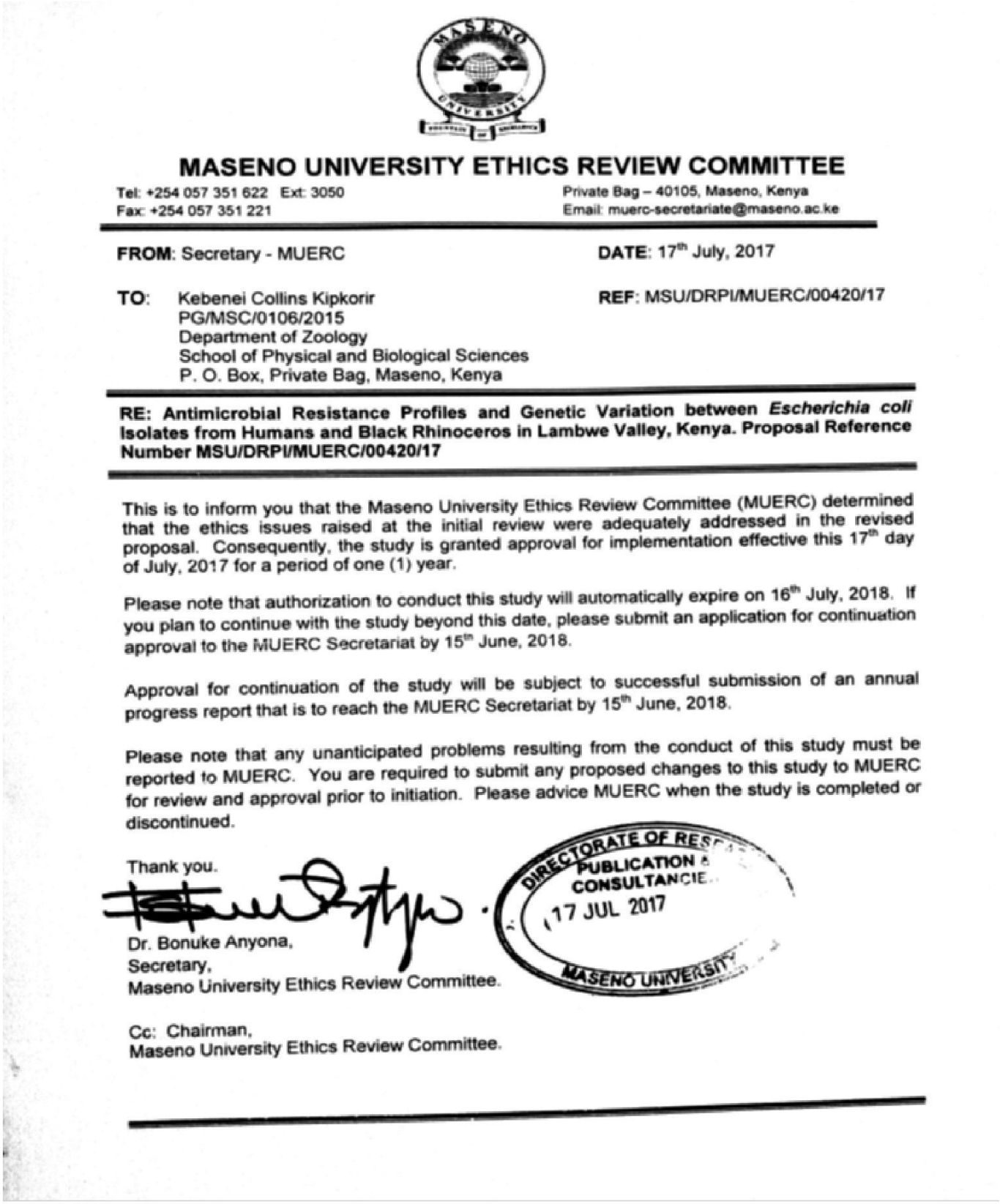
Bench top centrifuge capable of 20,000 x g

200 ul micropipette

1000 ul micropipette

vortexer

APPENDIX IV: Ethical Clearance Certificate



MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221

Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 17th July, 2017

TO: Kebenei Collins Kipkorir
PG/MSC/0106/2015
Department of Zoology
School of Physical and Biological Sciences
P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/00420/17

RE: Antimicrobial Resistance Profiles and Genetic Variation between *Escherichia coli* Isolates from Humans and Black Rhinoceros in Lambwe Valley, Kenya. Proposal Reference Number MSU/DRPI/MUERC/00420/17

This is to inform you that the Maseno University Ethics Review Committee (MUERC) determined that the ethics issues raised at the initial review were adequately addressed in the revised proposal. Consequently, the study is granted approval for implementation effective this 17th day of July, 2017 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 16th July, 2018. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 15th June, 2018.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 15th June, 2018.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

Dr. Bonuke Anyona,
Secretary,
Maseno University Ethics Review Committee.

DIRECTORATE OF RES... PUBLICATION & CONSULTANCIE... 17 JUL 2017 MASENO UNIVERSITY

Cc: Chairman,
Maseno University Ethics Review Committee.

APPENDIX V: Statistical Analysis Formulas

1. Mann-Whitney formula:

$$U = (n_1 - n_2) + ((n_1 (n_1 + 1) / 2) - R_1);$$

Where U is a measure of the difference between the ranked observations of the two samples, n_1 and n_2 are sample sizes and R_1 is the sum of ranks assigned to the values of the first sample.

2. Z-test for difference in proportion of two samples formula:

$$Z = (p_1 - p_2) / (\sqrt{(p_1 q_1 / n_1) + (p_2 q_2 / n_2)});$$

Where: p_1 and p_2 = sample proportion, $q = 1 - p$, n_1 = number of items in sample one, n_2 = number of items in sample two.