# PREVALENCE OF ANTIMICROBIAL RESISTANCE AND CHARACTERIZATION OF FECAL INDICATOR BACTERIA AND *Staphylococcus aureus* FROM FARM ANIMALS, WILDLIFE, PETS AND HUMANS IN TANZANIA

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## EXTENDED ABSTRACT

This thesis is prepared according to "Publishable manuscript" format of the Sokoine University of Agriculture. It is divided into four sections; the first section presents introduction, problem statement, objectives, rationale of the study and hypothesis while the second section is a review of literature in relation to the work performed. The third section contains four manuscripts which cover the research work done, whereas the last section details overall conclusions of the study. The first manuscript is about the findings on the awareness of risks associated with antimicrobials use and practices that contribute to selection for antimicrobial resistance in livestock. The second and the third Manuscripts present the prevalence of antimicrobial resistance in indicator bacteria namely *S. aureus* from nostrils, *E. coli* and *Enterococci spp* from faecal samples obtained from livestock, dogs and contact human beings, and the last manuscript presents findings on antimicrobial resistance in faecal indicator organisms from buffalo, wildebeest, zebra and cattle grazing with wild ungulates. The studies involved testing resistance using antibiotic discs, inclusion of antibiotics in the media and evaluating bacterial DNA for resistance genes against commonly used antibiotics in livestock. In response to the first objective presented in manuscript 1, awareness of human health risks due to use of antibiotics among livestock keepers and factors that contribute to selection of antibiotic resistance bacteria within livestock in Tanzania were determined. Looking at awareness on health risks among the livestock keepers surveyed, one hundred and sixty randomly selected Tanzanian small-scale livestock keepers were involved in the questionnaire-based survey. The results showed that 30% of the respondents were not aware of the concept of antibiotic resistance whereas 52% were not aware of the types of diseases that can be

treated with antibiotics, 22% did not know the essence of withdrawal period after treatment of animals with antibiotics and 40% did not know if antibiotics used in livestock pose risk to human health. Side effects of antibiotics to human beings known by the respondents include drug resistance, allergy, poisoning, skin rashes and cancer. Further, it was found that the most commonly used antibiotics were oxytetracyline (TE) (62.9 %) followed by sulphadimidine/ trimethoprim / (STX) (23.2 %), penicillin-streptomycin (13.4 %) and gentamycin (CN) (0.5 %).Only 54% of the respondents obtained their antibiotics through prescription by veterinarians. Potential risk factors were identified to be the sources of development of antimicrobials resistance. These included livestock management systems, antibiotics handling and types of antibiotics used in the study areas. Points of selling antibiotics and their prescriptions were found to be handled by agriculture and veterinary input shops, veterinarians, livestock markets and exhibition areas. The study concludes that there is a gross lack of awareness on health threats associated with antibiotics resistance among the farming communities thus posing serious public health threats in Tanzania.

On the other hand, prevalence of nasal carriers, population structure and antimicrobial resistance of *Staphylococcus aureus* and *S. pseudintermedius* in healthy humans, pigs and dogs in Tanzania were investigated in the same study area as the second objective and manuscript 2 of the study**.** Nasal swabs were taken from healthy humans, pigs and dogs (*n*=100 per species) in Tanzania and examined for growth of *S. aureus* on the Oxacillin (OX) Resistance Screening Agar Base (ORSAB) media. Presumptive *S. aureus* isolates were confirmed by *nuc* PCR tested for the presence of *mec*A, *mecC* and were *spa*-typed while antimicrobial susceptibility patterns were determined by disc diffusion, *S. aureus* was isolated from 22% of humans, 11% of dogs nasal bacteria isolates and 4% of pigs. *S. pseudintermedius* was isolated from 4% of dogs. A total of 21 *spa* types were identified: 13 from human isolates, 7 between dogs and one among pig isolates. Three *spa* types

(t314, t223 and t084) were isolated from both humans and dogs. Disc diffusion test revealed resistance to sulphamethoxazole/trimethoprim (STX) (3/37; 8.1%), rifampicin (RD) (2/37; 5.4%), amoxycillin-clavulanic (AMC) acid (1/37; 2.8%), oxacillin (OX) (4/37; 10.8%), chloramphenicol ( C) (3/37; 8.1%), gentamicin (CN) (3/37; 8.1%) and penicillin (P) (37/37; 100 %)**.** All the four isolates resistant to oxacillin were negative by PCR for *mecA* but positive for *mecC. mecC* encoded Methicillin Resistant *Staphylococcus haemolyticus* (MRSH) was detected in pigs, dogs and humans. The study presented the first report on strains with this gene in Tanzania. Moderate levels of antimicrobial resistance were observed irrespective of the host species from which the strains were isolated.

In addition, the Prevalence of antimicrobial resistance in indicator bacteria isolated from faecal samples of livestock, poultry and humans in Tanzania was studied as the third objective and presented in manuscript 3. In order to quantify the public health risks posed by handling animals, their waste and animal products, a total of 587 faecal samples were collected from humans, pigs, beef, dairy cattle and exotic and indigenous chickens to investigate the relatedness of antibiotic resistant bacteria from humans and animals in Tanzania. A sum of 485 *E. coli* isolates were obtained from faecal samples grown on MacConkey agar plates. Also, tetracycline (TE), s sulphadimidine/ trimethoprim (STX), ampicillin (AMP) and cefataxime (CTX) were included in the media to test the resistance of *E. coli* isolates, the results showed higher resistance to TE (385, 79.4%), STX (374, 77.1 %) and AMP (362, 74.6%) and less resistance was observed in CTX (194, 40%). Further, antibiotic discs were used to determine the resistance of 350 *E. coli* isolates, whereas higher resistance was also observed to TE (241, 68.8%), STX (214, 61.1%), S (195, 55.7%) and AMP (192, 54.8%). Moreover, resistance to CTX was observed in 143 isolates (40.9%) and ciproflaxin (CIP) 30.9 % (108) while less resistance was observed in AMC (48, 13.7%) and CN (37, 10.6%). Besides *E. coli*, a total of 550 *Enterococci spp*

isolates were also speciated and tested for resistance. Out of the 164 enterococci isolates tested, 151 (92.1%) were *E. faecium*, 5 (3.1%) *E. faecalis* and 8 (4.9%) *E. hirae*. Growth of resistant indicator bacteria colonies was carried out in AMP and VA in which higher resistance was observed in AMP (85, 15.5%) as compared to vancomycin (VA) (30, 5.5%). For 240 *Enterococci spp* tested with antibiotic discs, higher resistance was observed to rifampicin (RD) (174, 75.2%), TE (33, 13.8%) and e rythromycin (E) (36, 15%) whereas; there was no resistance to CN (120µg) and C (5, 2.1 %). Other antibiotics tested for this group included CIP (9, 3.8%), AMP (12, 5%) and VA (12, 5%). Also, sulII and *tetW* resistance genes were found in all livestock and humans samples (*n*=22 per species) with degree of variations in when calculating the proportion of resistance genes in relation to number of bacteria estimated from "total" 16s RNA. For all the sample percentages of *tetW* to the total of 16s genes were 0.06 % Humans, 11.88% Cattle, 2.4% Pigs, 2.15% local chickens and 0.98% exotic chickens; whereas, *sulII* represented; 0.22% Humans, 0.34 % Cattle, 0.2%Pigs, 0.3% local chickens and 0.02% exotic chickens. All isolates (n=194) which showed resistance to CTX on MacConkey agar (59 of which were non-lactose fermenters) revealed a band of the same size to that of a positive CTX-M control strain in all isolates analyzed, indicating that they all contained an ESBL gene of this class. Antibiotics that are commonly used in Tanzania had higher resistance as estimated from both colony forming unit (CFU) of resistant bacteria and resistance profiles determined by the use of antibiotic discs. Resistance genes were also demonstrated to commonly used antibiotics (TE, STX) in the country. The findings of this study indicate that prolonged use of TE and STX has caused indicator bacteria to develop resistance against these antimicrobials and the resistance genes observed for the same antibiotics suggest that the genes circulate between animals and humans sharing the same environment.

Furthermore, the forth objective presented in manuscript 4 was to determine the prevalence of antimicrobial resistance in faecal indicator organisms from buffalo, wildebeest, zebra and cattle grazing with wild ungulates in the study area. The study investigated the antibiotic resistance in *E. coli* and *Enterococci spp* isolated from faecal samples of wild ungulates and cattle grazing with them. Faecal samples were collected from 35 buffalos, 40 wildebeest and 40 zebra from two study sites namely Mikumi National Park (MNP) and Ngorongoro Conservation Area (NCA).Also, additional 20 faecal samples were obtained from cattle grazing together with the wild ungulates in NCA in order to determine whether co-grazing with cattle constitutes a risk factor for carriage of antibiotic resistant bacteria. AMP, STX, TE and CTX resistant *E. coli* were observed in 52.6%, 39.2 %, 31.9% and 17.8% of wildlife samples respectively. The mean of $\log_{10}$CFU antibiotic resistant bacteria did not differ significantly between the three wildlife species. $\text{Log}_{10}$ CFU of ampicillin resistant *E. coli* was significantly higher among wildlife grazing together with cattle, whereas, cefotaxime resistance was more common in samples from MNP. In antibiotic discs test of antimicrobials in the samples, one hundred and twenty *E. coli* colonies were purified and antibiotic resistance profiled. Wildlife isolates from MNP (without cattle) were more frequently resistant to TE, CN and ENR than isolates from NCA (with cattle). Notably, *E. coli* resistant to CTX and ENR were observed among the randomly obtained isolates from all wildlife but not from cattle. Out of 120 enterococci isolates speciated, 95 (79.2%) were *E. faecium*, 9 (7.5%) *E. faecalis*, 7 (5.85) *E. gallinarum* and 9 (7.5%) *E. hirae.* On the other hand, AMP and VA resistant *Enterococci* were detected in 7.41% of wildlife samples but not in cattle and one hundred and twenty eight purified colonies of *Enterococci spp* were antibiotic resistance tested. Isolates for wild animal grazing together with cattle showed higher levels of resistance to STX than cattle without. While,the average ratio of *sulII* gene-copies to 16s rRNA copies in faecal samples from wildlife did not differ between the two sampling sites, the ratio of *tetW* to

16s rRNA was significantly higher in samples from MPN than from NCA. In conclusion, grazing together with cattle was not found to be a risk factor for the presence of antibiotic resistant bacteria in faecal samples of wild ungulates.

Overall findings document for the first time in Tanzania that livestock keepers are unaware of antimicrobial resistance, uncontrolled antibiotic dispensing for animals presents high risk for spread of antimicrobial resistance among domestic animals with potential to contribute antimicrobial resistance to human beings and moderate levels of antimicrobial resistance observed in *S. aureus* isolates irrespective of the host species whose strains were isolated. Also different *spa* types of *S. aureus* circulating in Tanzania and for the first time detection of *mecC* encoded MRSH from *S. haemolyticus* in pigs, dogs and humans is reported in Tanzania. However, the use of antimicrobial resistant bacteria from farm animals and human beings and similarities in the resistance by phenotyping and genotyping were shown to tetracycline and sulphamethazole which are commonly used antimicrobials in Tanzania, the genotyping showed the similarity in genes coding for TE and STX in both human and farm animals isolates. Also the study in wildlife species showed that wild ungulates are reservoir of antibiotic resistance bacteria and resistance genes thus carrying risk of transfer of resistant genes to humans and livestock in the livestock-wildlife interfaces of MNP and NCA and humans are at public health risks by sharing the same environment with pets and farm animals. Further studies in molecular typing of the isolates obtained from humans, dogs and domestic animals sharing the same environment also cattle and wild ungulates grazing together in MNP NCA are indicated.

With regard to the general objective of the study; “to isolate, characterize and investigate the extent of antibiotic resistance among selected bacteria (*Escherichia coli*, *Enterococci spp* and *Staphylococcus aureus)* in humans, livestock, dogs and selected wild animals”,

the findings sufficiently and positively addressed the objective in terms of isolation, characterization and extent of antibacterial resistance in targeted species.

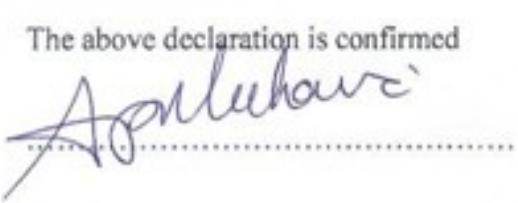
## DECLARATION

I, Abdul Ahamed Selemani Katakweba do hereby declare to the Senate of Sokoine University of Agriculture that, this thesis is my own original work done within the period of registration and that it has neither been submitted nor is being concurrently submitted for a degree award in any other institution.

15/01/2014

Abdul A.S Katakweba (PhD. Candidate) Date

The above declaration is confirmed



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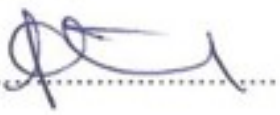

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## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| AMC | Amoxycillin-clavulanic acid |
| AMP | Ampicillin |
| AR | Antibiotic resistant |
| BLF | Buffalo |
| C | Chloramphenicol |
| CIP | Ciproflaxin |
| CN | Gentamicin |
| CTL | Cattle |
| CTX | Cefotaxime |
| DNA | Deoxyribonucleic acid |
| ESBL | Extended Spectrum β-Lactamase |
| LRRD | Livestock Research for Rural Development |
| MNP | Mikumi National Park |
| MRS | Methicillin Resistant Staphylococci |
| MRSA | Methicillin Resistant *Staphylococcus aureus* |
| NCA | Ngorongoro Conservation Area |
| OX | Oxacillin |
| P | Penicillin |
| RD | Rifampicin |
| S | Streptomycine |
| STX | Sulphamethoxazole/ trimethoprim |
| SUA | Sokoine University of Agriculture |
| TE | Tetracycline |

| | |
|---|---|
| VA | vancomycin |
| WLB | Wildebeest |
| ZBR | Zebra |

# CHAPTER ONE

## 1.0 INTRODUCTION

Domestic animals are ubiquitous in poor communities across the developing world, and high numbers are even found in the most densely populated, lower-income urban and peri-urban areas, where farm animals are in close contact with people (LID, 1999). The poor livestock keepers often stock a mixture of different species, trading-off specialization for better protection against risks (Waters-Bayer and Bayer, 1992; Randolph *et al*., 2012). Thus, livestock keeping will continue to be integral to improving the well-being of people in developing countries as seen from health, nutrition and socioeconomic perspectives (Mark and Lawrence, 2007). Regardless of their importance livestock expose the owners and consumers to zoonoses and food-borne diseases (WHO, 2007), and recently the use of antimicrobials in animals has been shown to contribute to increased resistance to antimicrobials in human beings (Lathers, 2002). According to WHO (2009), infectious and non infectious diseases are invariably treated with antibiotics thus with lack of control on the use of antimicrobials in animals in Tanzania (Katakweba *et al*., 2012); the spillage of antibiotic resistance from animals to human beings is likely to occur. Callie *et al*. (2012), reported that improper dispensing of antimicrobials also contributes to the increased antibiotic resistant strains of bacteria in both animals and human beings.

Consequent to trade liberalization policies availability of antibiotics became commonplace in human medicine and animal production in developing countries (Carlos, 2010), thus allowing the selection pressure to increase the advantage of maintaining resistance genes in diverse groups of bacteria. Through evolutionary progress reistance genes can be retained, and be dispersed among bacterial populations (Mathew *et al*., 2007). Subsequently these resistance genes can be transfered to closely related human pathogens

(JETACAR, 1999). This makes commensal bacteria a reservoir of resistance genes for (potentially) pathogenic bacteria. The disease-causing bacteria (pathogens) can be transferred directly from animals or animal products to humans (Box *et al*., 2005). Furthermore, resistant commensal bacteria of food animals might contaminate meat and milk (products) and so reach the intestinal tract of humans (Mathew *et al*., 2007).

Whether increased selection pressure from increased use of drugs, keeping mixed species of livestock, close contact with human beings, wildlife and domestic animals, interactions has a role in the epidemiology of AR genes in Tanzania is not known. Understanding the prevalence of AR in commensal bacteria and typing of the genes responsible for AR was envisaged to give initial indication of the problem in Tanzania.

Investigation of antimicrobial resistance can be safely assessed by using bacteria that are commensal of different body tracts (Hartl and Dykhuizen, 1984). These bacteria also known as indicator bacteria include *Escherichia coli* and *Enterococci spp* are which are native habitat of gastrointestinal tract while *Staphylococcus aureus* inhabits respiratory tracts of humans and other warm-blooded animals (Wertheim *et al*., 2005). These species of bacteria are important commensals and pathogens of human and animal and are regarded as an important source of antimicrobial resistance determinants (Neu, 1992; Nimalie *et al*., 2008).

### 1.1 Problem Statement

Dramatic increase in the occurrence of multiple antibiotic resistant strains among zoonotic bacterial pathogens has been reported since 1980s (Gallard *et al.*, 2001). This is the period when most of antimicrobials were developed. The development of antibiotic resistance (AR) among pathogenic bacteria is a major public health concern (David *et al*., 2002). The

appearance of AR has been directly linked to the use and overuse of antibiotics. Antibiotic resistant bacteria have been found in farm animals where antibiotics are heavily used, in associated food products, in environments contaminated with animal waste, and in farm workers (Barton, 2000; Ian *et al*., 2004; Viola and DeVincent, 2006). Drugs that are used therapeutically in animals may also generate a reservoir of AR bacteria (Mathew *et al*., 2007). Antibiotic resistant bacteria in food animals threaten the efficacy of human drugs if AR bacteria or bacteria or AR genes become incorporated into bacterial populations colonizing humans (Barton, 2000; David *et al*., 2002). Increased availability of antibiotics and uncontrolled use warrant the need for investigating the extent of AR with these practices (Katakweba *et al*., 2012).

There is no scientific proof in Tanzania from humans, livestock or wild animals or their food products for content of antimicrobial resistant bacteria. Also there is no evidence of resistant bacteria from animals being transferred to humans sharing the same environment. This created the need for gathering information that will help to address all these situations and protect human and animal health.

For informed decision making in supporting programs to track and prevent the spread of antimicrobial-resistant organisms in animals and humans data on prevalence and DNA based studies are imperative. This is important for infection-control programs and antimicrobial agent management programs and for guiding practitioners in veterinary and human medicine to use antimicrobial treatment more rationally.

### 1.2 Rationale of the Study

1. In Africa, and Tanzania inclusive, people traditionally keep livestock in close proximity to the homestead or even inside the domicile. Also there are interactions between

domestic animals, wildlife and humans in national parks and game reserves. These tendencies increase the risks of pathogen transmission through direct or faecal-oral route. Humans and livestock come into exceptionally close contact both directly and through cross-contamination of the shared environment. Resistance bacteria from livestock can therefore be spread to humans through this contact.

2. Poor hygiene in hospitals and communities augments the rapid spread of antibiotic resistant bacteria in vulnerable populations.
3. Poverty is also a major force driving the development of antimicrobial resistance. People use half of the recommended doses to treat themselves and their animal due to financial constraints. In turn the organisms develop resistance to the drugs in use.
4. The spread of resistance is also facilitated by use of animal food and food products by humans. Most of animal foods products that are used in our country are not totally protected from contaminations thus increase the possibility of contracting resistant bacteria from livestock products.
5. Improper use of both human and livestock antibiotics is evident in our country. Some of the practitioners in the pharmaceuticals are not trained and others prescribe the drugs that are not related to the disease. Hence this is also a source of drug resistance.
6. Most of studies in our country involved detection of antibiotic resistance in livestock or humans separately. There is no study that has been conducted to correlate the resistance in livestock and humans or crossover genes from animals to humans sharing the same environment.
7. Ignorance of the population and lay perosons (Pastrolalists) doing treatements of animals without respecting recommended antimicrobial dosages and their withdraw periods.

## 1.3 Hypotheses of the Study

Resistance to commonly used antimicrobials in cattle, pigs, and chickens medicine is present among *E. coli, Enterococci spp* and *S. aureus* isolated from faecal samples of healthy humans, cattle, pigs and chickens and nasal swabs of humans and dogs and wildlife in Tanzania.

*E. coli, Enterococci spp, S. aureus* from healthy humans, dogs, cattle, pigs, chickens and wildlife that share a common environment will have same antibiotic resistance patterns.

## 1.4 Objectives of the Study

### 1.4.1 Main objective

The broad objective of this study was to isolate, characterize and investigate the extent of antibiotic resistance among selected bacteria (*Escherichia coli, Enterococci spp* and *Staphylococcus aureus)* in humans, livestock, dogs and selected wild animals

### 1.5.2 Specific objectives

1. To investigate perception of human health risks associated with the use of antibiotics among livestock keepers from different management system and factors that contribute to selection of antibiotic resistance bacteria in Tanzania.
2. To establish the extent of AR to commonly used antibiotics in the populations by using isolates of *Staphylococcus aureus* from humans, pigs and dogs
3. Determine AR patterns of *Escherichia coli*, *Enterococci* and resistance genes and ESBL of *Escherichia coli* from livestock and human beings.
4. Isolate and characterize indicator bacteria (*Escherichia coli, Enterococci spp*) from wild animals and contact farm animals and determine their antibiotic resistance and molecular typing to trace resisntnce genes.

# CHAPTER TWO

## 2.0 RETERATURE REVIEW

### 2.1 Definitions, History and Discovery of Antimicrobials

#### 2.1.1 Definitions

Antimicrobials are defined as naturally-occurring, semi-synthetic and synthetic compounds with selective antimicrobial activity that can be administered orally, parenterally or topically and are used in human and veterinary medicine to treat and prevent diseases (Prescott *et al.,* 2000; Ian *et al.,* 2004). However, Codex Alimentarius (Codex Alimentarius, 2005) defined antimicrobials as veterinary antimicrobial drug(s) referring to naturally occurring, semi-synthetic or synthetic substances that exhibit antimicrobial activity that can kill or inhibit the growth of a microorganism. From World Organization for Animal Health, antimicrobials are referred to as naturally occurring, semi-synthetic or synthetic substance that at in vivo concentrations exhibits antimicrobial activity that can kill or inhibit growth of micro-organisms (OIE, 2012). Furthermore, DANMAP (2010), referred the term antimicrobial agents to cover antibacterial, antiviral, anti-coccidials and antimycotic agents while WHO (2002), defined as any substance of natural, synthetic or semi-synthetic origin which at low concentrations kills or inhibits the growth of micro-organisms but causes little or no host damage.

Antibiotics were defined by early workers as any substances produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution (Waksman, 1947; Waksman, 1973). However the common agreed definition by FAO (2005) and WHO (2001), antibiotics are drugs of natural or synthetic origin that have the capacity to kill or to inhibit the growth of microorganisms. The term antibiotic is used as a synonym for antibacterial used to treat bacterial infections in both people and animals (WHO,

2001). The word antibiotic came from the word antibiosis (Gr. anti, "against"; bios, "life"), a term coined in 1889 by Louis Pasteur's pupil Paul Vuillemin which meant a process by which life could be used to destroy life (Bellis, 2013). Apart from chemical classifications, Prescott *et al*. (2000), classified antibiotics in a functional ways of which is considered narrow if it inhibits only Gram negative or positive bacterial and broad spectrum if it kills both Gram negative and Gram positive bacteria. Davey (2000) classified antibiotics as bacteriostatic and bacteriacidal respectively if they are only inhibitory or with both killing and inhibition of microorganisms at near or the same concentrations.

### 2.1.2 *Discovery of modern antimicrobial drugs*

Despite the evidence of ancient antibiotic exposure in human fossils (Bassett *et al*., 1980; Aminov, 2010) actual search and use of antibiotics began in late 1880s. This was subsequent to the discovery of germ theory on disease which linked bacteria and other microbes to the causative **agents of the diseases. Scientists began to devote time for searching of drugs that would kill these disease-causing bacteria. The goal was to find so-called "magic bullets" that would destroy microbes without toxicity to the person taking the drug (Aminov, 2010;** Bellis, 2013). Accidentaly, Alexander Fleming found that mould (*Penicillium notatum*) that contaminated *Staphylococcus* culture plates did not have bacterial growth around it and had inhibition zone congruent to the moulds' (*P. notatum*) growth ring (Bellis, 2013). Further research by Fleming proved that even diluted broth cultures of *P. notatum* prevented growth of Staphylococcus (Fleming, 1929; Todar, 2004).

In the late 1940s and early 1950s, new antibiotics, including streptomycin, chloramphenicol and tetracycline were introduced, and the age of antibiotic chemotherapy came into full action (Bellis, 2013). These antibiotics were effective against Gram-positive and Gram-negative pathogenic bacteria, intracellular parasites, and the tuberculosis

bacillus. Synthetic antimicrobial agents such as the "sulfa drugs" (sulfonamides) and anti-tuberculosis drugs, such as para aminosalicylic acid (PAS) and isoniazid, were also developed (Todar, 2004).

### 2.1.3 Development of synthetic antibiotics and other antimicrobials

Development of antibiotics is associated with ideas of Ehrlich and Fleming targeting on only disease-causing microbes and not the host (Aminov, 2010**)**. They were targeting a chemical compound that will be able to exert their full action exclusively on the parasite harbored within the organism. In 1904 Ehrlich found a drug against syphilis (Ehrlich and Hata, 1910 ; Aminov, 2010). The drug was marketed by Hoechst under the name Salvarsan together with a more soluble and less toxic Neosalvarsan, until its replacement by penicillin in the 1940s (Mahoney *et al*., 1943). Fleming's research and study inspired him to discover naturally antiseptic enzyme in 1921 which he named lysozyme. Six years later, he stumbled on discovering penicillin (Todar, 2004). Fleming was trying to get chemists interested in resolving persisting problems with purification and stability of the active substance and supplied the *Penicillium* strain to anyone requesting it. Further investigations by Florey and Chain (1945) as reported by Chain *et al.* (2005) resulted in purification of sufficient quantities and later clinical testing of penicillin. This led to mass production and distribution of penicillin in 1945 (Todar, 2004; Aminov, 2010).

## 2.2 Use of Antimicrobial Drugs in Human and Veterinary Medicine

In human medicine, antimicrobials are approved for disease treatment and prophylaxis or prevention (Kagashea *et al*., 2010). In animals, antimicrobial indication include: therapeutic use to treat sick animals; prophylactic use to prevent infection in animals; as growth promoters to improve feed utilization and production (Barton, 2000; Viola and DeVincent, 2006; Mathew *et al*., 2007). Therapeutic treatment involves treatment of individual animals over a short period with doses of antibiotic exceeding the minimal

inhibitory concentration of the known or suspected pathogen. Prophylactic treatment again involves moderate to high doses of antibiotic, often given in feed or water for a defined period to a group of animals (Barton, 2000; David *et al*., 2002). Antibiotics used as growth promoters tend to be given in feed at subtherapeutic levels over extended periods to entire herds and flocks. Their use improves physiological performance of individual animals (Barton, 2000; Ian *et al*., 2004; Viola and DeVincent, 2006). The prophylactic and growth promotion use of antibiotics at low levels, have resulted in selection of antibiotic-resistant bacteria that contaminate both animal food products and the environment (Mathew *et al*., 2007).

Many of the drugs that are shared by both medical and veterinary practices are restricted to a very specific use, dose and duration and can only be administered by a qualified and certified personel. This means that if you have respiratory infection in a person, it can be treated with whatever the doctor determines to be the best drug, at the best dose, for the appropriate length of time. In food-producing animals regulations require to use approved drugs at a specific dose and for a specific length of time (Barber, 2001; DANMAP, 2008). According to WHO (2010), more than half of all medicines in developing countries are prescribed, dispensed or sold inappropriately, and that half of all patients fail to take them correctly. This incorrect use may take the form of overuse, underuse and misuse of prescription or non-prescription medicines. Humans in Tanzania have also free access to antimicrobials from the drug shops without any prescriptions, as is the common tendency in other Africa countries (Kagashea *et al*., 2010; Tagoe and Atar, 2010). Animal health services in the developed countries have been sub optimal with an increased tendency for animal owners to stock drugs in their houses and engaging unskilled people, such as farmers themselves and animal attendants to treat the animals (Katakweba *et al*., 2012). This could explain the use and misuse of antimicrobials because their relatively low cost

and ready availability for sale ‘over the counter’ (Karimuribo *et al*., 2005; Miralles 2010; Katakweba *et al*., 2012). In developed countries laws and by laws are more strictly adhered to, and proper use and disposal of antimicrobials are practiced (DANMAP, 2008).

## 2.3 Antimicrobial Resistance Definition and Emergence

### 2.3.1 Antimicrobial Resistance (AR)

Over time, micro-organisms (bacteria, yeast, and parasites) change so that they can protect themselves from the deleterious effect of the drug or medicine (Manitoba, 2001) which lead into AR. Studies have shown that probably antibiotic resistance emerged in nature prior to human use of drugs because some organisms produce antibiotic compounds to acquire resistance as means to survive in the presence of their own products and competing species (Davies, 1997). Thus, some resistance genes likely originated long before the modern medicine, and agricultural use of antibiotics (Mathew *et al*., 2007). After use of antimicrobials in human medicine and animal production, selection pressure increased and led to production of resistance genes in diverse groups of bacteria.

### 2.3.2 Emergency of AR

Soon after introduction of the first effective antimicrobials, namely, the sulfonamides in 1937, the development of specific mechanisms of resistance was reported (Lewis, 1995; Levy, 1998). In the 1940s’, several years before the introduction of penicillin as a therapeutic compaund, a bacterial enzyme penicillinase was identified by two members of the penicillin discovery team (Abraham and Chain, 1940). The first bug reported to battle penicillin was *S. aureus* in 1943 followed by *Pneumococcus (Streptococcus) pneumoniae* in 1967. In 1983, a hospital-acquired intestinal infection caused by the bacterium *Enterococcus faecium* joined the list of bugs that outwit penicillin (Lewis, 1995). Streptomycin was introduced in 1944 for the treatment of tuberculosis (TB) and mutant

strains of *Mycobacterium tuberculosis* resistant to therapeutic concentrations of the antibiotic were found to arise during patient treatment (Shah *et al*., 2007; Davies and Davies, 2010). Chloramphenicol, erythromycin and vancomycin were introduced to counter the rapidly emerging resistance to penicillin and to treat those infections caused by organisms intrinsically resistant to penicillin, such as tuberculosis (Powers, 2004). The unexpected identification of genetically transferable antibiotic resistance in Japan in the mid-1950s changed the whole picture by introducing genetic concept that collections of antibiotic resistance genes could be disseminated by bacterial conjugation throughout an entire population of bacterial pathogens (Davies, 1995; Helinski, 2004).

### 2.3.3 Factors contributing to AR

Larson (2007), categorized behavioral and environmental factors that are involved in developing resistance in antimicrobials. Behavioral factors include inappropriate use of antibiotics, such as prescribing for nonbacterial infections and community member self-prescribing of antimicrobials. Moreover contact including animals, crowding, contaminated items, compromised skin integrity, cleanliness and failure to vaccinate for vaccine-preventable diseases also add to AR (WHO, 2010). Poor hygiene in hospitals as well as in the community augments the rapid spread of antibiotic resistant bacteria in vulnerable populations (Otto *et al*., 2008). Once the food from animal origin is contaminated with resistant bacteria then they can be easily spread to humans and animals (Butaye *et al*., 2006). In European countries, the relationship between companion animals (cats and dogs) and humans has radically changed. Dogs usually were maintained outside households, today they are often kept inside houses (Luca *et al*., 2004). In Africa, people traditionally keep livestock in close proximity to the homestead or even inside the domicile. These situations increase the risks of pathogen transmission through direct or faecal-oral route (Rwego *et al.*, 2008). In such circumstances, humans and livestock come

into exceptionally close contact both directly and through cross-contamination of the shared environment. The emergence of antibiotic resistance is further complicated by the fact that bacteria and their resistance genes are travelling faster and further (WHO, 2000; Grundmann *et al*., 2006). Airlines now carry more than two billion passengers annually, vastly increasing the opportunities for rapid spread of infectious agents worldwide including antibiotic resistant bacteria (WHO, 2007).

Environmental and policy factors include the continued use of antibiotics in agriculture and the lack of new drug development. Antimicrobials used in agriculture for growth promotion and major source of environmental contamination (Larson, 2007). Improper use of drugs for prophylaxis in animal husbandry increases antibiotic resistant isolates in the environment (Kariuki *et al*., 1997). Specific environmental conditions generate different evolutionary selection pressures and environmental pollution is potential selective pressure favoring the evolution of resistance in bacteria (Barkay *et al*., 2003). Environmental contamination with drug-resistant bacteria from animal wastes significantly contributes to resistance of either humans or animals if they are sharing the same environment (Osterberg and Wallinga, 2004; Roess *et al*., 2004; Carlos, 2010).

### 2.3.4 Antimicrobials used in animals and AR in human beings

Use of growth-promoters in animals is probably the area of highest concern. Some of the antimicrobials used in animals are regarded to compromise the efficacy of some key human antibiotics (Barton, 2000). Antibiotic-resistant bacteria (AR) can easily transfer their resistance traits to unrelated bacteria once inside the human body (Shoemaker *et al*., 2001). There are three ways in which AR bacteria from animals can pose risk to human beings one way is through food of an animal origin (meat, milk and eggs). Antibiotic resistant bacteria can be transferred from animals to humans and vice versa, and food-

borne transmission through food chain of animal origin is a recognized risk. The second way is through working with animals. Resistant bacteria may be picked up by workers in the livestock industry through handling animals, feed, and manure (Levy *et al*., 1976; Van den Bogaard *et al.,* 2002). Moreover, farming communities and slaughterhouse workers are routinely exposed to antibiotics or antibiotic-resistant bacteria, or both. Lastly, is through the environment that is shared between animals and humans (Davies and Davies, 2010). Manure that contains resistant bacteria creates an immense pool of resistance genes available for transfer to bacteria that cause human and animal diseases. Farm wastes are spread on agricultural fields as fertilizers, and waste run-off can enter rivers, lakes, and ground water (Osterberg and Wallinga, 2004). With increasing evidence of highly antimicrobial resistant animal-borne pathogens (White *et al*., 2001), human beings are exposed to high risk transfer of these genes from animals.

### 2.3.5 Antibiotic resistant bacteria and resistant gene in the soil and Environment

The soil pathogenic and non pathogenic mocrorganisms have all genetic components of antibiotic resistant that are present in a given natural microbial community (Kozhevin *et al*., 2013). Detection of resistant microorganisms in unimpaired natural habitat was found in ancient microbial community from 10 000 to 3 million years in marine sea sediments, ancient, permafrost, pure high mountain lakes, soil depth 170 – 259 m and in microbiota of caves (Kozhevin *et al.,* 2013). The above biotypes were not known to geologically to have been exposed to anthropogenic intervation and did not come into contact with medical preparations of antimicrobials. Antimicrobial resistant microbs existed long before the beginning of the antibiotic era not related with antibiotic pressure but is a trait that is inherent to microorganism in nature (Kozhevin *et al*., 2013).

With current modern antimicrobials use, antibiotic-resistant bacteria and antibiotic resistance genes are excreted into the environment with faeces of humans and animals that are treated with antibiotics. Resistant bacteria and AR genes enter the environment through discharge of untreated or partially treated wastewater from hospitals, territories of agriculture farms or slaughterhouses; sewage overflows during heavy rainfall, application of activated sludge from waste water treatment plants as fertilizer to agricultural soil, and run off of animal manure or faeces of pasture animals. Once in the environment, bacteria of different origin come into physical contact and the environment is considered to be the natural reservoir of resistance genes from those bacteria (Genthner *et al*., 1988; Xu *et al*., 2007). Soil bacteria, especially bacteria belonging to the family of Actinomycetes, produce antibiotic resistance proteins to protect themselves from their own produced antimicrobials (Hopwood, 2007; Tahlan *et al*., 2007). Antibiotics produced by these bacteria exert selective pressure on other microorganisms in the same habitat as well. Bacteria excreted by humans and animals acquire these genes through horizontal gene transfer, resulting in bacteria resistant to antimicrobials that were not yet seen previously in human or animal bacterial isolates (Wright, 2007). Even though the resistant bacteria of human and animal origin may die off in the environment, the endogenous environmental bacteria may pass the acquired resistance genes on to their progeny (Andersson and Levin, 1999). Resistant genes may remain present in the environment for a long time due to the presence of trace amounts of antibiotics in the environment, originating from treated humans and animals or from soil bacteria and will exert selective pressure. In the absence of antibiotics AR genes may be stable in the environment, due to compensatory mutations that restore fitness and co-selection of the AR resistance genes with other genes on the same mobile genetic element that increase fitness in certain environments, such as the presence of heavy metals. Hence, the environmental bacteria represent a long-lasting reservoir of AR genes, fed by bacteria that are secreted by man and animal (Andersson,

2003; Allen *et al*., 2010; Zhang *et al*., 2009). Apart from humans, livestock and the environment a few additional reservoirs of antibiotic resistance and resistance genes have been identified. These reservoirs can be divided into three categories namely, reservoirs in which antibiotic resistance develops due to administration of antibiotics to cure or prevent disease (e.g. pets, zoo animals, ornamental fish, fish for consumption grown in tanks); reservoirs that pick up antibiotic resistance from the environment (e.g. wild life, fish cultivated in natural waters); and reservoirs to which bacteria and genes are introduced actively, and antibiotic resistance may be introduced as accidental or unwanted byproduct (e.g. fermented food and genetically modified food) (Geenen *et al*., 2010).

### 2.3.6 Evolution and ecology of antibiotic resistance genes

The issue of antibiotic resistance has been exclusively associated with the use and overuse/misuse of antibiotics in humans and animals (Aminov and Mackie, 2007). This is true for the clonal dissemination of pathogenic bacteria with resistance mechanisms. This is based on altering target molecules through mutational events and selection of mutants. Majority of antibiotic resistances are most likely cases of acquired resistance, through the lateral transfer of antibiotic resistance genes from other ecologically and taxonomically distant bacteria (Aminov and Mackie, 2007). A new perspective on antibiotic resistance is also based on a broader evolutionary and ecological understanding rather than from that of the traditional use of antibmicrobials. Phylogenetic insights into the evolution and diversity of several antibiotic resistance genes suggest that at least some of these genes have a long evolutionary history of diversification that began well before the “antibiotic era”. The primary antibiotic resistance gene pool originated and diversified within the environmental bacterial communities, from which the genes were mobilized and penetrated into taxonomically and ecologically distant bacterial populations, including pathogens. Besides direct selection by antibiotics, there is a number of other factors that

may contribute to dissemination and maintenance of antibiotic resistance genes in bacterial populations (Aminov and Mackie, 2007).

The presence of DNA encoding drug resistance in antibiotic preparations has been a factor in the rapid development of multiple antibiotic resistances due to the uptake of this DNA by bacteria. Evolution of the residual DNA with a more sensitive fluorescence detection technique demonstrated once more that many antibiotic preparations, of both research and clinical grade, contain detectable amounts of DNA (Woegerbauer *et al*., 2005). Another factor is Phylogeny of antibiotic resistance genes. Phylogenetic analyses helped to identify the non antibiotic-producing environmental bacteria. A research was conducted to tetracycline and vancomycin regarding their resistance. The phylogenetic approach was applied to analyze the evolutionary history of antibiotic resistance genes, encoding ribosomal protection proteins (RPP) that function as alternative elongation factors and confer resistance to tetracyclines (Connell *et al*., 2002, 2003). Phylogenetic analysis strongly supported a scenario of early branching and long independent diversification of eight (at that time) clusters of RPPs well before the modern ‘antibiotic era’ (Aminov *et al*., 2001). Monophyletic origin of the *tet* genes, with an early branching event separating them from the other group of elongation factors, EF-G and encoded by the fusA genes *Staphylococcus aureus*. Until the late 1980s, the glycopeptide antibiotic vancomycin was considered as the drug of last resort for treatment of diseases caused by gram-positive bacteria such as enterococci, MRSA, Streptococcus pneumoniae, and Clostridium difficile (Cunha, 1995). The emergence and extremely rapid dissemination of VRE and a recent arrival of a high-level vancomycin-resistant (Weigel *et al*., 2003) make the phylogenetic reconstruction of this scenario a particularly valuable example of antibiotic resistance gene evolution. The mechanism of high-level vancomycin resistance, widespread in enterococci and now acquired by *S. aureus*, is target modification, which includes the alternative

peptidoglycan synthesis pathway terminating in D-alanyl-D-lactate instead of D-alanyl-D-alanine (Walsh *et al*., 1996).

Factors contributing to dissemination and maintenance of antibiotic resistance include evolution that contributes to the dissemination of antibiotic resistance genes. There is ever-increasing production and consumption of antibiotics for various purposes, from treatment of disease to the questionable practice of feeding them to food-production animals at sub therapeutic levels for growth promotion. Another factor is acquisition of the antibiotic resistance genotype. Increase the fitness of certain bacteria in the absence of antibiotic selective pressre thus allowing the rapid emergence and dissemination on a worldwide scale (Enne *et al*., 2004; Luo *et al*., 2005). In this case, the dissemination of antibiotic resistancue becomes a self-perpetuating process, replacing the antibiotic susceptible genotype in the absence of any antibiotic selective pressure. The amelioration of the fitness cost of antibiotic resistance carriage may be one of the reasons why the antibiotic resistance genes are persistent in the apparent absence of selection imposed by the presence of antibiotics, for example, in wild animals (Gilliver *et al*., 1999). Another aspect of ecology of antibiotic resistance that recently emerged with the advent of molecular ecology tools in antibiotic resistance studies in the environment is the realization of the fact that the microbial ecosystems are not isolated and there is extensive gene exchange between different compartments from humans, animals, soils and environment (Aminov and Mackie, 2007).

### 2.4 Detection of AR in the Population and Clinical Samples

Guidelines for standard methods of broth and agar dilatational susceptibility testing, disc diffusion and molecular testing has been developed (CLSI 2006; Biomerieux, 2008). These guidelines describe all aspects of the testing procedure including media

specifications, bacterial inoculums, solvents and diluents for each antimicrobial, incubation time and temperature and interpretive criteria for categorical analyses (CLSI, 2006; CLSI, 2008). The validity of Results can be objectively evaluated by testing quality control organisms along with the isolates under investigation (CLSI, 2008). The selection of quality control organisms is based on the drugs tested and each drug should have a corresponding organism with a quality control range including the concentrations tested. Commonly used quality control organisms for testing non-fastidious aerobic bacteria include *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (CLSI 2008). Faecal samples, nasal swabs, abscesses and urine are normally collected from animals and human being for detection of AR (Bager, 2000). Bacterial isolation is carried out and the isolates are subjected to a panel of antibiotics and detection of resistant genes (Stobberingh *et al*., 1999; van den Bogaard *et al*., 2001).

### 2.4.1 Phenotypic methods of detecting AR

Both quantitative and qualitative tests have been described for phenotyping bacteria for AR (Prescott *et al.,* 2000). Quantitative tests include disk diffusion, agar dilution, broth macrodilution, broth microdilution, and a concentration gradient test (Prescott *et al*., *2000). The tests are inhibitory rather than killing of bacteria and the end results are reported as susceptible, intermediate or resistant. Qualitative Results are reported as minimal inhibitory concentration in µg/ml or mg/l (Prescott et al.,* 2000). Of these, the disk diffusion and the broth microdilution tests are the most commonly used in both human and veterinary medicine. Disk diffusion method is used to determine the antimicrobial agent sensitivity profiles of the bacteria. The minimum inhibitory concentration is measured and recorded as whether the organism is susceptible (S),

intermediately susceptible (I), or resistant (R) to the antibiotics (Prescott *et al*., *2000;* CLSI, 2008).

### 2.4.2 Molecular methods

After the discovery *of DNA based techniques, elucidation of AR genes in bacteria has been of great use and these have been shown to complement phenotypic results (Rolain et al*., 2004). Though phenotypic methods have been used for antibiotic susceptibility testing, molecular detection is found to be more rapid and efficient. Nucleic acid-based detection systems are able to detect the presence of resistance genes and play a critical role in the elucidation of resistance mechanisms (Fluit *et al*., 2001; Rolain *et al.*, 2004). Furthermore, processing of large numbers of samples is inhibited by phenotypic characterization compared to molecular method (Jackson *et al*., 2004).

## 2.5 Techniques Used In Identification of Antibiotic Resistant Indicator Bacteria

There are many techniques available which are variably suited to particular research questions, and associated with their own set of technical advantages and disadvantages (Savelkoul *et al*., 1999; CLSI, 2008). Multilocus sequence typing (MLST), *spa* typing**,** multiplex PCR, and Matrix-assisted laser desorption-ionization (MALDI) time-of-light mass spectrometry (MALDI – TOF/MS), are the most commonly used techniques depending on the type of bacteria to be identified. Apart from molecular techniques, colony morphology on blood agar and selective media, biochemical tests such as (IMViC), catalase, coagulase and oxidase tests can also be carried out for the identification of respective bacteria (Kubitschek, 1990; Winn *et al*., 2006; Rubin, 2011).

### 2.5.1 Multilocus Sequence Typing (MLST)

As a relatively low-resolution, high-fidelity technique, MLST is best suited to big picture of evolutionary studies. While MLST will not detect divergence among closely related *S.*

*aureus*, it is valuable for evolutionary studies and for broadly clustering the finely resolved types identified with other techniques (Rubin, 2011). The online *S. aureus* database as well as protocols for other species can be found at www.mlst.net 38 (Aanensen and Spratt, 2005). MLST of *S. aureus* is based on the sequences of seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*) (Enright *et al*., 2000). Each unique sequence for each gene is assigned a number in the MLST database and the numeric profile from all seven genes defines the sequence type (Enright *et al*., 2000). Unique sequence types are also assigned numerical identities in the database. New sequence types are numbered sequentially as they are described. The relatedness of sequence types (ST) is based on the seven-gene numeric profile rather than the sequence type number itself (Cheng *et al*., 2011).

### 2.5.2 *spa* typing

This is fast and inexpensive technique based on sequencing of the hyper-variable x-region of the staphylococcal protein A (*spaA*) (Harmsen *et al*., 2003). Unlike MLST, *spa* typing discriminates between closely related isolates; its resolving power is somewhere between PFGE and MLST (Rubin *et al*., 2011). Within a MLST sequence type there may be many *spa* types (SpaServer, 2011). Like MLST, *spa* types are cataloged in a central, web-based database allowing easy access to the details of each *spa* type and global epidemiological information (Harmsen *et al*., 2003). The hyper-variable x-region contains a variable number of short (21-30 base pair) sequences (Hallin *et al*., 2009; SpaServer, 2011). An isolate is assigned a *spa* type according to which and how many repeats are present and their order (Hallin *et al*., 2009). For example the 24 base pair sequence 5’ AAA GAA GAC AAC AAA AAA CCT GGT-3’ was the thirty fourth repeat deposited in the database and is therefore identified as “r34” (SpaServer, 2011). *spa* types are identified numerically in the order of their discovery, no genetic relationship can be inferred from sequentially

numbered types; relatedness is dictated by the repeat sequence of each type. For example t034 (r08-r16-r02-r25-r02-r25-r34-r24-r25) is more closely related to t4652 (r08-r16-r02-r25-r02-r25-r34-r24) than t035 (r26-r17-r13-r12-r17-r17-r16) (SpaServer, 2011; Rubin, 2011).

### 2.5.3 Mutiplex PCR

Multiplex-PCR was first described in 1988 as a method to detect deletions in the duchenne muscular dystrophy (DMD) genes from humans in USA (Chamberlain *et al*., 1988). This is becoming a rapid and convenient screening assay in both the clinical and the research laboratories and time and effort can be saved (Markoulatos *et al*., 2002). The identification of *Enterococci spp*, using biochemical tests based on their phenotypic characteristics, is complicated and time-consuming (Devriese *et al*., 1993). This method in not only used in clinical microbiology, but also in food microbiology, the methods of molecular biology focused on the genus or species identification of enterococci (Dutka-Malen *et al*., 1995; Ke *et al*., 1999). Intra-generic typing of enterococci from foods (Suzzi *et al.*, 2000; Andrighetto *et al*. 2001) and from faecal samples and rectal swabs of farm and pet animals (Devriese *et al*., 1996) are now being identified using this method (Cupakova *et al*., 2005). It is a process whereby simultaneously amplifying multiple sequences in a single reaction is taking place (Markoulatos *et al*., 2002). Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences (Chamberlain *et al*., 1988). It requires primers that lead to amplification of unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of reaction conditions (Markoulatos *et al*., 2002). Some of the applications of multiplex PCR include: Pathogen Identification, Mutation Analysis, Gene Deletion Analysis, Template Quantitation, Linkage Analysis, RNA Detection, Forensic Studies and Diet Analysis (Chamberlain *et al*., 1988). Ke *et al*. (1999) developed

a PCR method for rapid genus-specific identification of *Enterococci spp*, which is based on the detection of *tuf*-gene encoding the elongation factor Tu (EF-Tu). EF-Tu is a GTP-binding protein playing a central role in the protein synthesis. The *tuf*A-gene was proved to be present in all tested species, which enables its successful application as the genus-specific PCR method (Ke *et al*., 2000). This technique can also be used to other bacteria provided multiplex PCR assay is prepared for specific bacteria to be identified (Alvarez *et al*., 2004).

### 2.5.4 MALD – TOF

Matrix-assisted laser desorption-ionization (MALDI) time-of-light mass spectrometry (TOF/MS); MALDI-TOF/MS (Jackson and Lay, 2001) is a rapid method used in characterization of bacteria or bacterial taxonomy. Generally its applications involve whole cells, simple cell lysates, or crude bacterial extracts (Lynn *et al*., 1999). This method has one clear advantage, speed of analysis. MALDI-TOF mass spectrometry is applied directly to crude cellular fractions or cellular suspensions and that the resulting data from such complex mixtures provide evidence for chemotaxonomic classification (Winkler *et al*., 1999). MALDI provides unique insights into bacterial biology and chemistry based on the detection of specific chemicals or in the measurement of chemical changes in response to environmental, temporal, or other external influences (Jackson and Lay, 2001). This method has been also involved in the detection of isolated bacterial proteins (e.g., bacterial toxins), bacterial DNA or RNA, and bacterial metabolites (Nordhoff *et al*., 1992). Most early MALDI-TOF/MS studies of bacteria involved *E. coli*, but has been also used for the analysis of PCR amplified DNA and RNA in studies that involve other bacteria and bacterial genes (Hurst *et al*., 1996). Temporal characteristics must be taken into account when MALDI-TOF/MS is applied to identify bacteria, and that

bacterial incubation time is one of the variables that should be carefully controlled in experiments aimed at bacterial identification (Jackson and Lay, 2001).

## 2.6 Antibiotic Resistant Indicators in Epidemiology Studies of AR

Antimicrobial resistance has been assessed by using bacterial indicators that are native/ commensal habitat of different body tracts (Hartl and Dykhuizen, 1984). *E. coli* and *Enterococci spp* are the native habitat of gastrointestinal tract while *S. aureus* is that of respiratory tracts of humans and other warm-blooded animals (Wertheim *et al*., 2005). These species of bacteria are important commensal and pathogens of human and animal and are regarded as an important source of antimicrobial resistance determinants (Neu, 1992; Nimalie *et al*., 2008). Due to permanent habitation in the gut and respiratory tract, these bacteria have been used as indicator bacteria to gauge the extent of AR in a population (Hartl and Dykhuizen, 1984**;** Nimalie *et al*., 2008). Furthermore, these bacteria have potential ability to acquire and disseminate resistance that could be transmitted to pathogenic or zoonotic bacteria (Kim *et al*., 2001).

### 2.6.1 *E. coli* bacteria as antibiotic resistant indicator

There is a large body of literature reviewed by Novick, 1981, demonstrating that the sub therapeutic use of antibiotics in the mass production of poultry, eggs, and pork has promoted the emergence of and maintains the prevalence of multiple-antibiotic-resistant (MAR) *E. coli* in the faecal environment of these animals. The wide use and abuse of antibiotics in human therapy has produced multiple AR *E. coli* in the faeces of humans as well (Isenberg and Berkman, 1971; Novick, 1981). These practices have resulted in the coexistence of MAR *E. coli* within these major reservoirs of enteric disease for humans. In Spain, *E. coli* is used as indicator bacteria in pigs, poultry and cattle (Moreno *et al.*, 2000) as it is in Denmark and other European countries. Jordi *et al.* (1999) studied the prevalence and antimicrobial susceptibility of diarrheagenic *E. coli* in children under 5 years of age in Ifakara, Tanzania and Carl *et al*. (2002) carried out susceptibility testing of *E. coli* isolates recovered from humans, cattle, swine, and food during the years 1985 to 2000 from

different countries and were stored in *E. coli* reference centre at Pennsylvania State University. The isolates were resistant to tetracycline, sulfamethoxazole, cephalothin ampicillin. Highest frequencies of resistance occurred among swine isolates. Findings from the study suggested antimicrobial resistance to be widespread among *E. coli* O26, O103, O111, O128, and O145 inhabiting humans and food animals. *E. coli* and related bacteria possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population (Brüssow *et al*., 2004).

### 2.6.2 Enterococci spp bacteria as antibiotic resistant indicator

In Denmark and other European countries, *Enterococcus spp* is used as indicator bacteria, especially *E. faecium* and *E. faecalis* (Bager, 2000; Jordi *et al.*, 1999). Due to Enterococci *spp* bacteria being a cause of nosocomial infection and their increasing resistance to a wide range of antibiotics; they have been found to demonstrate both intrinsic and acquired resistance (Hunt, 1998). Cristina *et al.* (2007) did direct detection of antibiotic resistance genes in specimens of chicken and pork meat and faecal samples. The genes detected included that of tetracycline and erythromycin resistance. A molecular approach based on PCR amplification of bacterial DNA directly extracted from specimens was applied. Lester *et al.* (2006) showed that transfer of the *vanA* gene from an *E. faecium* isolate of animal origin to an *E. faecium* isolate of human origin can occur in the intestines of humans.

### 2.6.3 *S. aureus* bacteria as antibiotic resistant indicator

Methicillin-resistant *S. aureus* (MRSA) is a type of *S. aureus* that is resistant to the beta-lactams group of bacteria, which include methicillin and other more common antibiotics such as oxacillin, penicillin and amoxicillin. *S. aureus* infections, including MRSA, occur

most frequently among persons in hospitals and healthcare facilities, where the inhabitants have weakened immune systems (Kim and Simor, 2001; Wertheim *et al*., 2005; Nimalie *et al*., 2008). MRSA has been found in cats, dogs and horses. Owners can transfer the organism to their pets and vice-versa, and MRSA in animals are generally believed to be derived from humans (Maree *et al*., 2007). Armand-Lefevre *et al*. (2005) described *S. aureus* (methicillin-susceptible and -resistant) carriage among pigs and pig farmers. Shitandi and Sternesjö, (2004) evaluated the prevalence of multidrug resistant *S. aureus* (Penicillin G, tetracycline, erythromycin, sulfamethazine, and chloramphenicol) in Kenyan milk between large- (>200 L/d) and small- (<50 L/d) scale producers. EFSA, (2013) when analyzing the antimicrobial resistance data among zoonotic and indicator bacteria in 2011, submitted by 26 European Union Member States, used methicillin-resistant *S. aureus* (MRSA) from animals and food as antibiotic resistant indicator.

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# CHAPTER THREE

## 3.0 RESEARCH METHODOLOGY

### 3.1 Awareness of Human Health Risks Associated with the Use of Antibiotics among Livestock Keepers and Factors That Contribute to Selection of Antibiotic Resistance Bacteria within Livestock in Tanzania (Manuscript No 1)

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## 3.2 Abstract

Awareness of human health threats due to the use of antimicrobials in livestock and the factors that can contribute to selection of resistant bacteria were assessed among 160 randomly chosen Tanzanian small-scale livestock keepers. Thirty percent of the respondents were not aware of the concept of antibiotic resistance, 52% were not aware of which types of diseases can be treated with antibiotics, 22% did not know the principle of withdrawal period after treatment of animals with antibiotics and 40% did not know if antibiotics used in livestock pose risk to human health. Side effects of antibiotics to human beings stated by the respondents included drug resistance, allergy, poisoning, skin rashes, cancer and resistance to treatment. The most commonly used antibiotics were oxytetracyline (62.9%), sulphadimidine (23.2%), penicillin-streptomycin (13.4%) and gentamycin (0.5%). Points of selling antibiotics and their prescriptions were found to be agriculture and veterinary input shops, veterinarians, livestock markets and exhibition areas. Only 54 % of respondents obtained antibiotics through prescription by veterinarians. Livestock management systems, antibiotics handling and types of antibiotics used in the study areas were identified as potential risk factors to the development of antimicrobials resistance. This study has revealed gross lack of awareness on health threats associated with antibiotics resistance among the farming communities posing serious public health threats in Tanzania.

**Key words:** antibiotic prescriptions, antibiotic sources, education, management systems

## 3.3 Introduction

Domestic animals are found everywhere in poor communities across the developing world, and high rates are even found in the most densely populated, low-income urban areas, where farm animals are in close contact with people. The poor livestock keepers often stock a mix of different species, trading off specialization for better protection

against risks (Randolph et al 2007). Regardless of the important role on the economy and social life, livestock expose the animal keepers, the environment and consumers to agents of zoonotic infections and food-borne diseases.

Infectious zoonotic agents, as well as non-zoonotic diseases that are affecting livestock, are commonly treated with antibiotics (WHO 2007). All antibiotics used in veterinary medicine are the same or closely related to antibiotics used in human medicine, or they may induce cross-resistance (Ungemach et al 2006). The veterinary use of antibiotics, including the use growth promoters in livestock, is therefore thought to be a risk factor for the emergence of antibiotic resistance in human pathogens (Lathers 2002), and the use of antibiotics in livestock is considered as one of the reasons for increased occurrence of antibiotic resistant strains of bacteria in both animals and human beings (Callie et al 2012). Antibiotic resistant bacteria can be transferred from animals to humans and vice versa, and food-borne transmission through food of animal origin is a recognized risk. For example, a study conducted in the Washington DC found 20 % of the sampled meat was contaminated with *Salmonella spp* and 84 % of those bacteria were resistant to antibiotics used in human medicine and animal agriculture (Swartz 2002). Workers in the livestock industry may pick up resistant bacteria by handling animals, feed, and manure, and they can then transfer the bacteria to family and community members (Van den Bogaard et al 2002). Manure that contains resistant bacteria creates an immense pool of resistance genes available for transfer to bacteria that cause human and animal diseases. Farm wastes are spread on agricultural fields as fertilizers, and waste run-off can enter rivers, lakes, and ground water (Osterberg and Wallinga 2004).

Antibiotic abuse is prevalent in most developing countries; however, the extent to which it contributes to antibiotic resistance has not been quantified. According to Komolafe (2003)

and Carlos (2010) treatment and handling of infectious diseases in most developing countries is left in the hands of undertrained physicians, drug sellers, livestock keepers, owners of input shops and untrained personnel. However, there is no published work on the awareness of livestock keepers in developing countries about antibiotic resistance as a problem and their role in controlling this problem. The aim of this study was to find out how livestock keepers in Tanzania generally obtain antibiotics, what determines the choice of antibiotic, if they were aware of any potential health risks due to the use of antibiotics in their animals, and more generally to identify risk practices that could contribute to development of antimicrobial resistance.

## 3.4 Materials and Methods

### 3.4.1 Study area and livestock composition

The study was carried out in both Kinondoni (urban and peri urban) and Morogoro (urban, peri urban and rural) districts in Dar-es-Salaam and Morogoro regions respectively (Appendix 3). The study was conducted between July 2010 and January 2011. The sampling frame was district, wards, and finally a household with livestock. Random sampling was used to pick the districts and wards with large numbers of dairy and beef cattle, layers and broiler chickens, pigs and indigenous scavenging chickens. This type of sampling was very useful because livestock keepers were rearing livestock commercially, used a lot of antibiotics and all targeted livestock species were found in the study areas. In each district four wards were selected, hence, a total of 8 wards were surveyed in both districts. Within a ward, the list of livestock keepers was used as a sampling frame from which respondents were randomly picked using a Table of random numbers. The heads of the households (father and mother) were the main respondents; however, other members of the household attended the interview in the absence of heads of households. Where the

livestock project belonged to a group of people the group leader was interviewed on behalf of others.

### 3.4.2 Data collection

Data collection followed the approach described by Gonsalves et al (2005). Briefly, the work included individual interviews using semi-structured questionnaire and participant observation. Individual interviews of selected livestock keepers were conducted using questionnaires (Appendix 1) targeting household heads. Both closed and open-ended questions were included in the questionnaires. The information sought included household bio data characteristics (age, education, family position and employment), livestock (types of livestock, experience on livestock keeping, purpose of keeping livestock, duration of keeping livestock), herd size, types of antibiotics commonly used and stored, sources of antibiotics, involvement in antibiotic prescription, knowledge on the effects of using animal products treated with antibiotics to humans.

### 3.4.3 Data analysis

Data derived from questionnaires were recorded into the spreadsheets for statistical analysis. The data were analyzed using EPI INFO statistical software Release 3.5 (June 2008) and descriptive statistics such as: means, standard deviations, frequencies and percentages were generated.

## 3.5 Results

### 3.5.1 Respondents demographic information

Demographic information on the 160 respondents regarding sex, age, family role, education and occupation is summarized in Fig. 3.1. The majority of the livestock keepers (84%) with ordinary and primary school education levels were mainly dependent on the

income from livestock, while post secondary school (degree and diploma) commonly had other occupations.

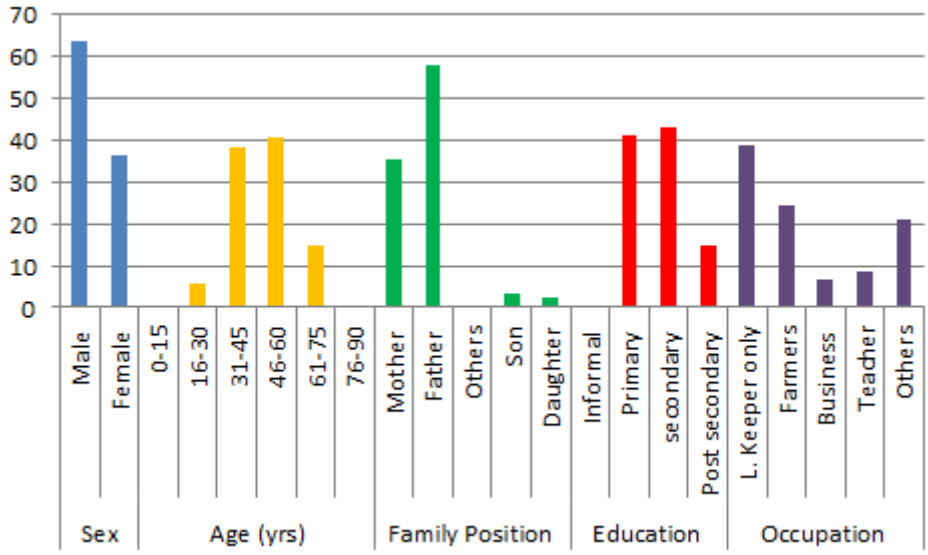


**Figure 3.1: Demographic information of respondents in the study area ("Y" axis is percentage)**

Respondents in the study area mentioned the types of livestock they were keeping. The majority of the livestock keepers were rearing chickens. Other species included pigs, dairy cattle, beef cattle, goats and rabbits. Some of the respondents had more than one species of animals in the same area with different management systems (Table 1).

**Table 3.1: Types of livestock reported by the respondents**

| Types of livestock | Number of respondents | % of respondents |
|---|---|---|
| Commercial (layers and/or broiler) chickens | 15 | 9 |
| Indigenous chickens | 25 | 15 |
| Commercial and indigenous chickens | 8 | 5 |
| Commercial and/or indigenousl chickens and cattle | 40 | 25 |
| Commercial and/or indigenous chicken and pigs | 19 | 12 |
| Commercial and/or lindigenous chicken and cattle and pigs | 16 | 10 |
| Commercial and/or indigenous chicken and other animals[1] | 6 | 4 |
| Dairy cattle | 9 | 6 |
| Beef cattle | 3 | 2 |
| Pigs | 14 | 9 |
| Others[2] | 5 | 3 |
| | **160** | **100** |

[1]. *Other animals include rabbits and goats.* [2]. *Others include respondents who reared more than four different types of animals*.

Management of animals in the studied area was divided into four systems namely indoor, free range, grazing and both grazing and indoor. Different types of livestock were associated with different management systems. Beef cattle had free range, grazing and indoor, dairy cattle had grazing, indoor and both indoor and grazing, Broiler, layers and also pigs had indoor systems. Indigenous chicken had free range. Both family and hired labor were used in the study area. The hired labor was sometimes shared (8.9 % of cases) by neighbors.

### 3.5.2 Use of antibiotics and antibiotics stored in the farms during the study

The most used and commonly reported antibiotics were tetracycline, sulphadimidines and penicillin-streptomycin (Table 2). The same drugs were found in the stores of individual livestock keepers. The information obtained from respondents and antibiotics found in stores confirmed that antibiotics were used in the study area for treating livestock.

**Table 3.2**: **Antibiotics used and stored in the study area**

| Type of antibiotic | Antibiotics in use stated by respondents[#] | | Number of farms where antibiotics were found in farm stores[b] | |
|---|---|---|---|---|
| | **Number of respondents** | **% of 160 respondents** | **Number of farms** | **% of 160 farms** |
| Tetracycline injectable | 118 | 61 | 38 | 70 |
| Tetracycline sprays | 4 | 13 | 6 | 12 |
| Sulphadimidine | 45 | 23 | 4 | 7 |
| Penicillin - Streptomycin | 26 | 2 | 6 | 11 |
| Gentamycin | 1 | 1 | 0 | 0 |
| | **194** | **100** | **54** | **100** |

*# Some respondents reported more than one antibiotic, for which reason the recording totals more than 160 respondents. b): not all farmers stored antibiotics, for which reason the total number of farms is below 160. In both columns percentage is relative to the total number of respondents (n=160)*

### 3.5.3 Knowledge on antibiotic resistance and rules for use of antibiotics in animals

Thirty percent (48) of respondents had never heard of antibiotic resistance in humans and animals (Table 3). Other respondents had heard the information from different sources including seminars and workshops (10), brochures and books (13), exhibitions (19) radio and TVs (26), Veterinarians (35) and livestock field officers (9). Thirty six of respondents (22.5%) had not heard of withdrawal periods after use of antibiotics and did not observe such a period.

**Table 3.3**: **Sources of information on antibiotic resistance**

| Source of information | Respondents | % Respondents |
|---|---|---|
| From Veterinarian | 35 | 22 |
| From field livestock officers | 9 | 6 |
| From TV and Radio | 26 | 16 |
| From Exhibitions | 19 | 12 |
| From brochures and books | 13 | 8 |
| From seminars and workshops | 10 | 6 |
| Had never heard of antibiotic resistance | 48 | 30 |
| **Total** | **160** | **100** |

### 3.5.4 Prescription (recommendation) of antibiotics and sources of antibiotics for use in livestock

Livestock keepers in the study area received services of prescription of antibiotics to treat their livestock from different sources (Table 3.4). The majority used veterinary services, others obtained the services from the input/agricultural shops that were involved in selling livestock antibiotics. Field officers within the study area also provided clinical veterinary services. Other livestock farmers used their own knowledge without consulting other experts. Few livestock keepers depended on their neighbors for the same service and the rest from drug sellers in the markets.

As can be seen from Table 3.4, most of livestock keepers purchased their antibiotics from agriculture and veterinary input shops and Veterinarians, but also livestock field officers, neighbors, livestock markets; mobile vet drug shops and exhibition areas.

**Table 3.4: Prescription (recommendation) and sources of antibiotics in the study area**

| Activity | Source | No of respondents | % of respondents |
|---|---|---|---|
| **Antibiotic prescription (recommendation)** | Drug sellers | 7 | 4 |
| | Field officer | 44 | 27 |
| | Livestock experts | 6 | 4 |
| | Fellow livestock keeper | 9 | 6 |
| | Neighbors | 1 | 1 |
| | Vet and agric. inputs shops | 7 | 4 |
| | Veterinarian | 86 | 54 |
| | | **160** | **100** |
| **Antibiotic sources** | Agric. & vet. inputs shops | 47 | 29 |
| | Veterinarians | 28 | 18 |
| | Livestock field officers | 15 | 9 |
| | Neighbors | 8 | 5 |
| | Livestock markets | 31 | 19 |
| | Veterinary drug shops | 19 | 12 |
| | Exhibition areas | 12 | 8 |
| | | **160** | **100** |

### 3.5.5 Actions taken by livestock keepers when antibiotics failed

The respondents were asked how they reacted to failure of antibiotic treatment. The responses that were given included change of medications without consulting anybody (25 %). They also used wood ash, kerosene and herbs (12.6 %) (*Aloe vera* and neem tree (*Azadirachta indica*)) to treat their animals. Others consulted farm and veterinary input shops (7.6 %), field officers (14.3 %), veterinarians (35.1 %), pharmacists (3.6 %) and neighbors (1.8 %).

### 3.5.6 Awareness about possible impact on human health from the use of antibiotics in livestock

When livestock keepers in the study area were asked if they knew any possible effects on human health from the use of antibiotics in animals, forty percent (64) of respondents said they knew nothing regarding any effect, while sixty percent (96) said that there were possible effects when animal products from animals treated with antibiotics were

consumed (Fig. 3.2). The most commonly mentioned effects were deaths to humans, body swelling, diarrhea, and drug residues in animal products, itching, poison to humans, resistance of bacteria to drugs, skin lashes, stomach problems, body swelling, cancer and flue.

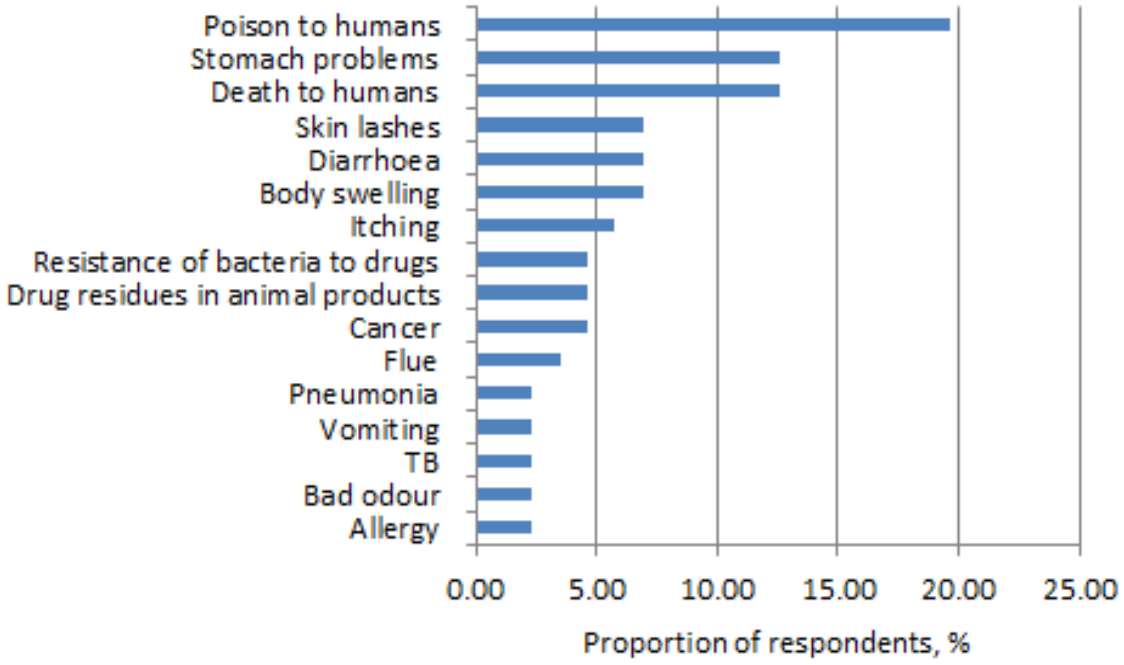


**Figure 3.2: Health effects on human after eating animal products from livestock treated with antibiotics – as mentioned by respondents**

### 3.6 Discussion

Livestock keepers in the study area used antibiotics to treat their livestock when they felt sick, and in addition, those who were keeping layers and broilers also used antibiotics for prophylaxis. Forty percent of livestock keepers were not aware of possible human health threats caused by the use of antibiotics in livestock, few had heard what antibiotic resistance meant, and few observed a withdraw periods after using the antibiotics.

Together, these Results show that a selective pressure for antibiotic resistant bacteria was present, but that the majority of livestock keepers were not aware of the health threats that can emanate from their use of antimicrobials, nor that their livestock management systems and handling of antibiotics is the most important risk factors for the development of antimicrobials resistance in livestock in Tanzania.

The Results confirm previous reporting from a rural district in China on lack of knowledge on prudent use of antibiotics and antibiotic resistance in developing countries (Chenggang *et al.*, 2011). This could be due to low level of education of the respondents as majority of them had primary and secondary school education. A socio-demographic analysis conducted by SPECIAL EUROBAROMETER 338 in 2010 within European countries (European Commission 2010) reveals that women seem to be better informed than men on this topic and age also plays a role and as regards to knowledge of antibiotics. Respondents who have gone on to higher education are also more likely to have a better knowledge of the effects of antibiotics. Another factor could be the lack of formal livestock keeper education and good advisory services in most developing countries. In the developed countries, formal education and good communication measures through advisory services have been used by health professionals to communicate this issue of antibiotic resistant to lay people that are lacking biomedical knowledge (Chenggang *et al.*, 2011).

The most important factors that may contribute to antibiotic resistance problems in developing countries, such as Tanzania, have been proposed to include 1) lack of access to appropriate antimicrobial therapy, 2) lack of regulation in use of antibiotics for human and animal, 3) lack of surveillance of antibiotic use and resistance levels, 4) lack of updated antibiotic use and treatment guidelines, and 5) lack of continuing medical education on antibiotic use for prescribers (Mtenga et al 2011). Based on the current study, lack of basic

knowledge on the concept of antibiotic resistance among livestock keepers should be added to that list.

WHO (2001) has defined prudent (or optimal) use of antibiotics in food animal production as the cost-effective use of antimicrobials which maximizes clinical therapeutic effect, minimizes drug-related toxicity and minimizes the development of antimicrobial resistance. In order to have proper use of antibiotics, prescription, delivery and record keeping of antibiotics used in livestock should be under the care of the prescribing veterinarian, and all therapeutic antibiotics should be supplied by, or with a prescription from a veterinary surgeon. The present findings show that only half of the livestock keepers in the study area obtained antibiotics for animal treatments with veterinary prescription. The remaining antibiotics were supplied in the shops without any prescription and were dispensed by untrained personnel in the shops. Furthermore livestock keepers without consulting veterinarians changed the antibiotics once they thought that the former ones used were not effective; over and above they also used indigenous herbs.

Komolafe (2003) and Carlos (2010) found that antibiotic abuse is one or perhaps the most important cause of the high prevalence of resistance among bacteria. This includes the use of wrong antibiotics, wrong doses, or the use of antibiotics for diseases that cannot be treated with antibiotics. Findings by Karimuribo et al (2005) stressed that this may be a particularly relevant problem in the least developed countries, since animal health services have been sub optimal with an increased tendency for animal owners to stock drugs in their houses and engaging unskilled people such as farmers themselves and animal attendants to treat the animals. In another study Mmbando (2004) found a high degree of drugs abuse by livestock keepers through failure in observing the recommended

therapeutic doses, use of wrong routes of administration, arbitrary drug combinations and non observance of withdraw periods, all known causes of developing and promoting spread of resistance to antibiotics (Iruka and Ojo 2010). This study supports these observation, and it has shown that there is a need for better information to uneducated livestock keepers on how, when and why to use antibiotics. Further, regulation on antibiotic use and prescription needs to be improved. Recommendations for policymakers should stress those antimicrobial agents should not be used in agriculture in the absence of disease. Use of antimicrobials for economic purposes such as growth promotion or feed efficiency should be discouraged. Antimicrobials should be administered to animals only when prescribed by a veterinarian and improved surveillance and national regulation is needed in both human medicine and food animal production to ensure that antibiotics are used prudently.

Although the extent of antibiotic use in animals in developing countries is unknown, one study from Kenya reported that tetracyclines, sulfonamides, and aminoglycosides were the most commonly used antimicrobials for veterinary purposes. Over 90 percent of the antibiotics used were for therapeutic purposes. This study has shown that tetracyclines, sulphadimidines and penicillin-streptomycin were the most commonly used antibiotics in the study area. The same drugs were found in the stores of individual livestock keepers for the first time in Tanzania confirming the validity of the responses given by the livestock keepers. Frequent use of same antibiotics for long time has been shown elsewhere to cause selective pressure to bacteria resistant to those specific antibiotics (Lathers 2002; Serrano 2005; Carlos 2010).

## 3.7 Conclusions

- Misuse of antibiotics in livestock in a developing country such as Tanzania is high, and the current study shows that this may in part simply be due to lack of knowledge.
- Many of the livestock keepers were not aware of the concept of antimicrobial resistance and did not know that use of antibiotics can have effects on health of their animals and their own health.
- Many factors wereidentified, which could contribute to development of antibiotic resistance in the study area, not least that antibiotics are prescribed, sold and used by unskilled personnel.

## 3.8 Acknowledgements

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# CHAPTER FOUR

## 4.0 ISOLATION AND CHARACTERIZATION OF *Staphylococcus aureus* and *S. pseudintermedius* AND THEIR ANTIMICROBIAL RESISTANCE FROM NASAL SWABS OF APPARENTLY HEALTH HUMANS PIGS AND DOGS IN TANZANIA

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Target Journal: *Journal of Infection in Developing Countries*

### 4.1 Abstract

### 4.1.1 Introduction

*Staphylococcus aureus* and *S. pseudintermedius* are opportunistic pathogens that cause a wide range of infections in humans and animals. Zoonotic transmission and antimicrobial

resistance are frequently reported and represent a health and economical concern in countries worldwide. The aim of this study was to investigate prevalence of nasal carriage, population structure and antimicrobial resistance of *S. aureus* and *S. pseudintermedius* from humans, pigs and dogs in Tanzania and to investigate the presence of methicillin resistant Staphylococci (MRS).

### 4.1.2 Methodology

Nasal swabs were taken from 100 healthy humans, 100 pigs and 100 dogs, and examined for growth on non-selective media. Presumptive *S. aureus* and *S. pseudintermedius* isolates were confirmed by *nuc* PCR and spa-typed. Isolates of *S. pseudintermedius* were further MLST typed. Antimicrobial susceptibility patterns were determined by disc diffusion. Staphylococci colonies were characterized on MRSA Brilliance agar. All colonies showing blue growth on this media, whether positive with the *nuc* PCR or not were tested for the presence of *mecA* and *mecC* by PCR.

### 4.1.3 Results

*S. aureus* was isolated from 22 % of humans, 4 % of pigs and 11 % of dogs. *S. pseudintermedius* was only isolated from dogs and only from 4 out of 100 of these. A total of 21 *spa* types of *S. aurues* were identified: 13 among human, 7 among dogs and one among pig isolates. Three *spa* types (t314, t223 and t084) were isolated from both humans and dogs. nuc PCR and MLST typing for *S. pseudintermedius* were carried in all samples and only 4 samples from dogs were positive to PCR and positive to four housekeeping genes used. Disc diffusion test revealed resistance to sulphamethoxazole/trimethoprim (3/37; 8.1%), rifampicin (2/37; 5.4%), amoxycillin-clavulanic acid (1/37; 2.8%), oxacillin (4/37; 10.8%), chloramphenicol (3/37; 8.1%), gentamicin (3/37; 8.1%) and penicillin

(37/37; 100%). The isolates of *S. pseudintermedius* were all resistant to penicillin and ampicillin. Four isolates that showed blue growth on Brilliance agar were negative by PCR for *mecA* but positive for *mecC*. These all belonged to the species *S. haemolyticus*. This is the first report on strains with this gene in Tanzania and of the presence of this gene in this species.

### 4.1.4 Conclusion

The *S. aureus spa* types circulating in the community in Tanzania were generally not shared by the different hosts. *MecC-encoded* MRS of species *S. heamolyticus* were detected in pigs, dogs and humans. Moderate levels of antimicrobial resistance were observed irrespectively of the host species from which the strains were isolated.

**Key words:** Antimicrobials; *S. aureus;* MRSA; humans; pigs; dogs; Tanzania

## 4.2 Introduction

*Staphylococcus aureus* is an opportunistic pathogen often carried asymptomatically by humans and animals (Lee, 2003; Shittu ***et al.*, 2011; Espinosa-Gongora, 2012**). Approximately 25-30 % of healthy humans carry *S. aureus* in their nasal cavity and nasal carriage is a recognized risk factor for *S. aureus* infection (Wertheim *et al*., 2005; Gorwitz *et al*., 2008). Other hosts of *S. aureus* include pigs, where a similar prevalence of carriage has been reported (Oppliger, 2012). *S. aureus* can also be found in dogs and cats; however, in dogs a related coagulase-positive staphylococcal species, S. *pseudintermedius*, is more prevalent than *S. aureus* (Paul *et al*., 2011). According to Boost *et al.* (2008), dogs could be a possible source of *S. aureus* infection to humans, and it was demonstrated by Rubin and Chino-Trejo (2011) that genetically related *S. aureus* strains can be shared between human and dogs from the same geographical area (Rubin *et al*., 2011).

Methicillin-resistant *S. aureus* (MRSA) includes those strains that have acquired a gene giving them resistance to methicillin and essentially all other beta-lactam antibiotics (Weese *et al*., 2005; Espinosa-Gongora, 2012). Livestock associated MRSA (LA-MRSA) have been reported worldwide in pigs. LA-MRSA with sequence type (ST) 398 is found most frequently in Europe (Huber *et al*., 2010; Espinosa-Gongora *et al*., 2012), whereas ST9 is the prevalent clone in South East Asia (Guardabassi *et al*., 2009; Wagenaar *et al*., 2009). *mecA* and *mecC* genes encode methicillin-resistant in *S. aureus* (MRSA) and other Staphylococci. *MecC-MRSA* poses a particular public health concern as it cannot be identified by the use of routine MRSA identification methods. PCR for amplification of *mecA* and SCC*mec* require different primers due to nucleotide divergence, and the penicillin-binding protein encoded by *mecC* is not recognized by the latex-agglutination test and shows a higher affinity for oxacillin than for cefoxitin (Kim *et al*., 2012). Initial discovery of *mecC*-MRSA in the United Kingdom revealed that isolates from cattle and humans in the same geographic area were genetically highly associated, indicating the possibility of transmission between the two (Garcia-Alvarez *et al*., 2011). MRSA lineages carrying *mecC* have been described in livestock and humans in Denmark (Harrison *et al*., 2013), Ireland (Shore *et al*., 2011), UK (Paterson *et al*., 2013), Germany (Cuny *et al*., 2011) and Sweden (Unnerstad ***et al*., 2013)**. ST130 MRSA isolates with the *mecC* gene have spread from cows to humans resulting in clinical infections (Harrison *et al*., 2013). Livestock can act as a reservoir for the emergence of new human bacterial pathogens in the agricultural setting (Spoor *et al*., 2013, EFSA & ECDC, 2013). It is currently not known whether back yard management of pigs, as performed in some parts of Tanzania, poses a potential danger for spread of LA-MRSA to livestock keepers. Zoonotic transmission of MRSA has been previously shown between humans and animals including

pet animals (Moodley *et al*., 2006); livestock (Voss *et al*., 2005) and horses (van Duijkeren *et al*., 2011).

In Tanzania there is a general lack of information on the lineages of *S. aureus* that are present in human and animal populations, as well as on their patterns of antimicrobial susceptibility. This study therefore aimed to investigate occurrence of *S. aureu*, *S. pseudintermedius* and MRS colonizing healthy humans, pigs and dogs in Tanzania and to characterize population structure and antimicrobial resistance of human and veterinary isolates from this country.

## 4.3 Materials and Methods

### 4.3.1 Ethical clearance and human subjects' consent

All human participants were informed of the objective and methods of the study and voluntarily signed an informed consent form (Appendix 2). The National Institute for Medical Research of Tanzania (NIMR) approved the study (ethical clearance certificate No 936 dated 01/4/2010 (NIMR/HQ/R.8a/Vol.IX/936)). The Ethical Committees of Sokoine University of Agriculture (SUA) approved the animal study.

### 4.3.2 Sample collection

Nasal swabs were collected from 100 epidemiologically unrelated healthy humans, 100 pigs and 100 epidemiologically unrelated dogs from urban and peri-urban Morogoro Municipal, Tanzania, from December 2011through March 2012. The pigs were sampled at different pens and houses within eight rearing places representative of the study area. A single sterile cotton swab was inserted 1- 2 cm into both nostrils and rotated along the nasal mucosa for 5 -10 s. Swabs were rubbed well by rotating 5 times over the inner wall of the ala and nasal septum. Swabs were stored in sterile tubes containing Cary Blair (Oxoid, UK) transport medium and were transported in a cool box to the laboratory.

### 4.3.3 Isolation, identification and typing of S. aureus

Swabs were enriched in Mueller Hinton Broth (Oxoid, UK) containing 6.5% NaCl for 24 h at 37°C, 10µl of the enrichment culture were streaked on Oxacillin Resistance Screening Agar Base (ORSAB) (Oxoid, UK) without selective supplement. Blue colored colonies were harvested and stored at -80°C in Muller-Hinton Broth (Oxoid, UK) with 50% glycerol. Subsequently isolates were streaked on blood agar and presumptive Staphylococci (appendix 5) were tested for coagulase activity in horse plasma. Coagulase-positive isolates (Appendix 6) were screened by *nuc* PCR to confirm *S. aureus* and *S. pseudintermedius* (Sasaki *et al*., 2010). Confirmed isolates as well as non-coagulase colonies were plated onto MRSA Brilliance agar (Oxoid, UK) and isolates showing growth on this medium were tested by PCR for the presence of *mecA* according to Oliveira and de Lancaster **(**2002) and for *mecC* according to Stegger *et al*. (2012). Positive control strains 68529-2 and 11-2 (Eriksson *et al*., 2013) were included and negative vials without DNA were likewise included as controls. Strains that were shown to be MRS and which were not positive by the *nuc* PCR were further characterized by matrix-assisted laser desorption-ionization (MALDI) time-of-flight mass spectrometry (TOF/MS (Jackson and Lay, 2001) for species identification.

All *S. aureus* isolates were *spa*-typed according to (Harmsen *et al*., 2003) and *S. pseudintermedius* according to (Perreten *et al*., 2010). Sequence and cluster analysis were performed using the Ridom StaphType software (Ridom GmbH, Würzburg, Germany) as previously described (Harmsen *et al*., 2003). The *spa* server database (http://spa.ridom.de/spatypes.shtml, last accessed on 13/02/2013) was consulted to extrapolate the *spa* types to previously reported STs or clonal complexes (CC). Alternatively, presumed STs and CCs were assigned based on associations with *spa* types

reported in the scientific literature. Multi-locus sequence typing (MLST) for *S. pseudintermedius* was performed according to Bannoehr *et al*. (2007). House genes used for MLST were 16sRNA, tuf, pta, agrD and cpn60. Allele numbers were determined by comparison with those allele sequences deposited in Genbank. Sequence types (STs) were determined using the key table kindly provided by the curator of the *S. pseudintermedius* MLST database, Vincent Perreten (vincent.perreten@vbi.unibe.ch). Associations between spa types and STs in *S. pseudintermedius* were determined according to Perreten *et al*. (2010).

### 4.3.5 Antimicrobial resistance testing

Antimicrobial susceptibility testing was performed by disc diffusion according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2008). The test was performed on Muller Hinton agar (Oxoid, Uk) using sulphamethoxazole/ trimethoprim (STX 25), ampicillin (AMP 10), rifampicin (RD 5), amoxycillin-clavulanic acid (AMC 30), oxacillin (OX I), chloramphenicol (C 30), gentamicin (CN 10) and penicillin (P 1) (Oxoid, UK). For quality control, *S. aureus* ATCC 29213 and ATCC 25923 were used in the study.

## 4.4 Results

### 4.4.1 S. aureus and S. pseudintermedius carriage

*S. aureus* was isolated from 22/100 humans, 4/100 pigs and 11/100 dogs. Four dogs were shown to carry *S. pseudintermedius* and this bacterium was not obtained from any other species. Four colonies showed blue growth on Brilliance agar. None of the isolates was shown to carry *mecA* genes, while *mecC* genes were detected in one isolate from dog, one from human (1/100) and two from pigs (2/100) (Fig. 4.1). The two isolates from pigs originated from different farms. These four isolates were demonstrated to belong to the species *S. haemolyticus* by Maldi/Toff.

### 4.4.2 Spa-typing

The overall Results of *spa*-typing of *S. aureus* are summarized in Table 1. Human isolates had 13 distinct *spa* types, including a new *spa* type (t10779). The most frequently found types were t714 (n=4), t148 (n=4), t084 (n=3), t311 (n=2) and t223 (n=2). Dog isolates showed seven distinct lineages with t127 (n=4) being the most frequent type. All four *S. aureus* isolates from pigs belonged to t131. Human and dog isolates shared three *spa* types: t314 (ST121), t084 (ST15 and ST18) and t223 (ST22). *S. pseudintermedius* were not assigned to *spa* or the ST type, except for MLST (Fig. 4.2).

### 4.4.3 Antimicrobial resistance

Results of antimicrobial susceptibility testing of *S. aureus* are shown in Table 4.2. All isolates were resistant to ampicillin and penicillin, while resistance to other agents was more sporadic. Most isolates (75.7 %) were resistant to two antimicrobials, and one pig isolate was resistant to six antimicrobials (Table 4.3). Resistance generally appeared higher in strain from humans than from other sources, but due to the relatively low number of isolates, no statistical analysis of this was attempted. The four strains of *S. pseudintermedius* were all resistant to penicillin. Besides that one isolate (D89) was resistant to ampicillin.

## 4.5 Discussion

The present study provides novel information about carriage frequency and diversity of *S. aureus* and *S. pseudintermedius* in healthy humans, dogs and pigs outside hospital and clinical settings in Tanzania. The observed isolation frequency of *S. aureus* from humans (22 %) was within the range shown in other studies, where 10-35 % has been found to be persistently colonized, 20-70 % intermittently colonized and a further 5-50 % never colonized (Hamdan-Partida *et al.*, 2010). Since this study was designed as a cross-sectional study, we were not able to distinguish between permanent and intermittent carriers.

In African countries very little is reported on the *S. aureus* prevalence and population structure with only a few studies being carried out. *S. aureus* colonization rate of 57.5% has been reported among food handlers in Gaborone (Truong *et al*., 2011). Recent studies have identified *S. aureus* as the main etiological agent and have reported *S. aureus* as the most frequently encountered bacterial species in microbiology laboratories in Nigeria (Shittu ***et al*., 2011).**

The prevalence of *S. aureus* carriage in dogs (11%) was in agreement with previous studies reporting isolation frequencies between 9-12 % in this animal reservoir (Griffeth *et al*., 2008) though Boost *et al*. (2008) reported a lower prevalence of 8.8% in the same species. Rubin *et al*. (2011) reported *S. aureus* carriers of 5.9%, 17.6% and 17.6% in pharyngeal, rectally and nasally colonized respectively. This is valuable information to understand the epidemiology of *S. aureus* in this animal species, since most previous studies in dogs focused on MRSA (Moodley *et al*., 2006; Griffeth *et al*., 2008) or *S. pseudintermedius* (Rubin *et al*., 2011). Rubin (2011) found multiple hot spots for colonization (nares, pharynx and rectum) of both *S. aureus* and *S. pseudintermedius*

carriage in dogs. The sensitivity of sampling increased when multiple sample sites were used (Paul *et al.*, 2011; Rubin 2011), and our estimate of carriage is therefore probably below the true prevalence of these bacteria in dogs in Tanzania. The frequency of *S. pseudintermedius* colonization shown in dogs is below the expected (Rubin *et al*., 2011). At present, the reason for this is unknown and deserves further attention.

*S. aureus* was isolated from 3 out of 8 pig farms and only 4 pigs were carriers. This prevalence may be regarded as low considering the available data on the occurrence of MRSA in intensive pig farming in Europe and South East Asia (Leonard and Markey, 2008; Oppliger *et al*., 2012). However, this difference may reflect that the semi-outdoor production system used by smallholder pig farmers in Tanzania creates fewer opportunities for *S. aureus* to colonize and or persist on the animal and the environment. Epidemiology studies on MRSA in intensive pig farming have shown that once *S. aureus* is present in the farm, it colonizes a large proportion of the pigs (Broens *et al*., 2012). This was not shown in our study where the low in-farm prevalence (0-3%) suggests low transmission rates. A longitudinal study elsewhere showed the highest transmission rates in young pigs (Broens *et al*., 2012). Our sample was withdrawn from pigs of approximately 4-6 months of age, and this may have contributed to the apparent low prevalence. The back yard pig management system may also contribute to the low prevalence of the bacterium. Producers who use pasture (extensive system) furrowing have lower health expenses than those using confinement systems, where close proximity encourages disease transmission (Appleby, 1996). A study by Temple *et al*. (2012) in France and Spain on different production systems, found Mollarcan Black pigs and Iberian pigs reared in extensive system to have low prevalence of infections, severe wounds and tail bites compared to conventionally housed pigs.

To the best of our knowledge, *spa* type t131has not been reported in pigs in Tanzania prior to this study where it was found on two farms. This *spa* type has been associated with ST80, an important community-acquired MRSA lineage in humans (Deurenberg *et al*., 2007; Witte, 2009), and has previously been found in cats and dogs and rarely in humans as MRSA (Sing *et al*., 2008). It has been suggested that cats might be a possible reservoir of this MRSA lineage (EFSA, 2009). MRSA ST80 has been reported in Europe, North Africa (Witte, 2009), Asia, Europe and Middle-East (Deurenberg *et al*., 2007) and is associated to Staphylococcal Cassette Chromosome (SCC) *mec* type IV (Tristan ***et al*., 2007)**. Skin and soft tissue infections and severe necrotizing pneumonia have been described in humans in association with this MRSA lineage (David and Daum, 2010). Altogether these data suggest that this lineage could have broader host specificity than previously thought, even though the MRSA lineage is clearly associated with humans. Apart from t131 found in Tanzania in pigs other lineages that are commonly found in pigs include, ST398, ST1, ST29, ST97, ST1476 (CC97), ST433 and CC30 (Espinosa-Gongora, 2012). CMRSA2 (EMRSA3), which belongs to CC5, is also common among pigs in Canada (Loeffler and Lloyd, 2010).

The isolation of only one *spa* type in pigs in the current study indicates the circulation of one lineage among farms. However, they bought piglets from different sources and did not share veterinarians, feed supply or other common utensils. Our human sample collection only contained three pig farmers, and these were all negative for *S. aureus* (data not shown).

Three *spa* types were common to humans and dogs: t314 (ST121), t084 (ST15 and ST18) and t223 (ST22). Since none of our samples were paired samples from owners and their dogs, this observation indicates that people and dogs to some extend share a common

population of *S. aureus*, probably maintained by bidirectional transmission. *S. aureus* isolates from canines and humans in the same geographic area have previously been found to be genetically similar, indicating a common population and probable interspecies transmission (Rubin and Chirino-Trejo, 2011). Apart from one new *spa* type (t10779), all other *spa* types have previously been reported in other studies in Canada (Rubin *et al*., 2011) where t084 was also found in human and dogs as MSSA. In that study t311 and t002 were found in both humans and dogs while in our study t311 was found in dogs and t002 in humans. *spa* type t127 was found in dogs in our study while Rubin *et al*. (2011) reported it in humans, indicating that dogs and humans are sharing these lineages. Some clonal lineages of *S. aureus* have a tendency to colonize specific species, and may be adapted to either humans or animals (e.g. MRSA ST22-IV) (Cuny *et al*., 2010). The rest of *spa* types isolated in humans and dogs could be of regional specificity circulating in Tanzania (Loeffler and Lloyd, 2010).

Tanzanian isolates of *S. aureus* and *S. pseudintermedius* from humans, pigs and dogs were all resistant to penicillin and ampicillin, while resistance against aminoglycosides, rifampicin, sulphonamides and phenicols was infrequent or absent. The high level of resistance to penicillin and ampicillin reported to be widely spread in *S. aureus* in other parts of the world (Blomberg *et al*., 2007; Boost *et al*., 2008; Mshana *et al*., 2009; Rubin and Chirino-Trejo, 2011) is therefore also found among *S. aureus* strains in Tanzania. Resistance to gentamicin was reported in 41.5% of human isolates derived from hospital in Tanzania (Urassa *et al*., 1999), but this appears to be much less among human and animals in the community based on our Results.

The epidemiology of MRSA in Africa is poorly documented (Breurec *et al*., 2011) and its frequency in most African countries has not been reported (Kesah *et al*., 2003). No MRSA

were detected in the present study, however the sample size is insufficient to have high confidence in this estimate. However, compared to reporting from other studies performed in African countries with values of 21-30% in human isolates from hospital settings in Nigeria, Kenya, and Cameroon, below 10% in Tunisia (Breurec *et al*., 2011) and 16.3% in Tanzania (Mshana *et al*., 2009) (n=26), community levels of MRSA in Tanzania appears low.

The present study reports *mecC*-MRSA in Tanzania for the first time. The strains isolated from human, dog and pig isolates belonged to *S. haemolyticus* and this report is the first documentation of *mecC* in this species. In Tanzania and other Africa countries, food animals, pets and humans are sharing the same environment. Through this sharing, the transmission of pathogens and antibiotic-resistant strains from one group to another is possible (Rwego *et al*., 2008; Katakweba *et al*., 2012), and the presence of *mecC* in a closely related bacterium to *S. aureus* makes it likely that spread to this bacterium can happen..

## 4.6 Conclusion

Humans, pigs and dogs were found to carry *S. aureus* with frequencies that are within the range of reports from other countries, while MRSA was not demonstrated while *mecC* was found in 4 colonies of *S. haemolyticus.* Resistance to antimicrobials was more frequent in human isolates than in isolates from pigs and dogs. The population structure of *S. aureus* showed similar *spa* types in humans and dogs, suggesting possible transmission between these two host species. This is the first time that a typical community-acquired *spa* type t131 isolated from pigs has been previously reported as a cause of infection in veterinary and human medicine. It remains to be studied the possible role of pigs as a reservoir of this lineage in Tanzania.

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**4.8 Author Contributions**

Abdul Katakweba took part in the planning, performed sampling and typing, evaluated Results and drafted the manuscript. Carmen Espinosa-Gongora participated in the design of the laboratory analysis performed typing and approved the final manuscript. Luca Guardabassi took part in the planning and approved the final manuscript. Amandus Muhairwa took part in the planning and approved the final manuscript. Madundo Mtambo took part in the planning, evaluated the Results and approved the final manuscript. John Olsen planned the study, evaluated Results and took part in drafting of the manuscript.

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**Table 4.1: *spa* type, associated STs and *spa* cluster analysis of *S. aureus* isolated from healthy humans, dogs and pigs in Tanzania**

| ***spa* Type** | **Associated ST or CC*** | ***spa* cluster*** | **Human isolates** | **Dog isolates** | **Pig Isolates** | **Total number of isolates** |
|---|---|---|---|---|---|---|
| t084 | ST15, ST18 | | 3 | 1 | | 4 |
| t127 | ST1 | I | | 4 | | 4 |
| t131 | ST80 | | | | 4 | 4 |
| t714 | None | | 4 | | | 4 |
| t148 | ST72 | | 3 | | | 3 |
| t223 | ST22 | | 2 | 1 | | 3 |
| t314 | ST121 | | 1 | 1 | | 2 |
| t311 | ST5 | II | 2 | | | 2 |
| t002 | ST5, ST231 | II | 1 | | | 1 |
| t015 | ST45 | | 1 | | | 1 |
| t267 | CC97 | | | 1 | | 1 |
| t451 | ST8 | III | 1 | | | 1 |
| t508 | None | | | 1 | | 1 |
| t690 | None | | 1 | | | 1 |
| t1476 | None | III | | 1 | | 1 |
| t1849 | None | I | 1 | | | 1 |
| t2030 | None | | 1 | | | 1 |
| t10779 | New | | 1 | | | 1 |

* STs and Clusters were assigned based on known associations with *spa* types (Harmsen *et al*., 2003).

**Table 4.2: Antibiotic resistance in isolates of *S. aureus* from Tanzania**

| Source | Antibiotics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **STX** | **AMP** | **RD** | **AMC** | **OX** | **C** | **CN** | **P** |
| | **Number of isolates showing resistance** | | | | | | | |
| **Pigs (n=4)** | 0 | 4 | 1 | 0 | 2 | 1 | 1 | 4 |
| **Dogs (n=11)** | 0 | 11 | 0 | 0 | 1 | 0 | 0 | 12 |
| **Humans (n=22)** | | 22 | 1 | 1 | 1 | 2 | 2 | 21 |
| **Total (n=37)** | 3 (8.1%) | 36 (97.3%) | 2 (5.4%) | 1 (2.8%) | 4 (10.8%) | 3 (8.1%) | 3 (8.1%) | 37 (100%) |

sulphamethoxazole/ trimethoprim (STX ), ampicillin (AMP), rifampicin (RD), amoxycillin-clavulanic acid (AMC ), oxacillin (OX ), chloramphenicol (C ), gentamicin (CN ) and penicillin (P )

**Table 4. 3: Number of *S. aureus* isolates showing resistance to one or more antimicrobials**

| | **Number of antimicrobials** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Source** | **0** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| **Humans (n=22)** | 0 | | 16 | 3 | 1 | 2 | 0 | 0 | 0 |
| **Pigs (n=4)** | 0 | | 2 | 1 | 0 | 0 | 1 | 0 | 0 |
| **Dogs (n =11)** | 0 | 1 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total (n=37)** | 0 | 1 | 28 | 4 | 1 | 2 | 1 | 0 | 0 |

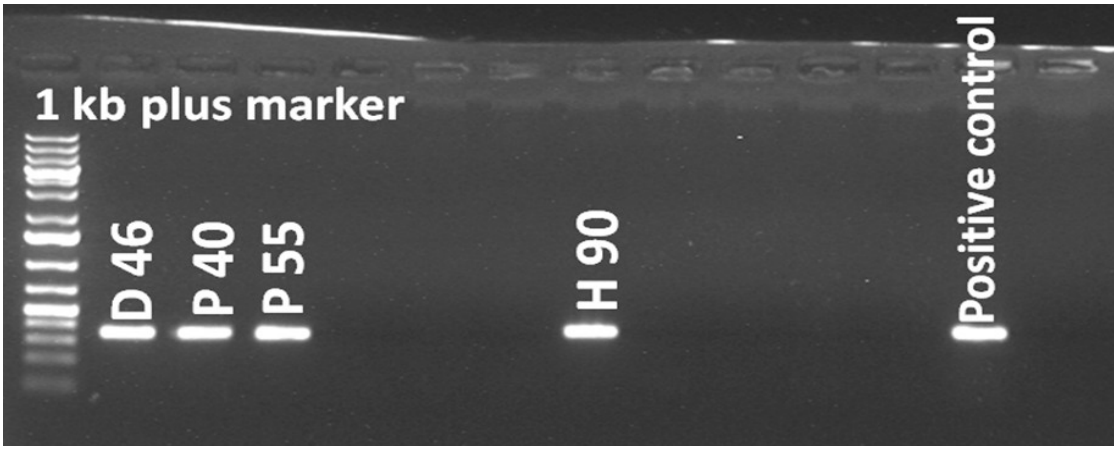


**Figure 4.1:** **Detection of *mecC* genes by PCR. Lane 1: D = Dog, P = Pig, H = Human Positive control: 86529 & 11-2**




**Figure 4.2**: **MLST of *S. pseundintermedius* with four housekeeping genes:pta, *tuf*, *agrD, cpn60***

# CHAPTER FIVE

## 5.0 ANTIMICROBIAL RESISTANCE IN INDICATOR BACTERIA FROM LIVESTOCK, POULTRY AND HUMANS IN TANZANIA (Manuscript No 3)

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## 5.1 Abstract

Five hundred and eighty seven faecal samples were collected from humans, pigs, beef, dairy cattle, exotic and indigenous chickens to investigate the relatedness of antibiotic resistant bacteria from humans and animals in Tanzania. The aim was to quantify the public health risks posed by handling animals, their waste and using animal products. Four hundred eighty five *E.coli* isolates were obtained from faecal samples grown on MacConkey agar plates. Tetracyline (TE), Sulphamethoxazole/ trimethoprim (STX), ampicillin (AMP) and cefataxime (CTX) were included in the media to test the resistance on *E. coli* isolates. Higher resistance was observed to TE (385, 79.38%), STX (374, 77.11 %) and AMP (362, 74.64%). Less resistance was observed to CTX (194, 40%). Samples from humans had the highest average number of coliforms resistant towards AMP ($\log_{10}$ CFU 3.9) and CTX ($\log_{10}$ CFU 2.2), while samples from chickens showed the highest resistance towards STX ($\log_{10}$ CFU 3.8) and TE ($\log_{10}$ CFU 3.83). Antibiotic discs were also used to determine the resistance from 350 *E. coli* isolates obtained from plates without antibiotics. Higher resistance was observed to TE (241, 68.8%), STX (214, 61.1%), S (195, 55.7%) and AMP (192, 54.8%). Resistance to CTX was observed in 143 isolates (40.9%) while CIP 30.9 % (108). Less resistance was observed to AMC (48, 13.7%) and CN (37, 10.6%). Apart from *E. coli*; 550 isolates of *Enterococci spp* were also speciated and tested for resistance. Out of 164 enterococci isolates speciated; 151 (92.07%) were *E. faecium*, 5 (3.05%) *E. faecalis* and 8 (4.88%) *E. hirae*. Growth of resistant indicator bacteria colonies was carried out in AMP and VA. Higher resistance was observed in AMP (85, 15.46%) compared to VA (30, 5.45%). Average Log $_{10}$ CFU of AMP resistant Enterococci were highest in humans (0.9) followed by dairy cattle (0.9) while the highest number in VAN resistant Enterococci surprisingly was recorded in exotic chickens (0.4) and less surprisingly in humans (0.39). For 240 *Enterococci spp* obtained from plates without antibiotics were tested with antibiotic discs, higher resistance was observed to RD (174, 75.2%), TE (33, 13.8%) and E (36, 15%). There was no resistance to CN (120µg) and C (5, 2.1 %). Other antibiotics tested for this group included

CIP (9, 3.8%), AMP (12, 5%) and VA (12, 5%). *sulII* and *tetW* resistance genes were found in all livestock and humans samples (*n*=22 per species) with degree of variations when calculating the proportion of resistance genes in relation to number of bacteria estimated from "total" 16s RNA. Gene copy numbers/gram sample were determined and related to the number of 16s rRNA gene-copies. Samples from cattle showed the highest numbers of *sulII* gene-copies in ($Log_{10}$ 5.7) while the lowest were observed in pigs ($Log_{10}$ 5.4) and there was no significant different between sample sources. For *tetW* genes, there was a significant different in sample sources and cattle had higher gene copy number ($Log_{10}$ 7.4) while lowest number was observed in humans ($Log_{10}$ 6.4). For all samples percentage of *tetW* to the total of 16s genes were 0.06 % Humans, 11.88% Cattle, 2.4% Pigs, 2.15% local chickens and 0.98% exotic chickens; whereas *sulII* represented; 0.22% Humans, 0.34 % Cattle, 0.2% Pigs, 0.3% local chickens and 0.02% exotic chickens. All isolates (n=194) that had shown resistance to CTX on MacConkey agar (59 of which were none-lactose fermenters) revealed a band of the same size as that of a positive CTX-M control strain in all isolates analyzed, indicating that they all contained an ESBL gene of this class.

Antibiotics that are commonly used in Tanzania had higher resistance as estimated from both CFU of resistant bacteria and resistance profiles determined by use of antibiotic discs. Resistance genes were also demonstrated to commonly used antibiotics (TE, STX) in the country. These findings indicate that prolonged use of these antibiotics has cause indicator bacteria to developed resistance against them and resistance genes observed from the same antibiotics suggest that they circulate between animals, chickens and humans sharing the same environment.

**Keywords**: Human, food animals, chickens, antibiotics, antibiotic resistance, resistance genes, bacteria, Tanzania

## 5.2 Introduction

Antimicrobioal resistance (AR) is a threat to treatment of bacterial diseases all over the world and dramatically increases health costs due to hospitlization, expensive drug choices and lost lives (WHO, 2012). In sub-Saharan Africa, lack of effective monitoring and enforcement on the control of the sale and use of antibiotics has led to widespread misuse of antibiotics both in human and veterinary medicine, and developing countries are increasingly a source of new clones of resistant bacteria (Byarugaba, 2004; Carlos, 2010). In the combat of AR, information on levels of resistance and spatial changes in resistance patters in bacteria from humans as well as food animal reservoirs forms the fundament for risk management. However, there is generally a shortage of scientific data to demonstrate trends of AR in animal or human populations in developing countries, and there is an urgent need for systematic surveillance to compensate for this lack of information.

Due to permanent habitation in the gut, *Escherichia coli* and *Enterococci spp* are commonly used as indicator bacteria to gauge the extent of AR in a population (Jordi *et al*., 1999; Bager, 2000). The importance of these bacteria is their ability to acquire and disseminate resistances that could be transmitted to pathogenic or zoonotic bacteria (Kim *et al*., 2001), and there is good evidence of strong correlation between resistance levels in these indicator bacteria and the general selection pressure in the habitat from which they were isolated (Aarestrup *et al*., 1999; Byarugaba *et al*., 2011). The potential of these organisms as indicators of AR has been widely demonstrated in developed countries, and surveillance is routinely based on the use of these bacteria (EFSA, 2009; EFSA, 2013).

Tanzania has a large livestock industry, and most production systems are extensive characterized by a low capital input and a low production level. Misuse of antibiotics, mainly due to lack of knowledge on health risks and a badly controlled prescription system is widespread (Katakweba *et al.*, 2012). No study has so far has been carried out in

Tanzania trying to establish a baseline of resistance levels in humans as well as in important food animals as to quantify the general level of AR and the public health risks humans can encounter by sharing the same environment with their animals, handling animals and animal wastes and using animal products. Therefore, the aim of this study was to estimate the extent of AR in a random collection of faecal samples from humans and the most important food animals.

## 5.3 Materials and Methods

### 5.3.1 Ethical clearance and human subject's consent

All human candidates were informed of the experiment and voluntary accepted the sampling and were provided and signed informed consent forms (Appendix 2). The National Institute for Medical Research of Tanzania (NIMR) approved the part of human study (certificate NIMR/HQ/R.8a/Vol.IX/936). The Ethical Committees of Sokoine University of Agriculture (SUA) approved the other part of animal studies.

### 5.3.2 Sampling

Freshly voided faecal materials (3-5 g) were collected in 2011/12 from one animal in randomly appointed pig farms, dairy cattle farms, beef cattle farms, commercial chicken farms and indigenous chickens in randomly appointed households. In addition, five to ten grams of morning faecal samples were collected from epidemiologically independent human beings. After collection, samples were stored in sterile containers, transported to laboratory and were stored at 4 °C before processing for bacteriology.

### 5.3.3 Enumeration of antibiotic resistant bacteria

One gram of sample was suspended in 9 ml of phosphate buffer saline (PBS) and tenfold dilution was made in PBS. 100 µl of appropriate suspensions were spread on agar plates

with and without antibiotics. Suspensions for isolation of *E. coli* were spread on MacConkey agar (Oxoid, Basingstoke, UK) and suspensions for isolation of *Enterococci spp* on Slanetz-Bartley agar (Oxoid, Basingstoke, Hants, UK). Antimicrobial used in MacConkey plates were Cefatexime (2mg/L), Tetracycline (16mg/L), Ampicillin (16mg/L) and Sulphamethoxazole (256mg/L) (appendix 7) while antibiotics used to enumerate resistant *Enterococci spp* included Ampicillin (16mg/L) and Vancomycin (16mg/L) (appendix 7). MacConkey plates were incubated for one and Slanetz-Bartley plates for two days at 37 °C. One to two colonies with typical colony morphology from each plate were randomly picked and sub-cultured in order to get pure cultures. These cultures were stored at -80 °C.

### 5.3.4 Bacterial species identification

Presumptive *E. coli* colonies (appendix 8) were identified to species level by Gram stain (appendix 9) and the IMVC tests (indole (appendix 10), methyl red, Voges Proskauer and citrate (Appendix 11 ) according to standard procedures (CSLI, 2008). Typical colonies of *Enterococcis* (appendix 12, Gram stain appendix13) were subjected to multiplexes PCR according to Dutka-Malen *et al.* (1995) and Ke *et al.* (1999). Isolates that showed atypical reaction in tests above were characterized by matrix-assisted laser desorption-ionization (MALDI) time-of-flight mass spectrometry (TOF/MS) (Jackson and Lay, 2001).

### 5.3.5 Antibiotic sensitivity testing

Antibiotic susceptibility was determined by Discs diffusion method according to CLSI guidelines (CSLI, 2008) using commercially available discs (Oxoid, Basingstoke, Hants, UK; appendix 14). For *E. coli* susceptibility was determined towards STX (25 µg), AMP (10 µg), AMC (30 µg), CN (10 µg), CIP (5 µg), TE (30 µg), S (10 µg) and CTX (30 µg). For *Enterococci spp*, susceptibility was determined for VA (30 µg), E (15 µg), CIP (5 µg),

AMP (10 µg), C (30 µg), RD (5 µg), CN (120 µg) and TE (30 µg). *E. coli* ATCC25922 and *Enterococcus faecium* ATCC 2912 were included as control strains.

### 5.3.6 DNA isolation

One gram from each faecal sample was weighed and mixed thoroughly with 9 ml of Phosphate Buffer Saline (PBS). 200 µl were taken and used for DNA purification with the QIA amp DNA Stool Mini Kit (Cat 51504; © 2001 QIAGEN) basing on the instruction of the manufacturer. DNA was eluted with 200 µl elution buffer and stored at -20 °C until use.

### 5.3.7 Detection of $bla_{ctx\text{-}M}$ in putative ESBL producing bacteria

*E. coli* colonies as well as isolates of lactose negative colonies growing on McConkey plates with CTX were analyzed for synergy between CTX and CA according to Lewis II *et al*. (2007) and for the presence of $bla_{ctx\text{-}M}$ by PCR according to the methods of O'Keefe *et al.* (2010). SYBR green PCR master mix (Invitrogen, Australia), CTX-M primer1 1354 (5'-ATGTGCAGYACCAGTAARGTKATGGC-3') and CTX-M primer 2 1380 (5'-TGGGTRAARTARGTSACCAGAATSAGCGG-3') were used.

### 5.3.8 Quantitative-PCR

Quantitative PCR (qPCR) was performed using previously designed primers for the quantification of *sulII*, *tetW* and 16s rDNA . The qPCR reactions were performed using a Stratagene MX 3000p PCR cycler with 25 µl reactions (12.5 µl Maxima SYBR Green/ROX qPCR Master mix (Fermentas). Primers used are listed in Table1. The standard curves of the respective genes were used for calculation of the gene copy number per gram sample. The calculations were performed by using the linear trend line for

standard calculations to obtain the mean value of the content in the qPCR tube. The prediction interval of the sample content was calculated using the formulas developed by.

### 5.3.9 Statistical analysis

SAS version 9.1 (SAS, 2011) was used to analyze overall variation in CFU and number of genes by qPCR using ANOVA with Duncan's post-modification test. SAS, Statistical Package for Social Science software (SPSS) version 16.0® and Graphpad Prism version 6.0 were used for analysis of antibiotic discs data by chi-square and Fisher's exact test. Excel sheets were used to determine the prevalence rates.

## 5.4 Results

### 5.4.1 Speciation of isolates obtained for resistance profiling

Resistance levels in commensal members of the gut flora are commonly measured to compare AR in different animal species and human beings (EFSA, 2009). In the current study we obtained random isolates on Slanetz-Bartley agar and McConkey agar without added antibiotics. Three hundred and fifty randomly obtained lactose positive colonies with the right colony morphology from McConekey agar were all shown to be *E. coli* by IMVC test. A random subset of 164 colonies from the collection of *Enterococcus spp.* was also characterized to species level by multiplex PCR and MALDI TOF/MS. The species distribution according to origin of the sample is shown in Table 2. The majority of isolates from all animal species and humans were shown to be *E. faecium* (92 %). Occasionally growth of non-lactose fermenters was observed on McConkey plates containing CTX. These were also purified and identified by MALDI TOF/MS. Out of 59 such isolates analyzed, 3 could not be assigned to any of current or new species, 49 were *Klebsiella pneumonia*, three *Ochrobactum sp.*, one *Achromobacter sp* and three were lactose negative *E. coli*.

### 5.4.2 Susceptibility testing of commensal *E. coli* and *Enterococci spp* by disc diffusion test

In order to assess the general level of resistance in human beings and food animals in Tanzania, we subjected purified isolates of *E. coli* and *Enterococci* to disc diffusion test (Appendix 15). Results for *E. coli* appear in Table 3. More than 50 % of all colonies were resistant towards TE (68.8 %), STX (61.1 %), S (55.7 %) and AMP (54.8 %) and the level of resistance was high in all animals species as well as humans for these antibiotic. Only isolates obtained from cattle showed significantly (P = 0.012) different level to STX and TE (low) compared to humans, while pig and cattle isolates had significantly less AMP resistance than human isolates. Surprisingly, indigenous chicken, which is considered the less intensive production system, generally was in the top most resistannce group followed by human isolates and with cattle in the lower end. Importantly this group included both dairy cattle and beef cattle, and isolates obtained from the latter group was the reason for the lower resistance (data not shown). CIP-resistance was found to be highest in isolates from chicken, while human, pig and cattle isolates showed the same level of resistance. Resistance to CTX was observed in 40.9 % of isolates, with more than 70 % of isolates from cattle showing this resistance.

### 5.4.3 *Enterococci spp.*

Showed the higher levels of resistance towards RD (75.2 %) and a much lower level of resistance to E (15 %) and TE (13.8 %). Resistance towards CN at a concentration of 120 µg was not observed. Notably, VRE were present in both humans and several food animals, and AMP resistance was observed a high level in cattle isolates (Table 5.4). One isolate from beef cattle and one from indigenous chicken were resistant to both VA and AMP. Nine out of ten isolates that showed resistant to AMP originated from cattle and belonged to *E. faecium*.

### 5.4.4 Quantification of resistant E. coli and *Enterococci spp*. in samples from food animals and humans

In order to investigate whether determination of AR levels based on single colonies obtained from randomly picked animals reflected the quantitative level of resistance we determined the CFU of resistant *E. coli* and *Enterococci* in relation to the total number of these bacteria in each animal and humans. In order to do so we spread dilutions of faecal samples directly onto McConkey and Slanetz-Bartley agar plates with antibiotics included in the agar. The detection level of this approach was 100 CFU/g and as seen in Table 5.5 and Table 5.6, not all samples contained this number of resistant bacteria. The proportion of samples that were above the detection level did not always match the prevalence estimates of resistances determined on single isolates (as shown in Table 3 and Table 5.4). Notably, 47/97 samples from humans contained CTX-resistant *E. coli* (Table 5.5) with a mean of 2.2 $Log_{10}$ CFU and more pig samples than anticipated from characterization of single colonies were positive for this antibiotic (40/97). On the other hand, few samples contained VRE above the detection level (Table 5.6), while plates with *Enterococcis spp*. with more than 100 CFU/ml of Ampicillin resistant colonies were obtained from 20/97 samples from humans.

From a public health point, the number of resistant bacteria per gram of feces constitutes more valuable information than the average resistance level of randomly obtained isolates. $Log_{10}$ CFU of *E. coli* (Table 5.7) revealed high numbers of TE, AMP and STM resistant *E. coli* which accounted for more than 70 % of all *E. coli* counted in all samples. Around 80 % of the lactose positive colonies from humans grew on McConkey containing these drugs. The CFU of both ampicillin and vancomycin resistant *Enterococci* was low, and always constituted less than 21 % of the total *Enterococci* count (Table 5.8).

### 5.4.5 ESBL determination from *E. coli* and non *E. coli* bacteria resistant to CTX

Colonies were obtained from all samples (n=194) that had shown resistance to CTX on MacConkey agar. In cases were both lactose positive and negative colonies were present, one colony of each were picted, and 59 out of the 194 colonies characterized were lactose negative. Isolates were analyzed by double disc diffusion for synergy between CTX and clavulanic acid, and synergy was observed for all isolates. They were then subjected to PCR with primers directed against the CTX-M group. This revealed a band of the same size as that of positive CTX-M control in all isolates analyzed (Figure 1), indicating that they all contained an ESBL gene of this class.

### 5.4.6 Quantification of resistance genes by qPCR

Gene copy numbers of two selected resistance genes were determined to be able to compare AR levels obtained by single colonies and quantification by culture methods to an estimate of AR levels obtained by a culture independent method. Quantification was based on standard curves derived from known number of amplicons. Standard curves can be seen from supplementary material (Fig. S 5.1- S 5.3).

Gene copy numbers/gram sample were determined and also related to the number of 16s rRNA gene-copies in the same sample (Table 5.7). The highest numbers of *sulII* gene-copies were observed in cattle ($Log_{10}$ 5.68) and the lowest in pigs ($Log_{10}$ 5.37) and there was no significant (P >0.05) different between sample sources. For *tetW* genes, there was a significant difference in sample sources and cattle had highest gene copy number ($Log_{10}$ 7.39) while the lowest number was observed in humans ($Log_{10}$ 6.41). 16rDNA copy numbers were significantly different between sample sources, indicating that the total numbers of bacteria were different between species. For that reason copy number of resistance genes was related to the number of this gene. In relative terms, cattle had the

highest relative proportion of *tetW* gene-copies followed by pigs, indigenous chicken, exotic chickens and humans. Likewise in the case of the *sulII* gene, dairy cattle had the highest relative copy number, followed by pigs, indigenous chickens, exotic chickens and humans.

### 5.5 Discussion

The aim of this study was to estimate the extent of AR in random collected faecal samples from humans and the most important food animals by determining the prevalence of antimicrobial resistance in indicator organisms, *E. coli* and *Enterococci spp*. The main approach was susceptibility testing of randomly obtained isolates of indicator bacteria. This is the most common approach in surveillance of antibiotic resistance (EFSA, 2009). Commensal bacteria constitute a reservoir of resistance genes and their level of resistance is considered to be a good indicator for selection pressure by antibiotic use (van den Bogaard and Stobberingh, 2000).

High levels of resistance were observed in *E. coli* to tetracycline, ampicillin and sulphonamide. No accurate account is available on drug use in Tanzania, but a small survey reported this class as the most commonly used drugs in agriculture in Tanzania (Katakweba *et al*., 2012). High level of resistance to streptomycin was also observed. Streptomycin or penicillin-streptomycin is a commonly used aminoglycoside in food animals in Tanzania. The selective pressure exerted in intramammary and injectable preparations for the treatment of mastitis and other bacterial infections in cattle and pigs can contribute to the resistance and account to this finding (Kivuki *et al*., 2006), however, the relatively high prevalence of resistance to streptomycin among isolates from commercial chicken production may not be accounted for by its use in these species, since oral formulations for mass medication are not available. There is a current product in the

market with a combination of oxytetracyline Hcl and neumycin sulphate and this product can cause cross reaction with streptomycin that was among the tested antimicrobials.

Tadesse *et al*. (2012) assessed historical changes in antimicrobial drug resistance in *E. coli* isolated from humans and food animals in United States. Significant upward trend in resistance was observed for ampicillin, sulfonamide, and tetracycline. Co-resistant phenotypes were observed between tetracycline and streptomycin and tetracycline and sulfonamide. These data described the evolution of resistance after introduction of new antimicrobial agents and provided information for resistance development over time.

Reports on resistance levels in *E. coli* isolates from humans and animals in other studies from Tanzania vary considerably from one study to another. Jordi *et al*. (1999) reported 38 % multiresistnace of *E. coli* isolates from faeces of children with diarrhoea. Moyo *et al*. (2010) reported a resistance of STX 38.5 %, AMP 57.7 % and CTX 37 % of *E. coli* isolated from urine. In animal studies, Kivaria *et al*. (2006) found a resistance of 25%. 42% and 63% towards S, TE and P respectively among *E. coli* isolated from milk samples. Blomberg *et al*. (2005) indicated that there is less antimicrobial resistance in *E. coli* isolates from rural area than in isolates from the commercial capital like Dar es Salaam. The large variation between studies is most likely reflecting the large variation in sampling strategies and methodology, and underlines the importance of a strict adherence to a common protocol, if data are to be used in the risk management content. The current study was initiated partly because of this situation.

A study on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food was submitted by 26 European Union Member States and analyzed by the European Food Safety Authority (EFSA) and the European Centre for Disease

Prevention and Control (ECDC) (EFSA, 2013). Among indicator *E. coli* from broilers and pigs, resistance to tetracyclines, ampicillin and sulfonamides ranged between 37 % and 57 % while resistance levels were lower in laying hens (14 % to 18 %). In the case of cattle, levels of resistance to the same antimicrobials fell within the range of 20 % to 74 % in younger age groups (fattening veal calves) but were much lower in older cattle (mainly adult cows). In general, resistance levels were lower among isolates from cattle and layers than in isolates from broilers and pigs (EFSA, 2013). Compared to the Results of the current study (Tables 5.3 up to 5.6 ), the Results of the EFSA (2013) report indicate that levels of antibiotic resistance is actually lower/the same/higher in countries with intensive livestock production. Misuse of antibiotics is common in Tanzania (Kagashea *et al*., 2010; Katakweba *et al*., 2012) and other African countries (Byarugaba, 2004; Carlos, 2010) are the main contributory factor for the resistance.

Less resistance was observed to antibiotics that are less commonly used in animal industry in Tanzania, like ciproflaxicin, cefotaxime and gentamycin. The same trend has been observed in Uganda (Byarugaba *et al*., 2011). The resistance detected in *E. coli* isolates from all three antibiotics may have been caused by the clonal spread of resistant isolates (Kijima-Tanaka *et al*., 2003), which are then maintained for other reasons than resistance. Co-resistance with other unrelated compounds or horizontal transfer of resistance genes from human derived bacteria may also be the reason as humans and animals may share the same environment (Rwego *et al*., 2008). In additions, illegal import and use of such these types of drugs cannot be ruled out.

Resistance levels determined in *Enterococci* from Slantez Bartley agar are based on several species of bacteria, and differences between animal species could be due to differences in inherent resistance. However, in speciation of *Enterococci spp*, more than

90 % were found to be *E. faecium*, followed by *E. faecalis* and *E.hiare*. These species distributions are both supported by previous reporting but also disagree with one report. A study by Moussa (1965) speciated 2,477 Enterooocci strains isolated from water, soil, vegetation, sewage and human and animal faeces and found two major groups: *E faecalis* and *E. faecium*, but in contrast to our study, *E. faecalis* was found to predominate in the human and poultry gut. In another study, Layton *et al*. (2010) screened 64 faecal samples from avian, mammalian and humans for the occurrence of *Enterococci spp*. They found *E. avium, E. casseliflavus, E. durans, E. gallinarum, E. faecium, E. faecalis, E. hirae and E. saccharolyticus* and like in our study, the majority of isolates from humans and animals were *E. faecium.* The high proportion of *E. faecium* in humans as well as animals allowed us to compare Results between species without considering the underlying species distribution.

In this study high resistance to rifampicin was observed among isolates of *Enterococci* on a much higher level than to other tested antimicrobials, which, on a note were lower compared to other reported studies (Ruzauskas *et al*., 2009). The observation on high levels of resistance to rifampicin in isolates from all animal species was surprising, since rifampicin is only used in humans. This may indicate a high transfer of bacteria from humans to animals, for example through environmental contamination, which is particularly relevant in developing countries because of crowding and inadequate excreta management, leading to recycling and amplification of resistance genes within and between the environment, food animals, and the human population (Kalter *et al*., 2001; Rwego *et al*., 2008). With the current situation of use and misuse of antimicrobials in delevoping countries the possibility of using human drugs to treat animals diseases cant be rulled out because there are cases where humans use livestock drugs to treat their chronic urinary tract infections (Katakweba personal observations).

The EFSA (2009) study on antimicrobial resistance in zoonotic and indicator bacteria also reported on resistance in *Enterococci spp.* Among these bacteria, resistance to tetracyclines and erythromycin was common in isolates from (domestic fowl), pigs and cattle with resistance levels of 23 % to 79 %. With exception of Rifampicin in our study that had up to 82.5% resistance, other levels were comparable to the ones reported in European countries. Resistance to vancomycin continued to be detected, albeit at very low levels (maximum 0.7 %), in Enterococcal isolates from animals (EFSA, 2013). Avoparcin, a glycopeptide showing cross-resistance to medically important glycopeptides, has been used in the European Community as a growth promoter in animal feeds, but it has not been used as growth promoter in Tanzania. It was therefore surprising to find VRE among isolates obtained from animal species, possible again indicating an influx of resistant bacteria from the human population. Vancomycin-resistant enterococci (VRE) in Europe are thought to have emerged partly due to the use of avoparcin in animal husbandry, and this has been documented in a study employing molecular analysis of isolates of *E. feacium* (Nilson, 2012; Klare *et al*., 1999). VRE in human-associated samples may also be a result of antibiotic use in hospitals (Kühn *et al*., 2005), and this is the likely explanation for the presence of vancomycin resistant Enteroccci in the human isolates in our study. Extended spectrum beta lactamase (ESBL) producing bacteria constitute a particular and threatening human health problem, for which a food animal source may be present (Tham, 2012; Kluytmans *et al*., 2013). The types of bacteria that are most common associated with ESBL are *E. coli* and *Klebsiealla* species (Lewis *et al*., 2007; Pallecchi *et al*., 2007). In this study all bacteria shown to be CTX resistant were shown to be ESBL and to contain $ctx_{bla\text{-}M\ type}$ of gene. This constitutes the first report on the presence of ESBL in Tanzania from food animals, and further studies are needed to determine the distribution of genes and how this correlates with the genes present in isolates form humans. The $ctx_{bla\text{-}M\ type}$ is currently the most widespread and threatening ESBL, particularly in community-acquired

infections (Kariuki, 2010; Colomer-Lluch *et al*., 2011). A study by Pallecchi *et al*. (2007) found 88 % (44 isolates) from a remote community with minimal antibiotic exposure harbored a CTX-M-type and 12 % (6 isolates) had SHV-type (SHV-2 or SHV-12). They also observed an increased diversity of CTX-M-types. In this study a genuine PCR method was used, and no further sub-division was attempted.

Quantitative estimates of AR are important in relation to risk assessment of AR bacteria in food animals (Codex, 2009), and this requires quantitative estimates of resistance levels, rather than susceptibility patterns. The food borne route is the major transmission pathway for resistant bacteria and resistance genes from food animals to humans, but other routes of transmission exist (Smith *et al*., 2002), and resistance risks to human health from non-human use of antimicrobials are inherently indirect and complex (FAO/WHO/OIE, 2003). In countries with industrial type of food chain, the study of resistant bacteria decline over the food chain. Some carcass processing steps are effective in reducing both the general number of *E. coli* and with this the number of resistant Wu *et al*. (2009). The numbers of resistant bacteria detected in the current study were generally at the same level; lower, higher than reported from countries with more industrial type of production (EFSA, 2013) which would indicate the same/lower/higher risk to the consumers. However, the food chains in Tanzania, as in many developing countries, are generally shorter and normally without a chilling step, and general observations on consumer risks from developed countries cannot easily be transferred to this country. More detailed studies on the fate of resistant bacteria in the food chains in developing countries are therefore needed.

Current surveillance systems use phenotypic methods to determine AR, however, molecular detection may be more rapid and efficient (Tanih and Ndip, 2013), and in particular nucleic acid-based detection systems offer rapid, sensitive and culture

independent methods to detect the presence of resistance genes by a culture independent approach (Fluit *et al*., 2001; Momtaz *et al*., 2012), since quantitative data can conveniently be obtained by PCR methods (Rolain *et al.*, 2004). In this study, the absolute abundance (gene copies per gram wet manure) of resistance genes *tetW* and *sulII* as well as relative number of genes in relation to the number of 16sRNA genes was determined. The choice of genes to study was justified by two factors. *sulII* is the most common gene encoding sulphonamide resistance in *E. coli* (Bean *et al*., 2009), and Results were comparable to CFU counts of sulphonamide resistant *E. coli* on McConkey plates. *tetW* is a ribosomal protection type of tetracycline resistance genes (Whittle *et al*., 2003) and is the most abundant tetracycline resistance gene in human faecal samples (Scott *et al*., 2000). Thus the culture independent quantification of this gene allowed us to estimate the value of culture based detection of tetracycline resistant bacteria based on human samples. The bases to human samples was due to the fact that farm animals and humans were sharing the same environment, tetracycline is most commonly used in humans and animals and *tetW* being most abundant tetracylines resistant gene in humans (Scott *et al*., 2000). Furthermore a study by Katakweba *et al*. (2012) in the study area, found sulphonamide and tetracycline to be commonly used and stored in livestock keepers stores. Cattle had higher numbers of *tetW* and *sulII* genes followed by pigs, indigenous chicken, exotic chickens and finally humans. This trend has a connection with treatment regimes of animals as dairy cattle are frequently treated with these two antimicrobials. McKinney *et al*. (2010) found the same trend when determining the prevalence of tetracycline *tetO* and *tetW* and sulfonamide genes *sulI* and *sulII* in livestock lagoons of various operation type. *tetW* and *tetQ* were also more commonly found in lagoon water samples when Smith *et al*. (2004) did a quantification of tetracycline resistance genes in feedlot lagoons by Real-Time PCR.

Three techniques namely colony forming units (CFU), antibiotic discs and quantitative polymerase chain reactions (qPCR) for resistant gene detection in gut flora bacteria were used in this study. The reason behind was to get stronger results and biologically looking at different aspects (Prescott *et al*., 2000; Rolain *et al.*, 2004; Pei *et al.*, 2006). All three methods are independent and gave the Results of resistant in different antimicrobials used to indicator bacteria. Both CFU), antibiotic discs had good and comparable Results in antimicrobials tested. A good example is the Results obtained from CTX in *E.coli*. *tetw* and *sulII* resistance genes detected confirmed prolonged use of both antimicrobials (Lathers, 2002; Carlos, 2010). The CFU method had a weakness of estimating the values of colonies from the samples that have zero colony at 100µl spread on plates. For the disc method, bacteria are picked from plates without antibiotics and there is a possibility of picking a colony that is non resistant to any of used antimicrobials. The gene method has a disadvantage of getting low levels of resistance genes if the bacteria are exposed to that particular antibiotic for a very short period compared to that exposed for a long period one.

The findings of this study suggest that commensal enteric *E. coli* and *Enterococci spp*. from healthy animals may be an important reservoir for tetracycline and other antimicrobial resistance determinants. This could be attributed by use and misuse of antimicrobials because of their relatively low cost and readily availability for sale ‘over the counter’, these drugs are widely used by farmers for therapeutic and prophylactic applications (Miralles 2010; Katakweba *et al*., 2012). The intense use and misuse of antibiotics are undoubtedly the major forces associated with the high numbers of resistant pathogenic and commensal bacteria worldwide. Both the volume and the way antibiotics are applied contribute to the selection of resistant strains. Still, other social, ecological and genetic factors affect a direct relationship between use and frequency of resistance

(Barbosa and Levy, 2000). Surveillance data show that resistance in *E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine (Tadesse *et al*., 2012). The antimicrobial susceptibility data from the present study on the other hand indicated that humans may also be an important source of AR bacteria in animals, since some resitances could only be explained by the common use of the selecting drugs in humans. This situation is most likely only relevant to developing countries where there is more direct contact between animals and the human population and where the environmental reservoir can be expected to play a bigger role due to waste mismanagement. Over and above, humans in Tanzania have also free access to antimicrobials from the drug shops without any prescriptions, as is the common tendency in other Africa countries (Kagashea *et al*., 2010; Tagoe and Atar, 2010), and the high resistance levels observed in the current study is likely caused by this factor. Future studies on this kind of situation are needed to prove if this factor is a main contributory one to current resistance found in indicator bacteria.

## 5.6 Aknowledgements

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## 5.7 Author Contribution

Abdul Katakweba took part in the planning, performed sampling and typing, evaluated Results and drafted the manuscript. Athumani Lupindu performed sampling and typing

and approved the final manuscript. Jesper Rosenkranzt performed qPCR and approved the final manuscript. Peter Damborg participated in the design of the laboratory analysis and approved the final manuscript. Uswege Minga took part in the planning and approved the final manuscript. Amandus Muhairwa took part in the planning, evaluated the Results, drafting of the manuscript and approved the final manuscript. Madundo Mtambo took part in the planning, evaluated the Results and approved the final manuscript. John Olsen planned the study, evaluated Results and took part in drafting of the manuscript.

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**Table 5.1: Primers used for detection of *sulII* and *tetW* genes in faecal flora**

| Gene | Primers | Sequence | Annealing temp. (°C) | Source |
|---|---|---|---|---|
| sulII | sulII-Fw | TCCGGTGGAGGCCGGTATCTGG | 57 | |
| | sulII-Rv | CGGGAATGCCATCTGCCTTGAG | | |
| Tet(W) | tet(W)-Fw | GAGAGCCTGCTATATGCCAGC | 60 | |
| | tet(W)-Rv | GGGCGTATCCACAATGTTAAC | | |
| 16s rRNA | 1369Fw | CGGTGAATACGTTCYCGG | 60 | |
| | 1492Rv | GGWTACCTTGTTACGACTT | | |

**Table 5.2: Species composition (N) of *Enterococci spp* obtained from Slanetz-Bartley agar**

| Sample sources | *E. faecium* | *E. faecalis* | *E.hirae* | Total |
|---|---|---|---|---|
| Human | 33 | 1 | 3 | 37 |
| Dairy cattle | 41 | 1 | 2 | 44 |
| Beef cattle | 13 | 0 | 0 | 13 |
| Pigs | 20 | 1 | 2 | 23 |
| Exotic chickens | 27 | 0 | 1 | 28 |
| Indigenous chickens | 17 | 2 | 0 | 19 |
| **Total** | **151** | **5** | **8** | **164** |

**N = Number**

**Table 5.3: Sensitivity and resistance pattern of E. coli from food animals, human and chickens tested for antibiotic resistance using disc diffusion method (%)**

| | Isolate status | Human (N=70) | Indigenous chickens (N=70) | Exotic chickens (N=70) | Pig (N=70) | Cattle (N=70) | Total (N=350) | χ2 | P value |
|---|---|---|---|---|---|---|---|---|---|
| STX | R | 48 (68.6) | 54 (77.2) | 42 (60) | 42 (60) | 28 (40) | 214 (61.1) | | |
| | I/S | 22 (31.4) | 16 (22.8) | 28 (40) | 28 (40) | 42 (60) | 136 (38.9) | 27.33 | 0.0006 |
| CN | R | 7 (10) | 5 (7.1) | 10 (14.3) | 4 (5.7) | 11 (15.8) | 37 (10.6) | | |
| | I/S | 63 (90) | 65 (92.9) | 60 (85.7) | 66 (94.3) | 59 (84.2) | 313 (89.4) | 23.48 | 0.0028 |
| CIP | R | 13 (18.6) | 37 (52.9) | 36 (51.4) | 7 (10) | 15 (21.4) | 108 (30.9) | | |
| | I/S | 57 (81.4) | 33 (47.1) | 34 (48.6) | 63 (90) | 55 (78.6) | 242 (69.1) | 85.96 | 0.0001 |
| AMP | R | 40 (57.1) | 48 (68.6) | 55 (78.6) | 27 (38.6) | 22 (31.4) | 192 (54.8) | | |
| | I/S | 30 (42.9) | 22 (31.4) | 15 (21.4) | 43 (61.4) | 48 (68.6) | 158 (45.2) | 50.13 | 0.0001 |
| CTX | R | 22 (31.4) | 22 (31.4) | 32 (45.7) | 17 (24.3) | 50 (71.4) | 143 (40.9) | | |
| | I/S | 48 (68.6) | 48 (68.6) | 38 (54.3) | 53 (75.7) | 20 (28.6) | 207 (59.1) | 48.17 | 0.0001 |
| S | R | 39 (55.7) | 49 (70) | 40 (57.1 | 35 (50) | 32 (45.7) | 195 (55.7) | | |
| | I/S | 31 (44.3) | 21 (30) | 30 (24.9) | 35 (50) | 38 (54.3) | 155 (44.3) | 20.09 | 0.01 |
| TE | R | 50 (71.4) | 54 (77.1) | 51 (72.9) | 51 (72.9) | 35 (50) | 241 (68.8) | | |
| | I/S | 20 (28.6) | 16 (22.9) | 19 (27.1) | 19 (27.1) | 35 (50) | 109 (31.2) | 15.96 | 0.043 |
| AMC | R | 10 (14.3) | 18 (25.7) | 7 (10) | 4 (5.7) | 9 (12.9) | 48 (13.7) | | |
| | I/S | 60 (85.7) | 52 (74.3) | 63 (90) | 66 (94.3) | 61 (87.1) | 302 (86.3) | 21.26 | 0.0065 |

R: Resistant, I: Intermediate, S: susceptible. Sulphamethoxazole/ trimethoprim (STX 25 µg), ampicillin (AMP 10 µg), amoxycillin-clavulanic acid 2:1 AMC 30 µg), gentamicin (CN 10 µg) Ciproflaxin (CIP 5 µg), Tetracycline (TE 30 µg), Streptomycine (S 10 µg) and Cefotaxime (CTX 30 µg),

**Table 5.4: Sensitivity and resistance pattern of *Eenterococci spp* from food animals, human and chickens tested for antibiotic resistance using disc diffusion method (%)**

| Antibiotics | Isolate status | Human (N=40) | Indigenous chicken (N=40) | Exotic chicken (N=40) | Pig (N=40) | Dairy cattle (N=40) | Beef cattle (N=40) | Total (N=240) | $\chi 2$ | P value |
|---|---|---|---|---|---|---|---|---|---|---|
| | R | 9 (22.5) | 6 (15) | 6(15) | 6 (15) | 3 (7.5) | 6 (15) | 36 (15) | | |
| E | I/S | 31 (72.5) | 34 (85) | 34 (85) | 34 (85) | 37 (92.5) | 34 (85) | 204 (85) | 31.17 | 0.0005 |
| | R | 30 (75) | 33 (82.5) | 33 (82.5) | 28 (70) | 27 (67.5) | 23 (57.5) | 174 (75.2) | | |
| RD | S | 10 (25) | 7 (17.5) | 7 (17.5) | 12 (30) | 13 (32.5) | 17 (42.5) | 66 (27.5) | 9.28 | 0.0984 |
| | R | 1 (2.5) | 5 (12.5) | 0 | 3 (7.5) | 0 | 3 (7.5) | 12 (5) | | |
| VA | I/S | 39 (97.5) | 35 (87.5) | 40 (100) | 37 (92.5) | 40 (100) | 37 (92.5) | 228 (95) | 17.18 | 0.0704 |
| | R | 5 (12.5) | 10 (25) | 1 (2.5) | 4 (10) | 3 (7.5) | 10 (25) | 33 (13.8) | | |
| TE | S | 35 (87.5) | 30 (75) | 39 (97.5) | 36 (90) | 37 (92.5) | 30 (75) | 207 (86.3 | 14.65 | 0.012 |
| | R | 0 | 6 (15) | 3 (7.5) | 0 | 0 | 0 | 9 (3.8) | | |
| CIP | S | 40 (100) | 34 (85) | 37 (92.5) | 40 (100) | 40 (100) | 40 (100) | 231 (96.2) | 21.82 | 0.0006 |
| CN | S | 40 (100) | 40 (100) | 40 (100) | 40 (100) | 40 (100) | 40 (100) | 40 (100) | 0 | 1 |
| | R | 0 | 4 (10) | 0 | 0 | 1 (2.5) | 0 | 5 (2.1) | | |
| C | S | 40 (100) | 36 (90) | 40 (100) | 40 (100) | 39 (97.5) | 40 (100) | 235 (97.9) | 15.73 | 0.0077 |
| | R | 0 | 2 (5) | 0 | 1 (2.5) | 0 | 9 (22.5) | 12 (5) | | |
| AMP | S | 40 (100) | 38 (95) | 40 (100) | 39 (97.5) | 40 (100) | 31 (77.5) | 228 (95) | 32.63 | 0.0001 |

R: Resistant, I: Intermediate, S: susceptible. Vancomycin (VA 30 µg), Erythromycin (E 15 µg), ciprofloxacin (CIP 5 µg), ampicillin (AMP 10 µg), chloramphenical (C 30 µg), rifampicin (RD 5 µg), gentamycin (CN 120 µg) and Tetracycline (TE 30 µg).N –number

**Table 5.5: Proportion of individuals harboring *E. coli* resistant to different antibiotics**

| Animal spp | Samples tested | Sulphameth. (%) | Cefotaxime (%) | Tetracyline (%) | Ampicillin (%) |
|---|---|---|---|---|---|
| Humans | 97 | 79 (81.44) | 47 (48.45) | 77 (79.38) | 81 (83.51) |
| Exotic chickens | 97 | 74 (76.29) | 32 (32.99) | 70 (72.16) | 75 (77.32) |
| Indigenous chickens | 97 | 78 (80.04) | 40 (41.24) | 87 ( (89.69) | 71 (73.20) |
| Dairy cattle | 68 | 55 (80.88) | 33 (48.53) | 58 (85.29) | 57 (83.82) |
| Beef cattle | 29 | 13 (44.83) | 2 (6.7) | 14 (48.28) | 10 (34.48) |
| Pig | 97 | 77 (79.38) | 40 (41.24) | 79 (81.44) | 68 (70.10) |
| **Total** | **485** | **374 (77.11)** | **194 (40)** | **385(79.38)** | **362(74.64)** |

**Table 5.6: Proportion of individual harboring *Enterococci spp* resistant to different antibiotics**

| Anaimal spp | Samples tested | Ampicillin (%) | Vancomycin (%) |
|---|---|---|---|
| Humans | 97 | 20 (20.62) | 1 (1.03) |
| Exotic chickens | 97 | 12 (12.37) | 8 (8.24) |
| Indiginous chickens | 97 | 17 (17.52) | 7 (7.22) |
| Dairy cattle | 68 | 13 (19.12) | 3 (4.41) |
| Beef cattle | 29 | 2 (6.7) | 2 (6.7) |
| Pig | 97 | 11 (11.34) | 5 (5.15) |
| **Total** | **485** | **75 (15.46)** | **26(5.36)** |

**Table 5.7: Mean $Log_{10}$ CFU per gram (± SE) of *E. coli* in feces from different sources**

| Sample source | No antibiotic | Ampicillin | Cefotaxime | Sulphamethoxole | Tetracyline |
|---|---|---|---|---|---|
| Beef cattle (N = 29) | $3.98 \pm 0.22^{b}$ | $1.33 \pm 0.40^{c}$ | $0.21 \pm 0.43^{b}$ | $1.36 \pm 0.40^{b}$ | $1.61 \pm 0.37^{c}$ |
| Dairy cattle (N = 68) | $4.71 \pm 0.14^{a}$ | $3.42 \pm 0.25^{ba}$ | $1.94 \pm 0.27^{a}$ | $3.39 \pm 0.25^{ba}$ | $3.31 \pm 0.23^{ba}$ |
| Exotic chicken (N = 97) | $4.93 \pm 0.11^{a}$ | $3.40 \pm 0.20^{ba}$ | $1.43 \pm 0.22^{ba}$ | $3.41 \pm 0.20^{ba}$ | $3.07 \pm 0.19^{b}$ |
| Human (N= 97) | $5.04 \pm 0.11^{a}$ | $3.87 \pm 0.20^{a}$ | $2.20 \pm 0.22^{a}$ | $3.56 \pm 0.20^{ba}$ | $3.41 \pm 0.19^{ba}$ |
| Indigenous chicken (N = 97) | $5.09 \pm 0.11^{a}$ | $3.15 \pm 0.20^{ba}$ | $1.90 \pm 0.22^{a}$ | $3.80 \pm 0.20^{a}$ | $3.83 \pm 0.19^{a}$ |
| Pig (N= 97) | $4.80 \pm 0.11^{a}$ | $3.07 \pm 0.20^{b}$ | $1.82 \pm 0.22^{a}$ | $3.52 \pm 0.20^{ba}$ | $3.37 \pm 0.19^{ba}$ |

N = Number of samples tested

Different superscripts letters in the same column indicate significant difference ($p<0.05$)

**Table 5.8: Mean $Log_{10}$ CFU per gram (± SE) of resistant *Enterococci* in faecal samples from different sources**

| Sample source | No antibiotic | Ampicillin | Vancomycin |
|---|---|---|---|
| Beef cattle (N = 29) | 3.65 ± 0.26c | $0.22 \pm 0.31^{b}$ | 0.31 ± 0.21a |
| Dairy cattle (N = 68) | $4.82 \pm 0.16^{a}$ | $0.89 \pm 0.19^{a}$ | $0.18 \pm 0.13^{ba}$ |
| Exotic chicken (N = 97) | $4.86 \pm 0.13^{a}$ | $0.56 \pm 0.15^{ba}$ | $0.40 \pm 0.11^{a}$ |
| Human (N= 97) | $4.30 \pm 0.13^{b}$ | $0.94 \pm 0.15^{a}$ | $0.39 \pm 0.11^{a}$ |
| Indigenous chicken (N = 97) | $4.56 \pm 0.13^{ba}$ | $0.69 \pm 0.15^{ba}$ | $0.28 \pm 0.11^{a}$ |
| Pig (N= 97) | $4.52 \pm 0.13^{ba}$ | $0.40 \pm 0.15^{ba}$ | $0.26 \pm 0.11^{a}$ |

N = Number of samples tested

Different superscripts letters in the same column indicate significant difference ($p<0.05$)

**Table 5. 9: LSmeans (±SE) of $Log_{10}$ copy gene number per gram and (% resistant genes contents/16rRNA)**

| Animal spp | N | *sulII* (%RGC/16rRNA) | *tetW* (%RGC/16rRNA) | 16rRNA |
|---|---|---|---|---|
| Human | 22 | $5.36 \pm 0.10^{a}$ ($0.002 \pm 0.10^{b}$) | $6.41 \pm 0.17^{c}$ ($0.059 \pm 1.88^{b}$) | $10.15 \pm 0.16^{a}$ |
| Exotic chickens | 22 | $5.44 \pm 0.10^{a}$ ($0.020 \pm 0.10^{b}$) | $6.55 \pm 0.17^{cb}$ ($0.980 \pm 1.88^{b}$) | $9.84 \pm 0.16^{b}$ |
| Indigenous chickens | 22 | $5.44 \pm 0.10^{a}$ ($0.002 \pm 0.10^{b}$) | $6.53 \pm 0.18^{cb}$ ($2.149 \pm 2.00^{b}$) | $9.33 \pm 0.17^{c}$ |
| Cattle | 22 | $5.69 \pm 0.10^{a}$ ($0.343 \pm 0.10a$) | $7.39 \pm 0.17^{a}$ ($11.881 \pm 1.88^{a}$) | $9.58 \pm 0.16^{cb}$ |
| Pigs | 22 | $5.37 \pm 0.10^{a}$ ($2.389 \pm 1.88^{b}$) | $6.97 \pm 0.17^{ba}$ ($0.002 \pm 0.10^{b}$) | $9.61 \pm 0.16^{cb}$ |

RGC = Resistance Genes Contents, Super script $^{a,b,c}$; means within each column bearing same letter are not significantly different at $P<0.05$

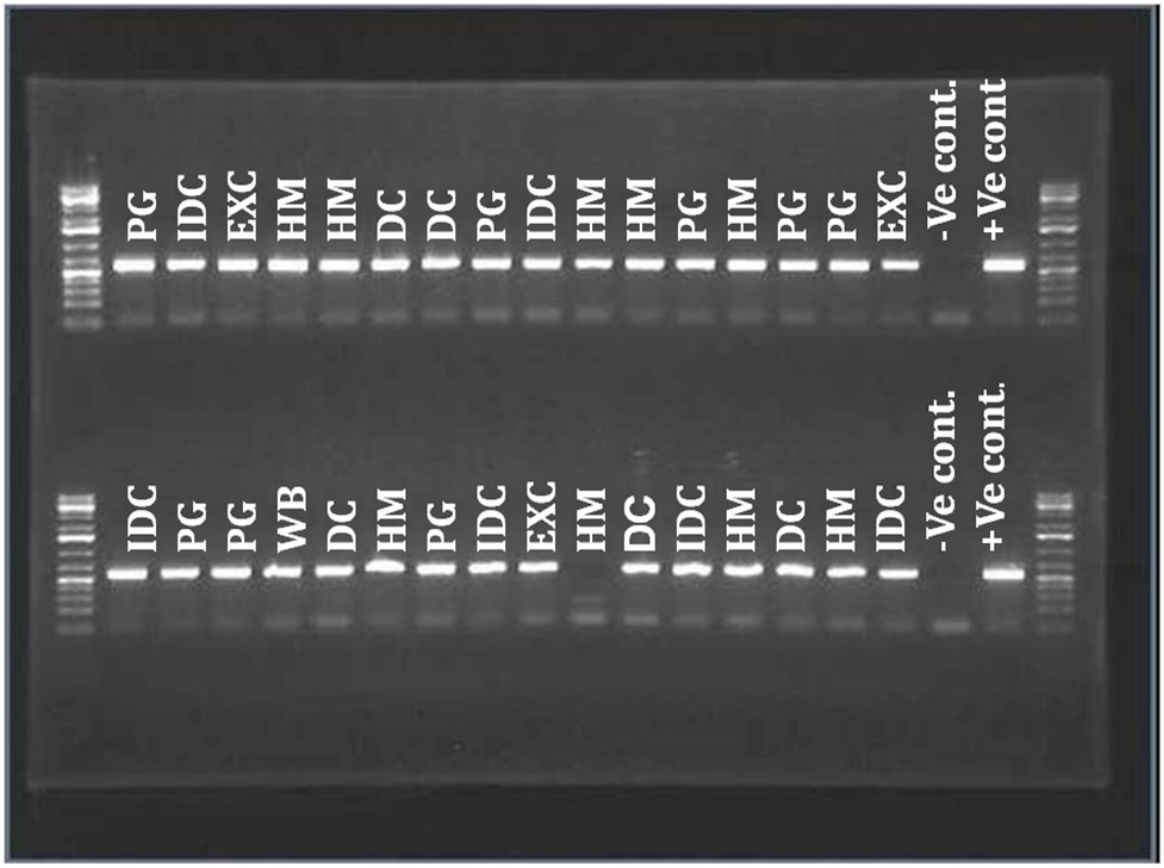


**Figure 5.1: Detection of ESBL genes of CTX-M-1 type from *E. coli* bacteria**

HM = Human, Pg = Pig, DC = Dairy, IDC = Indigenous chickens, BC = Beef cattle

EXC = Exotic chicken

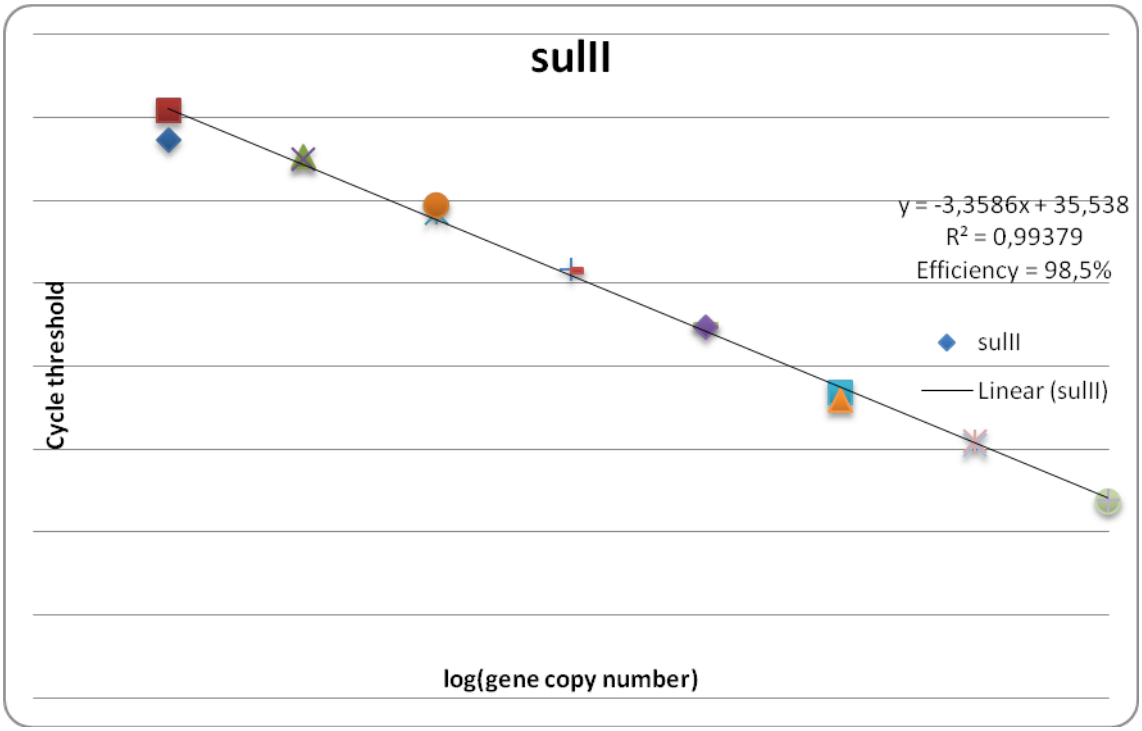


**Figure S 5.1: Standard curve for quantification of *sulII***

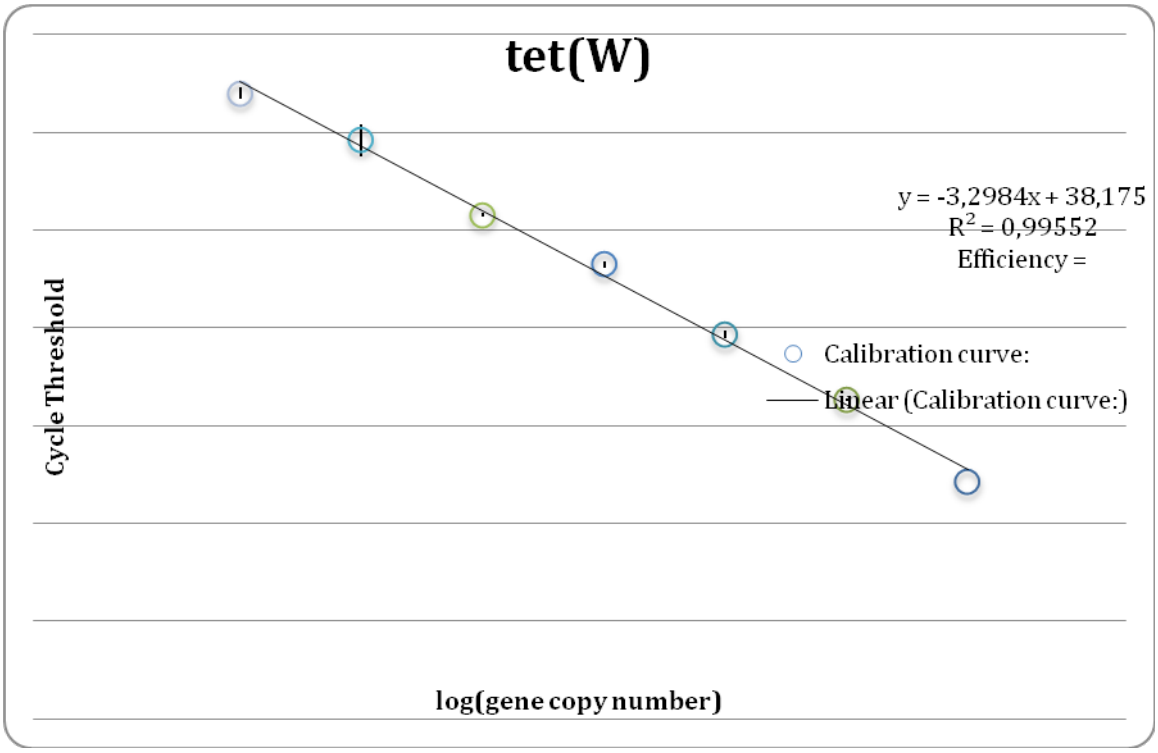


**Figure S 5.2: Standard curve for quantification of *tetW***

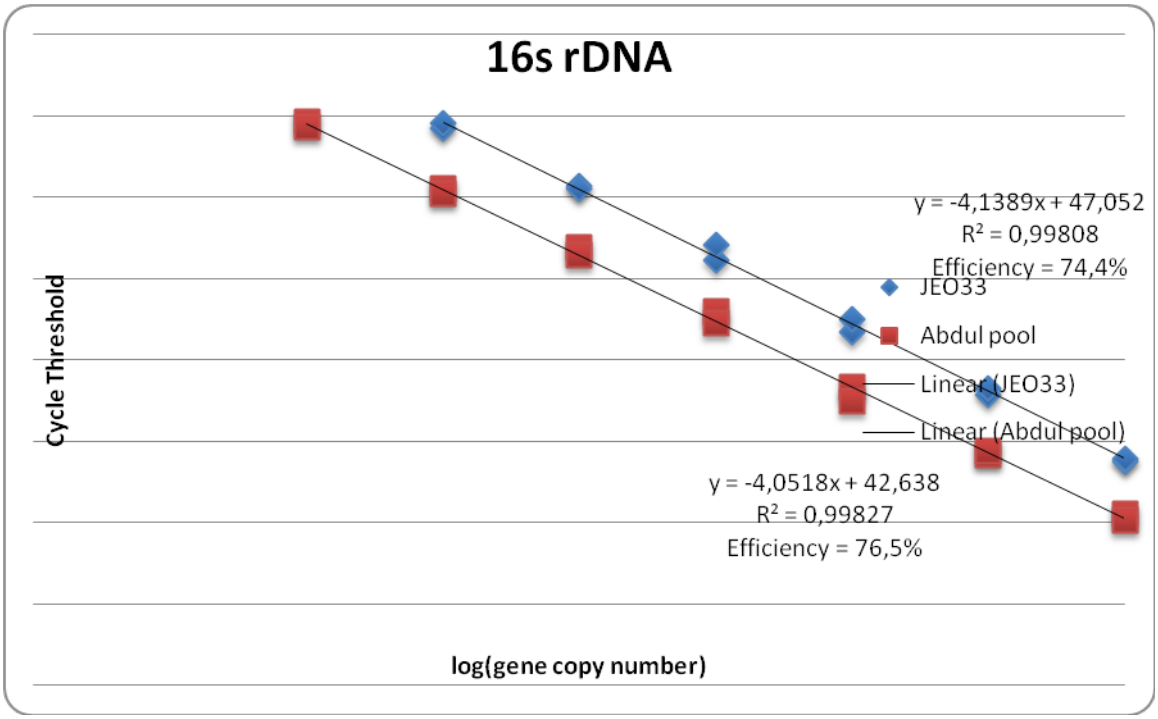


**Figure S 5.3: Standard curve for quantification of 16s rDNA**

# CHAPTER SIX

## 6.0 ANTIMICROBIAL RESISTANCE IN BUFFALO, WILDEBEEST AND ZEBRA GRAZING TOGETHER WITH AND WITHOUT CATTLE IN TANZANIA (Manuscript No 4)

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## 6.1 Abstract

**Aim**

In multipurpose conservation areas, wild life animals and pastoral people who keep livestock are in close contact. The aim of the current study was to determine whether the practice of co-grazing with cattle constitutes a risk of transmission of antibiotic resistant bacteria to wild ungulates.

## 6.2 Methods and Results

Fecal samples were collected from buffalo (n=35), wildebeest (n=40), zebra (n=40) and cattle (n=20) from Mikumi National Park, Tanzania (MNP), where cattle is prohibited and from Ngorongoro Conservation Area (NCA) where co-grazing is practiced. The number of coliforms and enterococci resistant to selected antibiotics was determined on MacConkey and Slantez Bartley agar with and without antibiotics. Wild life generally harbored higher number of resistant *E. coli* and Enterococci than cattle, but with no general influence in wild life of co-grazing with cattle, and isolates from wild life in NCA were not more resistant to the tested antibiotics than wild life from MNP. Vancomycin resistant enterococci were detected in 7.4 % of wild life samples and *E. coli* resistant to cefotaxime and enrofloxacin were observed among isolates from all wild life, but not from cattle. Culture independent estimates of the number of *sulII* gene-copies obtained by qPCR did not differ between wildlife from the two sample sites, while *tetW* was significantly higher in samples from MPN than from NCA.

### 6.2.1 Conclusions

Grazing together with cattle did not increase the likelihood of finding resistance bacteria in wild ungulates. Surprisingly, such animals were shown to carry bacteria resistant to

critically important antibiotics, such as 3rd generation cephalosporin, flourquinolone and vancomycin.

### 6.2.2 Significance and impact of study

The practice of co-grazing has been viewed as potentially dangerous to both cattle and wild-life, however the current study indicates that transmission or antibiotic resistant bacteria is an infrequent event. Wild ungulates may be viewed as a reservoir of bacteria resistant to critically important antibiotics.

**Keywords**

Buffalo, Wildebeest, Zebra, Cattle, antibiotic resistance, Tanzania

## 6.3 Introduction

In recent years it has been realized that wild animals often carry antibiotic resistant bacteria. Healthy wild scavenging birds such as seagulls (Radhouani *et al.*, 2009) and birds of prey such as buzzards (Radhouani *et al.*, 2012) have been found to carry surprisingly high numbers of antibiotic resistant bacteria in the absence of any known contact with antibiotics. Such bacteria are mainly believed to originate in humans and domesticated animals (Skurnik *et al.*, 2006), and as such they form an indirect proof of transmission of either the resistant bacteria themselves or horizontal gene-transfer of resistant genes. Intensification of human activities within the habitats of wild animals increases the risk of interspecies disease transmission, and areas of high human density or intense research and ecotourism activities expose wildlife to a high risk of disease spillover from humans and livestock (Benavides *et al*., 2012). It has been suggested that studies on the presence of acquired resistance traits in the commensal bacteria of wildlife animals can be used to obtain an indirect estimate of this risk (Pallecchi *et al*., 2008).

*Escherichia coli* and *Enterococci spp* bacteria are normal inhabitant of the gastrointestinal tracts of animals and human beings (Lester *et al.*, 2006**)**. Since they can be found in almost all animal species they have been used as indicator bacteria of antimicrobial resistance in a population (Vila *et al.*, 1999; Bager, 2000). The importance of these bacteria is their ability to acquire and disseminate resistance that could be transmitted to pathogenic or zoonotic bacteria (Kim *et al*., 2001), and in general resistance levels in these indicator bacteria has been found to be a good indicator of the selection pressure in an area (Aarestrup, 1999; Byarugaba *et al*., 2011).

Tanzania had a high number of wildlife animals, and wildlife tourism constitutes an important part of the economy. The animals are located in fifteen national parks as well as conservation areas, such as the world known Ngorongoro Conservation Area (NCA) and game reserves. The latter is Tanzanian's most visited protected area, and in addition it is a multiple land use zone inhabited not only by the wildlife but also by the pastoral Massai people who keeps Tanzania Short Horned Zebu cattle in high numbers. Within NCA the main wild ungulates, wildebeest (*Connochaetes taurinus)*, zebra (*Equus burchelli)* and buffalo (*Syncerus caffer)* interact with Massai people and their cattle in the grazing land and at water points (Voeten and Prins, 1999; Charnley, 2005). While antibiotic resistance has been observed in African wild primates (Rolland *et al*., 1985), mountain gorillas (Rwego *et al*., 2008), wild boars (Literak *et al*., 2009, and Mangoose (Pesapane *et al*., 2013), there is limited information available on the levels of antibiotic resistance from wild ungulates, and in particular how this is influenced by sharing grazing land with domesticated cattle and the general environment with the pastoral people. The aim of this study was to determine the prevalence of resistance to antimicrobial agents in indicator bacteria *E. coli* and *Enterococci spp* isolated from faecal samples of wild ungulates, cattle grazing with wild ungulates and whether this was influenced by grazing together with

cattle in NCA as compared to the same species of animals grazing in Mikumi Ntional Park (MNP), where cattle are strictly prohibited to graze and where there are little interactions with humans.

## 6.4 Materials and Methods

### 6.4.1 Research permit

The research permits needed to perform this study was obtained from Tanzania Commission for Science and Technology (COSTECH) (permit No. 2010-324-NA-2010-161 dated 1st November, 2010) to carry out research for one year in NCA and MNP in collaboration with Tanzania Wildlife Research Institute (TAWIRI).

### 6.4.2 Study area

The study was carried out in NCA located in northern part of Tanzania (Fig. 6.1; appendix 4) with GPS-coordinates of S3 11 13.992 E35 32 26.988 (UNESCO, 2011) between November 2010 and October 2011. The NCA has a population of about 25,000 large animals (Berry, 2009), including the three species researched in the current study, wildebeest zebra and buffalo. As a comparison, the same three species of animals were studied in MNP with GPS-coordinates of 7°12´S 37°08´E (Fig. 6.1; appendix 4). MNP is the fourth-largest park in Tanzania, and part of a much larger ecosystem centered on the uniquely vast Selous Game Reserve (TANAPA, 2013). Cattle are prohibited within the boarders of this park.

### 6.4.3 Sample sources and sampling

Freshly voided faecal materials (3-5 g) were collected from 35 Buffalo, 40 Wildebeest, 40 Zebra and 20 Zebu cattle (Table 6.1). After collection, samples were stored in sterile

containers, stored in the cool box at 4°C and transported to laboratory and analysis was carried out immediately.

### 6.4.4 Enumeration of antibiotic resistant bacteria

Ten fold serial dilutions of faecal material were made in phosphate buffered saline (PBS). 100 µl of the suspensions were spread on MacConkery agar (Oxoid, Basingstoke, Hants, UK) with CTX, TE, AMP and STX antibiotics in the following concentrations: 2mg/L, 16mg/L, 16mg/L and 256mg/L respectively or without antibiotics for enumeration of total coliforms. Enumeration of *Enterococci* was carried on Slanetz-Bartley agar (Oxoid, Basingstoke, Hants, UK) with AMP (16mg/L) and VA (16mg/L) antibiotics and on plates without antibiotics. McConkey plates were incubated for one day and Slantez-Bartley plates for two days at 37°C. In statistical comparisons, samples below the detection limit were given a valued of 0.1 $Log_{10}$ CFU.

### 6.4.5 Bacterial species identification

One to two colonies with typical colony morphology from each plate were sub-cultured in order to get pure cultures. These cultures were stored at -80 °C until further analysis. Identification of lactose positive colonies from McConkey agar to species level was performed using Gram stain and, the IMViC tests (indole, methyl red, Voges Proskauer and citrate test) using standard methods. *Enterococcus* species identification was obtained by multiplex PCR according to Dutka-Malen *et al.* (1995). Matrix-assisted laser desorption-ionization (MALDI) time-of-flight mass spectrometry (TOF/MS (Jackson and Lay, 2001) was used to identify isolates that did not show typical reactions in biochemical test or multiplex PCR.

### 6.4.6 Antibiotic sensitivity testing

Isolates obtained from McConkey and Slanetz Bartley plates without antibiotics were subjected to antimicrobial susceptibility testing by discs diffusion method (CLSI, 2008). For *E. coli* the antimicrobials constituted of sulphamethoxazole/trimethoprim (STX 25 µg), ampicillin (AMP 10 µg), amoxycillin-clavulanic acid 2:1 (AMC 30 µg), gentamicin (CN 10 µg) tetracycline (TE 30 µg), enrofloxacin (10 µg) and cefotaxime (CTX 30 µg). For *Enterococci spp*, antimicrobials were sulphamethoxazole/trimethoprim (STX 25 µg), erythromycin (E 15 µg), ampicillin (AMP 10 µg), rifampicin (RD 5 µg), gentamycin (CN 120 µg) and tetracycline (TE 30 µg). The reference strains *E. coli* ATCC 25922 and *Enterococcus faecium* ATCC 2912 *were* used for quality control (Byarugaba *et al*., 2011). Results were recorded as susceptible (S)/intermediately susceptible (I) or resistant (R) according to CLSI (2008).

### 6.4.7 DNA isolation

Ten fold serial dilutions of faecal material were made in phosphate buffer saline (PBS). After thorough vortex, 200 µl were used for DNA extraction with QIA amp DNA Stool Mini Kit (Cat 51504; © 2001 QIAGEN) following its manufactures instructions. Elution buffer (200 µl) was used to elute DNA and was stored at -20 °C until use.

### 6.4.8 Quantitative-PCR

Quantitative PCR (qPCR) was performed using previously designed primers for the quantification of *sulII* (*sulII*-Fw: TCCGGTGGAGGCCGGTATCTGG ; *sulII*-Rv: CGGGAATGCCATCTGCCTTGAG), *tetW* (*tet(W)*-Fw: GAGAGCCTGCTATATGCCAGC; *tet(W)*-Rv: GGGCGTATCCACAATGTTAAC) and 16s rRNA (1369-Fw: CGGTGAATACGTTCYCGG ; 1492-Rv: GGWTACCTTGTTACGACTT) .

The qPCR reactions were performed using a Stratagene MX 3000p PCR cycler with 25 µl reactions (12.5 µl Maxima SYBR Green/ROX qPCR Master mix (Fermentas). Standard curves (Supplementary material Fig. S 6.1 – S 6.3) of the respective genes were used for calculation of the gene copy number per gram dung sample. The calculations were performed by using the linear trend line for standard calculations to obtain the mean value of the content in the qPCR tube (Fig. S 6.4). The prediction interval of the sample content was calculated using the formulas developed by .

### 6.4.9 Statistical analysis

Statistical Package for Social Science software (SPSS) version 16.0 packages for windows ® was used for analysis of antibiotic discs data by chi-square test. The test for independence, with degree of freedom, where applicable, was used to compare the frequencies of resistance to antimicrobial agents amongst samples isolates from pairs of sources. SAS version 9.1 (SAS, 2011) and was used to analyze overall variation in CFU and number of genes from qPCR using ANOVA with Duncan's post-modification test. Excel sheets were used to determine the prevalence rates.

## 6.5 Results

### 6.5.1 Species composition of bacteria obtained from McConkey and Slanetz-Bartley agar

The value of indicator bacteria relies heavily on the assumption that bacteria compared from different animals are made up of the same species composition. To ensure that this was the case, 120 presumptive *E. coli* and 120 presumptive *Enterococcis spp* were randomly selected and identified to species level. All colonies with the typical morphology selected from McConkey agar were demonstrated to be *E. coli*. The species distribution of the *Enterocooci* isolates is shown in Table 2. Out of 120 isolates, 95 (79.2 %) belonged to

*E. faecium* the rest of 20.8% of isolates included *E.faecalis* (7.5%), *E. gallinarum* (5.8%) and *E.hirae* (7.5%). *E. gallinarum* was found in Wildebeest and Zebra and *E.hirae* was found in all wild ungulates, but not in cattle. No significant difference in species composition was observed between animals from NCA and MNP (data not shown).

### 6.5.2 Enumeration of resistant bacteria from wild animals and cattle

Where possible, enumeration of *E. coli* and *Enterococci spp*. resistant to the selected bacteria was performed by direct plating, and the number of bacteria resistant to each antibiotic was compared between samples obtained in NCA (interaction with cattle) and samples from MNP (without interaction). As can be seen in Table 6.3, the CFU was below the detection level of 100 CFU/gram in many samples, surprisingly most commonly in cattle, and levels of antibiotic resistant bacteria did not differ significantly between the three wildlife species. CFU of resistance bacteria were generally lower in cattle, although the differences were not statistically significant, but as the total number of *E. coli* was also lower from this animal species, the proportion of the total number that were made up of resistant coliforms was very similar in wildlife and cattle (data not shown). $Log_{10}$ CFU of ampicillin resistant *E. coli* was significantly higher ($P<0.05$) in wildlife samples from NCA (2.65±0.29) compared to wildlife samples from MNP (1.63±0.26), while cefotaxime resistance was more common in samples from MNP ($P<0.01$; NCA 0.36±0.23, MNP 1.14±0.21). There was no significant difference in tetracycline resistance in wildlife between the two study sites while $Log_{10}$ CFU of sulphamexazole resistant bacteria was higher in samples from MNP (1.84±1.71) compared to NCA (1.09±1.17). The total number of coliforms in wildlife from MNP (5.65±0.18) was significantly higher than the number of coliforms in wildlife from NCA (3.48±0.20).

Vancomycin and Ampicillin resistant *Enterococcus spp.* were not observed in samples from cattle, while a total of 10 wildlife animals carried VRE and ampicillin resistant enterococci (Table 6.4). The VRE were found in all three wildlife species. Though on average the resistance was low, the $Log_{10}$ CFU of ampicillin resistant *Enterococci spp* was found to be significantly higher ($P<0.05$) in samples from MNP (0.81±0.19) compared to NCA (0.09±0.23).

### 6.5.3 Susceptibility testing of commensal E. coli and *Enterococci spp* from wildlife and cattle

In addition to CFU determination, we determined the resistance profiles of individual isolates obtained from selective plates without antibiotics. Wildlife isolates of *E. coli* (Table 6.5) from MNP were more frequently resistant to tetracycline ($p=0.03$), gentamycin ($p<0.001$) and enrofloxacin ($p<0.0001$) than isolates from NCA. Significant differences were observed between animal species, for examples ampicillin resistance was significantly more common in isolates obtained from Buffalo than the other species investigated. Notably, *E. coli* resistant to cephalosporin and flourquinolone were observed among the randomly obtained isolates from all wildlife, but not from cattle.

Randomly obtained isolates of *Enterococci* from wildlife animals in NCA and MNP only differed with respect to sulfa-thrimethoprim resistance with isolates from NCA showing the highest prevalence (Table 6.6). Contrary to isolates of *E.* coli, there was no significant difference in resistance level between animal species.

### 6.5.4 Quantification of resistance genes tetW and sulII by qPCR

The copy number of *sulII* genes did not differ in wildlife between the two locations whether this was expressed as absolute numbers of as a proportion of 16s rRNA copy

number (Table 6.7). In contrast animals from NCA had a higher number of *tetW* genes compared to MNP, both expressed in absolute numbers and relatively to 16s rRNA copy number. The flora of cattle and buffalo was shown to contain a much higher proportion of *tetW* genes than the wildebeest and zebra, and in particular wildebeest had a low number of this gene.

## 6.6 Discussion

Wild animals, particularly species that live in close association with humans, may be exposed to resistant bacteria in their environment, and antimicrobial resistance has been detected in fecal bacteria from a variety of wild animals, including birds, reptiles, mammals, and fish, throughout the world (Rolland *et al.*, 1985, Sayah *et al.*, 2005, Jardine *et al.*, 2012). Surprisingly, in the current study, a quit high numbers of antimicrobial resistant *E. coli* and *Enterococcus spp*. strains were observed from free-ranging buffalo, zebra and wildebeest. Included in this was *E. coli* showing resistance to cephalosporin, and showing synergy with clavulanic acid, which is the classical test for ESBL production. Further studies are indicated to elucidate which ESBL genes are carried by these isolates. Also the study demonstrated the presence in wild life of flouroquinolone resistant *E. coli*, and enterococci with resistance to vancomycin or ampicillin.

Our observations cannot be explained by direct exposure to antibiotics, since the wild life authorities maintain a strict policy of none interference with wild life. Co-habitation with livestock in grazing and drinking water points was therefore hypothesized to be a contributing factor, and we designed the current study to investigate whether such interaction was a risk factor for carriage of antibiotic resistant bacteria in wild life. However, we did not observe a general higher level of antibiotic resistant bacteria in wild life from NCA, where such interaction is taking place, compared to MNP where wild life

does not normally interact with cattle. Based on the results, we concluded that the practice of co-grazing did not increase the risk of transmission of resistant bacteria or resistance genes from livestock to wild life. Taken to its full consequences, this observation indicates that disease transmission between cattle and wildlife may not be a major problem for disease-causing agents that require faecal-oral transmission, unless the infective dose is very low.

A likely explanation for the presence of antimicrobial resistant bacteria in Buffalo and Zebra is the behavior of these animals. Like has been reported for baboons (Rolland *et al.*, 1985), they were frequently found in close proximity of staff houses, offices, and lodges. It is possible that they come in contact with human refuse or even human excretes. Wildebeest on the other hand are much more timid animals and are afraid of human interactions. It is also known, however, that colonization of the intestinal tract by resistant bacteria can occur even in the absence such selection pressures, particularly as a result of contact with resistant bacteria in food or on environmental fomites (Adesiyun and Downes, 1999; Wright, 2010). There are a magnitude of flying birds in both NCA (over 500 species) and MNP (more than 400 species) (TANAPA, 2013) and around natural and artificial constructed water points, these come into contact with mammals. Healthy wild scavenging birds have been found often to carry surprisingly high numbers of antibiotic resistant bacteria (Radhouani *et al.*, 2009, Radhouani *et al.*, 2012). These birds could also be responsible of spreading resistant bacteria from one point to another (Adesiyun and Downes, 1999).

Grazing behavior of wild animals differ from that of cattle. They cover a large area within the NCA compared to that of cattle. They also cross the Tanzanian border and interact with other wild animals and cattle in Kenya. In this scenario, it is possible for wild animals

to feed on different vegetation (Ushimaru *et al.*, 2007) and get contact with more environments that have been contaminated by birds and animals faeces containing resistant bacteria (Adesiyun and Downes, 1999), and this may explain our observations. A more theoretical explanation is that plants rich in a variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids may select for antibiotic resistance, since they have been found *in vitro* to have antimicrobial properties (Cowan, 1999, Lewis and Ausubel, 2006), However, further studies are needed to investigate whether these groups of animals share resistance genes including humans within the same ecological niche.

Antibiotic resistant levels were found to be higher in wild animals compared to cattle, even though these are occasionally treated with antibiotics. A weakness of the current study is the lack of data on consumption of antibiotics in these cattle. However, such data does not exist and cannot be readily obtained due to lack of records. A recent study on antibiotic use in Tanzania showed that cattle are generally treated with oxy-tetracycline and sulphonamides as the most common drug, and that use of cefalosporin and flourquinolones cannot be afforded by the poor cattle keepers (Katakweba *et al.*, 2012). Hence, the presence of indicator bacteria resistant to such drugs is more likely to be caused by interaction with humans than with cattle.

Analogous to our investigation, Rwego *et al.* (2008) conducted a study to see if habitat overlap can increase the risks of anthroponotic and zoonotic pathogen transmission between humans, livestock, and wild apes, and found that contact with humans and/or livestock was indeed a risk factor for transmission of bacteria. *E. coli* were collected from humans, livestock, and mountain gorillas (*Gorilla gorilla beringei*) in Bwindi impenetrable National Park, Uganda. Gorilla populations that overlapped at high rates with people and livestock in their use of habitat harbored *E. coli* that was genetically

similar to *E. coli* from those people and livestock. The strains obtained from such animals were resistant to at least one antibiotic used by local people, and the proportion of individual gorillas harboring resistant isolates declined across populations in proportion to the decreasing degrees of habitat overlapping with humans. We have not performed genetic fingerprinting of the strains obtained in the current study, for the reason that we think the sampling was too insensitive to show more than general trends, i.e., it is very unlikely that we will detect genetically similar strains.

Human commensal and pathogenic bacteria with antibiotic resistance have been detected in wild animals, such as rodents (Gilliver *et al.*, 1999), primates (Rolland *et al.*, 1985; Rwego *et al.*, 2008), marine mammals (Schaefer *et al.*, 2009), and wild birds (Blanco e*t al.*, 2009; Bonnedahl *et al.*, 2009; Dolejska *et al.*, 2009). Since these animals do not receive antibiotics and direct contact with human or animal carriers of antibiotic-resistant bacteria is not likely, this finding suggest uptake of resistance from the environment. Through wild life antibiotic resistance can spread geographically, even to remote areas where antibiotics are not available (Gilliver *et al.*, 1999; Sjolund *et al.*, 2008).

To investigate whether resistance levels in general differed between species and between the two groups of wild animals, i.e. those grazing with and those grazing without cattle, quantification by culture independent qPCR was performed for one common tetracycline resistance gene and one sulphonamide resistance gene. qPCR has previously been used in several investigations to quantify levels of resistance genes (Pei *et al.*, 2006; Momtaz *et al.*, 2006). The chosen gene *tetW* is chromosomally encoded and common in Gram positive bacteria (Scott *et al.*, 1997). It has also been shown to be common in cattle (Peak *et al.*, 2007), and it is present in the human gut flora (Scott *et al.*, 1999). *sulII* represents the most common sulphonamide resistance genes in *E. coli* from many species of animals

and in man (Trobos *et al.*, 2008). No significant increase in gene pools were associated with co-grazing with cattle, supporting our observations from phenotypic based analysis, but in contrast to that investigation, the level of resistance (both expressed as absolute numbers of genes and number of genes relative to 16s rRNA) was not higher in wildlife compared to cattle, suggesting that the phenotypic tests highlight differences due to other genes that the two investigated by qPCR.

In conclusion, the results of the present study have demonstrated unexpected presence of antibiotic resistant *E. coli* and enterococci in wildlife animals in the absence of antibiotic pressure. Wild animals grazing together with cattle that are actively treated with antibiotics did not generally show higher levels of resistance, indicating that co-grazing does not result in transmission of resistant bacteria. This kind of situation need further research on both cattle and wildilife also humans who are carring their activities within that area.

### 6.7 Aknowledgements

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**Table 6.1: Number and sources of samples from wildlife and cattle**

| Sources of samples | NCA | MNP | Total |
|---|---|---|---|
| Buffalo | 15 | 20 | 35 |
| Wildebeest | 20 | 20 | 40 |
| Zebra | 20 | 20 | 40 |
| Zebu cattle | 20 | - | 20 |
| **Total** | **75** | **60** | **135** |

NCA: Ngorongoro Conservation Area. MNP: Mikumi National Park

**Table 6.2: Species composition (N) of *Enterococci* spp isolates**

| Source | *E. faecium* | *E.faecalis* | *E. gallinarum* | *E.hirae* | Total |
|---|---|---|---|---|---|
| Buffalo (N = 29) | 23 | 2 | 0 | 4 | 29 |
| Wildebeest (N = 37) | 29 | 3 | 2 | 3 | 37 |
| Zebra (N = 40) | 30 | 3 | 5 | 2 | 40 |
| Cattle (N = 14) | 13 | 1 | 0 | 0 | 14 |
| **TOTAL (%)** | **95 (79.2)** | **9 (7.5)** | **7 (5.8)** | **9 (7.5)** | **120** |

**N = Number**

**Table 6.3: Quantification ($Log_{10}$ CFU) per gram ±SE of resistant *E. coli* from wildlife species and cattle**

| Antibiotics/Species | AMP | TE | CTX | STX | No antibiotic |
|---|---|---|---|---|---|
| Buffalo | 2.42±0.33[a] (22/35) | 1.56 ±0.28[a] (17/35) | 1.00±0.25[a] (9/35) | 1.99±0.3[a] (21/35) | 4.84±0.25[a] (35/35) |
| Zebra | 2.04±0.33 [a] (21/40) | 1.15±0.28[a] 13/40) | 0.39±0.25 [a] (5/40) | 1.43±0.3[ba] 15/40) | 4.25±0.24[a] (40/40) |
| Wildebeest | 1.91 ±0.33[a] (20/40) | 0.78±o.28 [a] (9/40) | 0.93±0.25 [a] (8/40) | 1.27±0.3[ba] (14/40) | 4.60±0.25[a] (40/40) |
| Cattle | 1.95±0.52[a] (8/20) | 0.68±0.44 [a] (4/20) | 0.29±0.4 [a] (2/20) | 0.62±0.48[b] (3/20) | 3.14±0.39[b] (20/20) |

Groups with the same suprascript are not statistically different. Number in brackets shows how many samples out of tested samples that had CFU above the detection level of 100 CFU/g. Samples below this level were assigned a value of 1 CFU/g for statistical purposes.

**Table 6.4: Quantification ($Log_{10}$ CFU) per gram ±SE of resistant *Enterococci spp.* from wildlife species and cattle**

| Antibiotics/ Animal | Vancomycin | Ampicillin | No antibiotic |
|---|---|---|---|
| Buffalo | 0.21±0.23[a] (1/35) | 0.66±0.26[a] (4/35) | 3.76±0.34[a] (35/35) |
| Zebra | 0.60 ±0.19[a] (8/40) | 0.55±0.21[a] (4/40) | 3.85 ±0.28[a] (40/40) |
| Wildebeest | 0.24±0.2[a] (1/40) | 0.22±0.22[a] (2/40) | 4.35±0.29[a] (40/40) |
| Cattle | 0.00[a] (0/20) | 0.00[a] (0/20) | 2.29±0.51[b] (20/20) |

Groups with the same superscript were not statistically different. Number in brackets show how many animals out of tested that had CFU above the detection level of 100 CFU/g. Samples below this level were assigned a value of 1 CFU/g for statistical purposes.

**Table 6.5: Antibiotic susceptibility of *E. coli* from wildlife and cattle in NCA and MNP (%)**

| AB | Group | NCA | MNP | Pvalue | BFL | WLB | ZBR | P value |
|---|---|---|---|---|---|---|---|---|
| TE | R | 16 (24.6) | 23 (42.6) | 0.030 | 20 (64.5) | 8 (23.5) | 11 (26.8) | 0.0002 |
| | I/S | 49 (75.4) | 31 (57.4) | | 11 (34.5) | 27(76.5) | 30 (73.2) | |
| STX | R | 17 (25.8) | 15 (27.8) | 0.957 | 13 (41.9) | 11 (32.4) | 8 (19.1) | 0.0335 |
| | I/S | 49 (74.2) | 39 (72.2) | | 18 (58.1) | 23 (67.4) | 34 (70.9 | |
| CTX | R | 13 (19.7) | 9 (16.7) | 0.904 | 9 (29) | 8 (23.6) | 5 (11.9) | 0.231 |
| | I/S | 53(80.3) | 45 (83.3) | | 22 (71) | 26 (76.4) | 37 (88.1) | |
| CN | R | 4 (6.1) | 15 (27.8) | 0.0003 | 1 (3.2) | 7 (20.6) | 10 (23.8) | 0.050 |
| | I/S | 62 (93.9) | 39 (72.2) | | 30 (96.8) | 27 (79.4) | 32 (76.2) | |
| AMP | R | 32 (48.5) | 25 (46.3) | 0.352 | 12 (38.7) | 20 (58.8) | 20 (47.6) | 0.375 |
| | I/S | 34 (51.5) | 29 (53.7) | | 19 (61.3) | 14 (41.2) | 22 (52.4) | |
| ENR | R | 3 (4.6) | 23 (42.6) | <0.0001 | 8 (25.8) | 10 (29.4) | 8 (19) | 0.295 |
| | I/S | 63 (95.4) | 31 (57.4) | | 23 (74.2) | 24 (70.6) | 34 (81) | |
| AMC | R | 10 (15.2) | 9 (16.7) | 0.549 | 9 (29) | 5 (14.7) | 5 (11.9) | 0.236 |
| | I/S | 56 (84.8) | 45 (83.3) | | 22 (71) | 29 (85.3) | 37 (88.1) | |

STX = Sulphamethoxazole/trimethoprim, CTX= Cefotaxime, TE= Tetracycline, AMP= Ampicillin, CN= Gentamycin, ENR= Enrofloxacin, AMC= Amoxycillin-clavulanic acid A: category R: resistant, I: intermediate, S: Sensitive. B: NCA: Ngorongoro conservation area, wildlife only. MNP: Mikumi National Park, wildlife only. BLF: buffalo, CTL: cattle, WLB: wildebeest; ZBR: zebra. C: P-value for the nil-hypothesis that the groups are not significantly different.

**Table 6.6: Antibiotic susceptibility of *Enterococci spp* from wildlife and cattle in NCA and MNP (%)**

| AB | Group | NCA | MNP | P value | BFL | ZEB | WLDB | P value |
|---|---|---|---|---|---|---|---|---|
| TE | R | 29 (42) | 18 (30.5) | 0.338 | 15 (44) | 15 (36.6) | 10 (26.3) | 0.294 |
| | I/S | 40 (58) | 41(69.5) | | 19 (56) | 66 (63.4) | 28 (73.7) | |
| STX | R | 22 (31.9) | 14 (23.7) | 0.015 | 10 (29.4) | 14 (34.2) | 8 (21.1) | 0.625 |
| | I/S | 47 (68.1) | 45 (76.3) | | 24 (70.6) | 27 (65.8) | 30 (78.9) | |
| RD | R | 29 (42) | 28 (47.5) | 0.172 | 19 (55.9) | 19 (46.4) | 15 (39.5) | 0.329 |
| | I/S | 40 (58) | 31 (52.5) | | 15 (44.1) | 22 (53.6) | 23 (60.5) | |
| E | R | 26 (37.7) | 21 (35.6) | 0.774 | 14 (41.2) | 18 (43.9) | 10 (26.3) | 0.619 |
| | I/S | 43(62.3 | 38 (64.4) | | 20 (588) | 23 (56.1) | 28(73.7) | |
| CN | R | 24 (34.8) | 15 (25.4) | 0.363 | 10 (29.4) | 12 (29.3) | 13 (34.2) | 0.897 |
| | | 45 (65.2) | 44 (74.6) | | 24 (70.6) | 29 (70.7) | 25 (65.8) | |
| AMP | R | 16 (23.2) | 17 (28.8) | 0.554 | 12 (35.3) | 9 (22) | 10 (26.3) | 0.157 |
| | I/S | 53 (76.8) | 42(71.2) | | 22 (64.7) | 32 (78) | 28 (73.7) | |

R= Resistant I= Intermediate S= Susceptible, STX = Sulphamethoxazole/trimethoprim, CTX= Cefotaxime, TE= Tetracycline, AMP= Ampicillin, CN= Gentamycin, E= Erythromycin, RD= Rifampicin, AMC= amoxycillin-clavulanic acid A: category R: resistant, I: intermediate, S: Sensitive. B: NCA: Ngorongoro conservation area, wildlife only. MNP: Mikumi National Park, wildlife only. BLF: buffalo, CTL: cattle, WLB: wildebeest; ZBR: zebra. C: P-value for the nil-hypothesis that the groups are not significantly different.

**Table 6.7: $Log_{10}$ ±SE Gene copy numbers / gram sample**

| Sources and Animal spp | No of samples | *sulII* | *% of 16s rRNA* | *tetW* | *% of 16s rRNA* | 16rDNA |
|---|---|---|---|---|---|---|
| Mikumi | 49 | 5.59±0.10[a] | 0.36±0.30[a] | 6.08±0.12[b] | 0.56 ±1.2[b] | 8.84±0.10[b] |
| Ngorongoro | 56 | 5.39±0.10[a] | 0.41±0.27[a] | 7.14±0.11[a] | 5.32±1.14[a] | 9.49±0.10[a] |
| Zebra | 40 | 5.61±0.12[a] | 0.12±0.31[a] | 6.24±0.14[b] | 2.74±1.37[b] | 8.92±0.11[b] |
| Wildebeest | 32 | 5.54±0.13[a] | 0.47±0.37[a] | 6.44±0.13[b] | 0.79±1.49 [b] | 9.19±0.12[ba] |
| Buffalo | 33 | 5.30±0.13[a] | 0.63±0.36[a] | 7.29±0.15[a] | 4.31±1.47 [ba] | 9.51±0.13[a] |
| Cattle | 20 | 5.94±0.21[a] | 0.43±0.45[a] | 7.14±0.21[a] | 7.905±2.40[a] | 9.742±0.17[a] |

Superscript [a,b,c]: Numbers/proportions within each column bearing same letter are not significantly different at P<0.05


**Figure 6.1: Distribution and locations of Tanzania National Parks and NCA (TANAPA, 2013)**

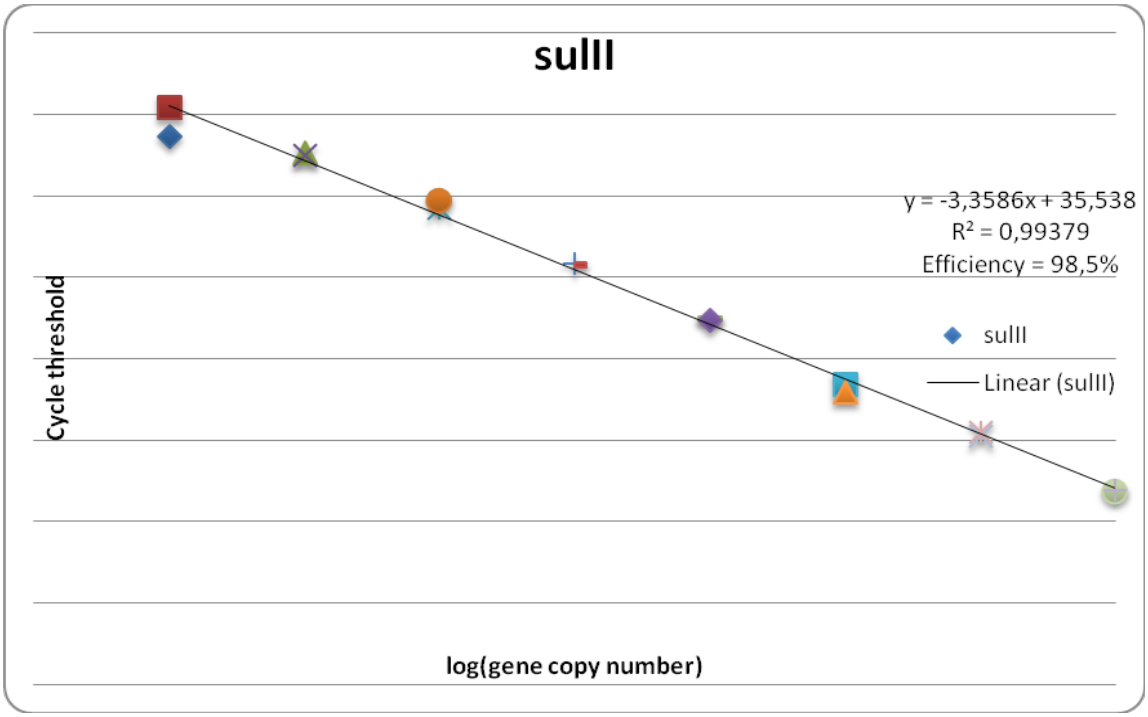


**Figure S 6.1: Standard curve for quantification of *sulII***

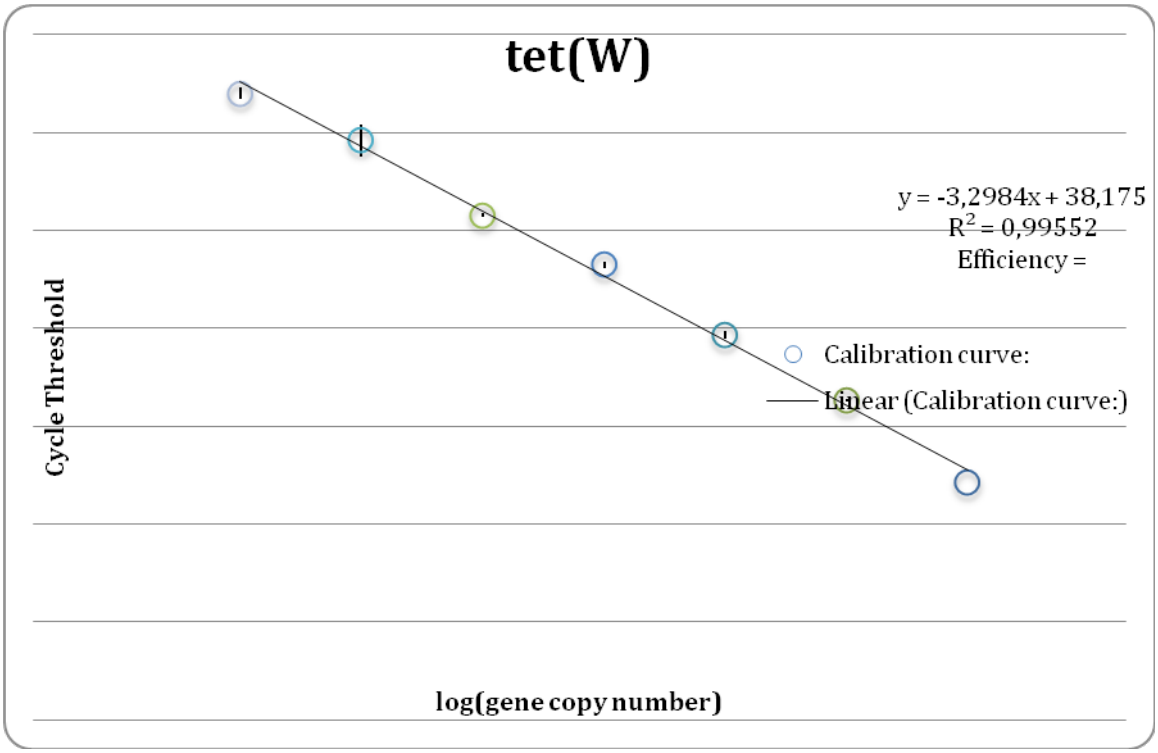


**Figure S 6.2: Standard curve for quantification of *tetW***

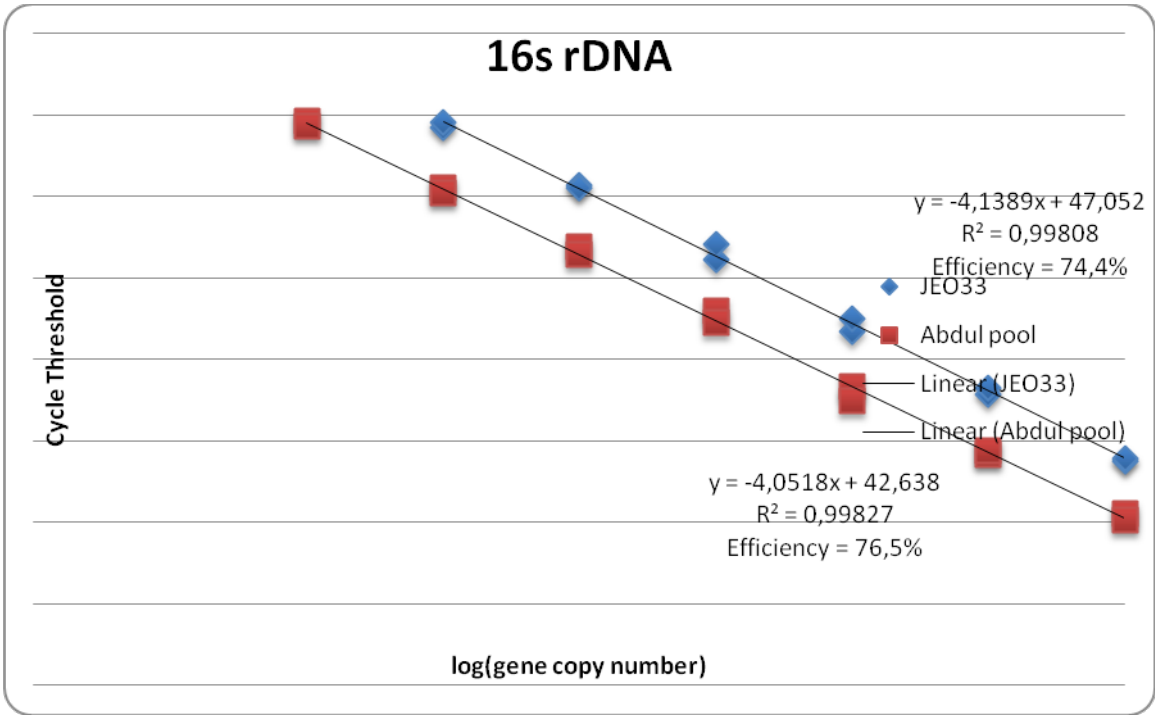


**Figure S 6.3: Standard curve for quantification of 16s rDNA**

# CHAPTER SEVEN

## 7.0 DISCUSSION

### 7.1 Overall Discussion

An overall understanding of antimicrobial resistance in a population can be obtained by studying the indicator bacteria namely *Escherichia coli, Enterococci spp* and *Staphylococcus aureus* isolated from apparently healthy animals (Nimalie *et al*., 2008; Manuscripts 2, 3 and 4). More insights on the antimicrobial resistance to commonly used drugs can also be investigated from pathogenic bacteria isolated from clinical and pathological samples (Mirzarazi *et al*., 2013). This further provides information about which antimicrobials the pathogens are susceptible to and can be prescribed for treatment (Kariuki, 2010; Byarugaba *et al*., 2011). Advent of DNA based techniques enabled epidemiologic typing of the strains which has allowed tracing of transmission of antibiotic

resistance genes across the bacteria species and animals species (Winokur *et al.*, 2001; Shoemaker *et al*., 2001; Osterberg and Wallinga, 2004). Apart from studying the science of AR understanding of knowledge of the people on the use of antimicrobials, perceptions and practices underlying their use (Katakweba *et al*., 2012) is also crucial to developing solutions to antimicrobial resistance. The present study investigated the antimicrobial resistance using indicator bacteria obtained from humans, chickens, livestock and selected wild animals (Manuscripts 2, 3 4). This provided baseline for risk management of the problem of antibiotic resistance in animals and on the other hand adding information to what is known about antimicrobial resistance in animals and human beings in Tanzania. This is the first study in Tanzania which provide data on prevalence and genomic studies of resistance of indicator bacteria to antimicrobials commonly used in human beings and animals (Manuscripts 2, 3).

A survey was carried out to livestock keepers from different livestock management systems (indoor, free range and grazing) found in Tanzania, to assess the perception of human health risks associated with the use of antibiotics among livestock keepers. It was also meant to unclear factors that contribute to selection of antibiotic resistance bacteria in Tanzania (Katakweba *et al*., 2012; manuscript 1). Findings of Chenggang *et al.* (2011), showed that lack of knowledge on prudent use of antibiotics and antimicrobial resistance in rural China influenced the use and misuse of antimicrobials. Rampant misuse of antimicrobials in Tanzania emerged as a potential risk factor for development of antimicrobial resistance to the commonly used drugs (Katakweba *et al*., 2012). Malpractices which included selling of veterinary drugs in open livestock markets, lack of prescription of veterinary drugs, and dispensing of drugs by untrained personnel were found in place across the different management systems of cattle which exist in Tanzania (Ong, 2011; Katakweba *et al*., 2012). Livestock management systems (indoor, free range and grazing), antibiotics handling and types of antibiotics used in the study areas were

identified as potential risk factors to the development of antimicrobials resistance in Tanzania (Katakweba *et al*., 2012). The findings concur with the observations by Chenggang *et al*. (2011) in rural China which framed a global health risk from the perception and practices regarding the use of antimicrobials in developing countries. Emergence of multidrug resistant pathogens from India (Nazir *et al*., 2012) and South Africa (Muller *et al*., 2011) has been shown in human pathogens, namely MRSA and *M. tuberculosis*. This multidrug resistance was through rapid genetic mutation which could be accounted to lack of prescription, non-compliance or unsupervised indiscriminate use of anti-tubercular drugs or other antibiotics. Sulphadimidines and tetracyclines were found to be the most commonly used antimicrobials in livestock in Tanzania (Katakweba *et al*., 2012). Subsequent demonstration of high prevalence of *sulII* and *tetW* genes among the human livestock and wild animals bacterial isolates suggest the selections of resistance to commonly used antimicrobials in the area (Manuscripts 2, 3, 4).

The findings fo this study underline the risk of transmission of resistance genes across the species, while shedding light for further investigations on the occurrence of these in wild animals with no history or evidence of antimicrobials exposure. Overt unawareness of the Tanzania livestock keepers about antibiotic resistance, and possible health effect that can happen when humans feed on the livestock products from the animal treated with antibiotics (Katakweba *et al*., 2012), call for deliberate measures be taken to educate the livestock keepers on antimicrobials use in animals. Addressing other management issues and feeding especially of farm animals would further reduce antimicrobials use without increasing cost to the farmers. The intensive use of antimicrobials agents in animal husbandry has spread into developing countries with consequent effect on human health and food safety (Garcés, 2002; Mayone *et al*., 2013). In the present study, antimicrobials were found to be intensively used in exotic chickens (Layers and broilers) and dairy cattle.

More use of antimicrobials was mainly due to indoor management system of these animals and to maximize the production for a short period of time (Katakweba *et al*., 2012). Antimicrobials are used in the livestock keeping systems in the study area for treatment (cattle, pigs and chickens), prophylaxis (pigs and exotic chickens) as growth promoters (exotic chickens). The impact of use of antimicrobials varies between countries and regions (Aarestrup, 1998). The variations are influenced by national and international trade, interaction between human and animal populations, contaminated land and water country national policies in relation to production, trade, food security and animal health (WHO, 2012; Mayone *et al*., 2013). The widespread and intensive use of antimicrobial agents in modern food production systems is a contributing factor to antibiotic resistance among bacteria. In Tanzania, it is more commonly found in urban and periurban areas compared to the pastoral communities due to the need of capture of the market of animal products (eggs, meat, animals and milk) in urban areas. Some developed countries such as Denmark have banned the use of growth promoters (DANMAP, 2010), where as in most of African countries including Tanzania there are no specific policies on growth promoters and are, still used (Katakweba *et al*., 2012; Mayone *et al*., 2013). Growth promoters have been proved to be a source of antimicrobial resistance to commensal bacteria and resistant commensal bacteria of food animals might contaminate, the environment and through animal products (meat, milk and eggs) can reach the intestinal tract of humans (van den Bogaard and Stobberingh, 2000; Rwego *et al*., 2008). This has great impact on human health because of transferring antibiotic resistant bacteria and resistance genes to human bodies (van den Bogaard and Stobberingh, 2000).

Three techniques namely colony forming units (CFU), antibiotic discs and quantitative polymerase chain reactions (qPCR) for resistance gene detection in gut flora bacteria were used in this study (Manuscripts No3 and 4) to get stronger results and biologically

looking at different aspects (Prescott *et al*., 2000; Rolain *et al.*, 2004; Pei *et al.*, 2006). All three methods were independent and gave the results of resistance to different antimicrobials against the indicator bacteria. Whereas CFU and antibiotic discs gave good and comparable results in antimicrobials tested, qPCR demonstrated the presence of *tetW* and *sulII* resistance genes which are linked to prolonged use of both tetracycline and sulphamethazole, respectively (Lathers, 2002; Carlos, 2010). Other antimicrobials like ampicillin, gentamycin and cefotaxime were also resisted by indicator bacteria although their genes were not quantified in this study (Manuscript No 3). Over and above these antimicrobials were also found in store in the study area (Katakweba *et al*., 2012). In the current study, it was surprised to find a quit high numbers of antimicrobial resistant *E. coli* and *Enterococcus spp*. observed from free-ranging buffalo, zebra and wildebeest. Co-habitation with cattle and other domestic animals in grazing and drinking water points was therefore hypothesized to be a contributing factor. It was finally found that co-grazing did not increase the risk of transmission of resistant bacteria or resistance genes from livestock to wild life.

Molecular typing studies were included to trace the possibility of transmission of antibiotic resistant bacteria by direct contact. *S. aureus* was used as an indicator organism; since numerous publications have demonstrated that this bacterium transfers from colonized animals to man, especially MRSA (Rubin *et al*., 2011; Espinosa-Gongora, 2012). MRSH was demonstrated in Tanzania in the current study through *mecC* gene (Manuscript No 2). These genes were detected in humans, pig and dog samples. There is a possibility of MRSH from one group to another as MRSA lineages that carry a novel *mecA* homologue (*mecC*) have been described in livestock and humans (Harrison *et al*., 2013; Spoor *et al*., 2013). Most of the types of *S. aureus* identified in Tanzania have been reported in other studies (Poeta *et al*., 2006; Rubin *et al*., 2011). A new *spa* type t10779

has however been identified and further studies on its *spa* and ST types are needed. Moreover, t084, t223 and t314 were found in both humans and dogs indicating crossing over of the bacteria from animals to humans (Rubin and Chirino-Trejo, 2010) and indicating that pet animals may be a hitherto overlooked reservoir for AR bacteria in man. In pigs only t131 *spa* type was isolated and was not shared by either dogs or humans beings (Manuscript No2).

The presence of *tetW* and *sulII* in the indicator bacteria in food animals and humans (Manuscripts 3, 4) is in line with previous findings which showed that *E. coli* from normal gut flora constitute an important reservoir of resistance genes. It has been suggested that these commensal bacteria constitute a reservoir of resistance genes for pathogenic and non-pathogenic bacteria (Kim *et al*., 2001; Nimalie 2008. These genes are also indicators for resistance against the types of antimicrobials used in the current study. Various studies have shown that the resistance genes are normally found soon after the introduction of antibiotics (Mathew *et al*., 2007). Further, these bacteria may act as reservoirs for genes that encode multidrug-resistant phenotypes which can be transferred to potential pathogens (Byarugaba *et al*., 2011). Finding antimicrobial resistant bacteria and resistant genes in the present studies strongly suggest that there is there is similarities of resistant strains and resistance genes between human and animal bacteria in the same enviroment (van den Bogaard and Stobberingh, 2000; Byarugaba *et al*., 2011; Manuscript No 3). Further studies should be conducted to find out if the isolates obtained from animals and humans sharing the same environment are genetically similar as it was carried out for *E. coli* isolated from children and poultry in Kenya (Kariuki *et al*., 1999), and Uganda (Rwego *et al*., 2008) which compared *E. coli* isolates from human and livestock sharing the same environment and found that the isolates were genetically similar.

Resistance in human diseases is strongly linked with the agricultural overuse of antibiotics and *antibiotic*-resistant bacteria (AR) can easily transfer their resistance traits to unrelated bacteria once inside the human body (Shoemaker *et al*., 2001). Resistant bacteria get into human bodies though food of animal origin (meat, milk and eggs), working with animals by handling animals, feed, and manure (Levy *et al*., 1976; Van den Bogaard *et al.*, 2002) and through the contaminated environment with human and animal wastes shared between animals and humans (Osterberg and Wallinga, 2004; Davies and Davies, 2010). Antibiotic resistant bacteria and antibiotic resistant genes are abundant in human and animal faecal material and can flow to and from environmental resistance reservoirs (Osterberg and Wallinga, 2004; Devies and Devies, 2010). Many resistance factors have been recruited from nonpathogenic bacteria that are circulating between animals, humans and environment (Bonomo and Szabo, 2006). In the present study food and pet animals were found sharing the same environment with humans. This could be one of the sources of antibiotic resistant bacteria to be trasfered from humans to animals and vice versa (Rwego *et al.*, 2008). Livestock attendants were handling animals, feed, and manure without protective gears (Lupindu *et al.*, 2012) and this could be another source of resistant bacteria and genes to be trasnfered from one group to another. Furthermore, animal products like milk, meat and eggs are used by humans and they contain or are contaminated with antimicrobial resistant bacteria or resistant genes that can be transferred to humans (Van den Bogaard *et al.*, 2002).

High levels of resistance was observed to the commonly used antimicrobials; tetracycline, penicillins, sulphadimidine and less resistance to those that are less commonly used in animal industry like cefotaxime and gentamycin (Katakweba *et al*., 2012). The same findings were also reported in Uganda by Byarugaba *et al*. (2011). Prolonged use of antibiotics exerts a pressure on the bacteria and resistance genes develop later on (Carlos,

2010). Levels of resistance to Gentamycin, Cefotaxime and Ciproflaxin observed in this study were comparable to the level of resistance in other countries (Bywater *et al*., 2004). With the exception of gentamycin used as intramammary infusions in cattle, no formulations of gentamycin, cefotaxin and ciproflaxin are available for use in livestock and chickens in Tanzania (Kivaria *et al*., 2006). The resistance may have been caused by the clonal spread of resistant isolates (Kijima-Tanaka *et al*., 2003) and co-resistance with other unrelated compounds or horizontal transfer of resistance genes, as humans and animals were sharing the same environment (Rwego *et al*., 2008).

High prevalence of antibiotic resistance and resistance gene were found in bacteria isolated from faecal samples of wild animals in NCA and MNP. It was surprising to detect antimicrobial resistant *E. coli* and *Enterococci spp* strains from free-ranging wildlife in buffalo, zebra and wildebeest. In the NCA ecotourism is more practiced compared to MNP and is important for conservation and economic growth. The association of human, cattle and other livestock wastes, which includes garbage as well as feces and waste water, may expose wildlife to human-associated pathogens and antibiotic resistance and vice versa, ultimately increasing future threats to human health (Pesapane *et al*., 2013). However, further investigations are required to explain the occurrence of resistance genes to commonly used antibiotics in wild animals with no history of drug usage. Buffalo and wildebeest are the preferred sources of bush meat, and they are utilized both legally and illegally in Tanzania. Household meat processing practices can provide widespread pathogen exposure risk to family members and the community, identifying an important source of zoonotic pathogen and antibiotic resistance bacteria transmission potential (Adesiyun and Downes, 1999; Alexander *et al*., 2012). There is a need for a unified approach in infectious disease research that includes consideration of both domestic and

wildlife sources of infection in determining public health risks from zoonotic disease invasions.

A study involving selected wild animals namely buffalo, zebra and wildebeest with no history of being treated with antibiotics (TANAPA, 2013) showed existence of antimicrobial resistant bacteria and resistant genes (manuscript No 4). There was further comparison of wild animals-livestock between the animals at Mikumi National Park and Ngorongoro Conservation Area. However the comparison did not show significant contribution of whether humans, domestic animals and birds are responsible for the spread in the environment with AR bacteria/genes to wild animals and therefore cannot be ascertained from the present study. Elsewhere, it has been shown that soil and water bodies are recipients, reservoirs, and sources of antibiotic resistance genes of clinical concern in the environment (Martinez, 2009; Wright, 2010). These bodies further receive inputs of antibiotics and antimicrobials from animals and humans, which can serve to amplify antibiotic resistant genes (CheeSanford *et al*., 2009; Heuer *et al*., 2011). The present study did not investigate the use or effect of these recipient bodies, further studies should be carried by taking soil, water and animal manure to gauge the of extent of antibiotic resistant bacteria and their resistant genes. Traces of metals such as Cu, Zn and As are commonly used in animal feeds as alternatives to antibiotics and antibiotic resistance can be co-selected by metals (Bolan *et al*., 2004; Berg *et al*., 2010; Knapp *et al*., 2011). Knap *et al*. (2011) using qPCR, quantified abundance antibiotic resistant genes (ARG) by comparing their levels with geochemical conditions in randomly selected soils from a Scottish archive. There was a significant correlation with soil copper levels also Chromium, nickel, lead, and iron significantly correlated with specific ARG. Therefore, metal abundances do influence the potential of AR in soil. There could be these factors at MNP and NCA in soils and water, however these were not investigated and future studies

like that of Knapp *et al*. (2011) can be conducted to ascertain their contribution to our current findings.

The findings of this study suggest that commensal enteric *E. coli* and *Enterococci spp* from healthy food animals and humans are an important reservoir for tetracycline and other antimicrobial resistance determinants used in the study (Sawant *et al*., 2007). The same trend has been reported in Kenya (Kivuki *et al*., 2006; Kariuki, 2010) and Uganda (Byarugaba *et al*., 2011). Slight differences that are observed between developing countries could be related to the different antibiotic regimens used for the different antimicrobial agents and livestock species (Mapenay *et al*., 2006; Ruzauskas *et al*., 2009). Resistance observed could be attributed to the use and misuse of antimicrobials because their relatively low cost and ready availability for sale 'over the counter' in many developing countries. In Tanzania there are over 1000 veterinary shops selling livestock drugs and out of that only 300 shops are registered. This kind of scenario aggravates the situation of antimicrobial misuse in the country (Masurli, 2014 personal communication) These drugs are widely used by farmers for therapeutic and prophylactic applications in most of developing countries (Tagoe and Atar, 2010; Miralles, 2010; Møller, 2011; Katakweba *et al*., 2012). This may also be attributed by the different formulations, the mode of administration, the different types of antibiotics used and the amounts of antibiotics used in the different food animal species and humans in the country.

The above mentioned informantion was based on the common perception, that the problems of antimicrobial resistance was primarily or exclusively being associated with the use and overused/misuse of antimicrobials in humans and animals (Katakweba et al., 2012; Aminov and Mackie, 2007). Recents studies have shown that resistant bacteria and resistance genes can also be contributed by the factors from environment, soils, water,

ecology and evolutions. Antimicrobial resistant microbe existed long before the beginning of the antibiotic era not related with antibiotic pressure but is a trait that is inherent to microorganism in nature (Kozhevin et al., 2013). The soil pathogenic and non pathogenic mocrorganisms have all genetic components of antibiotic resistant that are present in a given natural microbial community (Kozhevin et al., 2013). Even though the resistant bacteria of human and animal origin may die off in the environment, the endogenous environmental bacteria may pass the acquired resistance genes on to their progeny (Andersson and Levin, 1999). Resistant genes may remain present in the environment for a long time due to the presence of trace amounts of antibiotics in the environment, originating from treated humans and animals or from soil bacteria and will exert selective pressure (Zhang et al., 2009). Majority of antibiotic resistances are most likely cases of acquired resistance, through the lateral transfer of antibiotic resistance genes from other ecologically and taxonomically distant bacteria (Aminov and Mackie, 2007).

## 7.2 Conclusions and Future Perspectives

Misuse of antibiotics in livestock is high in Tanzania and is mainly attributed to lack of knowledge by the users in the study area. Antibiotics are prescribed, sold and used by unskilled personnel. Livestock keepers were not aware of the antimicrobial resistance, withdraw periods of antimicrobials and did not know that use of antibiotics can have effects on health of their animals and themselves. The situation is more alarming in the pastoral communities when compared to urbans and periurban areas because trained personnel are normally assisting in treatment regimes in these areas than in pastrol communities. These scenarios could contribute to development of antibiotic resistance in the study area. Dogs were shown to possibly be a risk factor for transfer of resistant *S, aurues* and *S. pseudintermedius.* Resistance to antimicrobials was more frequent in human isolates of *S. aureus* than in isolates from pigs and dogs.

The study has revealed that the most commonly used antibiotics in Tanzania including tetracycline, sulphamethaozole and ampicillin had higher antimicrobial resistance. These findings indicate that both resistance bacteria and genes are circulating in domestic animals, chickens and humans sharing the same environment due to use of those antimicrobials. There is high level of antibiotic resistance in the clones isolated and typed that circulate in the country in food animals, wild ungulates, pets and humans.

Wild ungulates including buffalo, zebra and wildebeest, their bacterial isolates indicators had been found to have resistant genes influencing resistance to commonly used antibiotic in Tanzania. This is an interesting finding as these animals have never been subjected to any kind of treatment regime. Therefore, wild ungulates are potential reservoirs for resistant bacteria and genes and can spread the resistance genes if they can share the same grazing pastures with cattle, sheep, goat and donkeys in the same environment with humans. Faecal samples from humans living in NCA and MNP should be accessed for their resistance patterns and compared to that found in cattle and wild ungulates.

There are no regular surveillances and monitoring of the use of antimicrobial in the country being most of veterinaty shops are not registered and livestock policies have no specific clauses emphasizing on how antibiotics should be used. Laws and the by laws are not emphasized in the use and misuse of antimicrobials in the livestock sector in the country. This has resulted in the selling of antimicrobials in the antimicrobial shops and livestock markets by untrained personnel. A national surveillance programme of antibiotics usage on the food-producing animals and antibiotic resistance is required to help in mitigating the problem of lack of availability of information. Local authorities have the mandate to oversia the whole issue of antimicrobial sales and usage as they

manage the livestock markets all over the country. Livestock markets have been famous as selling points of animal antimicrobials by untrained personel and this include handling and exposing the microbials in non professional contiditions.

Collaboration between veterinarians and human health professionals is essential to further our understanding of the ecology of this potential pathogen as the antimicrobial resistance in *E. coli*, *Enterococci* spp and *S. aureus* have little regard for species barriers. Monitoring the prevalence of resistance in indicator bacteria such as faecal *Escherichia coli*, *Enterococc*i *spp*, *S. pseudintermedius* and *S. aureus* should be conducted in different populations, animals, patients and healthy humans. Investigation of AR should also cover clinical, pathological and condemned carcasses. This will make it feasible to compare the prevalence of resistance and to detect transfer of resistant bacteria or resistance genes from animals to humans and vice versa.

Molecular studies should be conducted to ascertain the relationship of isolates from livestock, pets and humans if are genetically identical due to the fact that they are sharing the same environment.

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# APPENDICES

## Appendix 1: Questionaire used in Manuscript No 1

**A QUESTIONNAIRE TO LIVESTOCK KEEPERS ON VETERINARY ANTIBIOTIC USE IN MOROGORO AND DAR-ES-SALAAM REGIONS, TANZANIA**

**Personal particulars of the respondent**

Name of Participant_____ Date: _____ Region_____

1. Respondent biodata:
   Name: ……
   Sex: ……
   Age: ……
   Position in the family: Father ..... Mother ..... Daughter/son.....Others (Mention).....Occupation: ……..
2. Education level:
   a. Non formal education……
   b. Adult education……
   c. Primary education……..
   d. Secondary education (mention the level)……
   e. Certificate level.....
   f. Diploma level…….
   g. Degree level…….
   h. Others (mention) …….

4. What type of the following domestic animals/ chickens do you keep?

| Type animals/ birds | Number of animals/birds | Management system: specify | When did you start keeping animals? (yr) | Purpose of keeping animals / chickens? Commercial / subsistence |
|---|---|---|---|---|
| Layers | | | | |
| Broilers | | | | |
| Local chicken | | | | |
| Pigs | | | | |
| Dairy cattle | | | | |
| Dogs | | | | |

5. What are the common problems associated with the animals/ chickens you are keeping?
   a……
   b……
   c……..…..
   d……
6. (If diseases are mentioned in question 5); what are the major diseases/disease symptoms of animals/chickens do you encounter on your farm?
   ……
7. Who treats your animals/chickens once they fall sick?
……

8. What type of medications do you give to your animals/ chickens and

| Tick (√) | Type of medication | Who prescribes? | Who administers | Who buys the medications | Frequency per month |
|---|---|---|---|---|---|
| | Antibiotics | | | | |
| | Antihelminths | | | | |
| | Anticoccidial | | | | |
| | Growth promoters | | | | |
| | Vaccines | | | | |
| | Disinfectants | | | | |

9 (a). If antibiotics are mentioned in 8 above, mention the common antibiotic you use in your animals/chickens?
   ……
9 (b) If you normally buy and keep drugs of your animals, show the type of the drugs you have in the store
   ……
   10. Mention the possible health risks involved in humans.
   ……
11. Do you normally eat animal products like milk, meat, eggs and blood?
   Yes……No……..
12. Is there any health effects if a person eats animal/chicken products treated with antibiotics?
   Yes…….No……
13. If answered **Yes** in question 14, list down the health effects you know which may be caused by antimicrobials in animals/chicken food products
   ……
14. Have you ever heard anybody getting health effects after consuming livestock products with antibiotics Yes……..No……

15. If answered **Yes** what were the clinical signs reported?

……

16. Where do you get the drugs to treat the clinical signs…….

17. Does the drugs treat the problem completely Yes……..No……..

18. If answered **No** what steps do the individuals take……..

19. Is there any veterinary or medical practitioners that visits you and explain the effect of use of antibiotics in animals and humans?

    Yes……..No……..

20. If answered **No** what are the effects mentioned

    ……

21. Do you keep farm records?

Yes.....No.....

22. If answered **Yes**, which of the following records do you keep?

    (a) Disease

    (b) Treatment (……..)

    ( c) Slaughter date

    (d) Deaths

    (e) All above

    (f)

Others (specify)

……..

23. What kind of the labour is used in your animals/Chickens?

    Family…… Hired…… If hired how long have you been with the individual……..

24. If the labour is hired does it be shared with neighbours?

    ……

**THANKS FOR YOUR COOPORATION**

**Appendix 2: Informed Consent Form**

**INFORMED CONSENT FORM**

**Principal investigator**: Dr. Abdul.A.S.Katakweba
**Name of the organization**: Sokoine University of Agriculture – Morogoro Tanzania
**Research Title**: Prevalence and Molecular studies of Antibiotic resistant Bacteria from Animals and Contact Human beings in Tanzania.

**Purpose of the study**

The use of antimicrobial compounds in food animal production provides demonstrated benefits, including improved animal health, higher production and, in some cases, reduction in food borne pathogens. However, use of antibiotics for agricultural purposes, particularly for growth enhancement, has come under much scrutiny, as it has been shown to contribute to the increased prevalence of antibiotic-resistant bacteria of human significance.

In Tanzania however, no study has been done on the association between antibiotic resistant bacteria that have been isolated from humans and animals so as to quantify the public health risks humans can encounter by handling animals and animal wastes and using animal products.

Therefore, this study has been designed aiming at gauging the extent and dangers associated with antimicrobial resistance in humans and animals using indicator bacteria through prevalence and molecular typing.

**Procedures (Methodology)**

This study will be conducted in Morogoro and Dar-Es- Salaam regions. Permission to do the research will be requested from Regional and Districts administrative officers of the above mentioned regions. Preliminary survey will be conducted in the study areas to ascertain the type of prevailing production systems (commercialized and subsistence farming), population densities of target animals and type of drugs commonly administered to the animals and Farmers' knowledge on health risks. Seasonal sampling of animals and humans (faecal and nasal secretions) will be done to cover any changes in seasonal (Rainy and Dry) variations.

**Benefits from the study**

The results will lay the base line for this kind of public health problem in our country. Awareness on drug efficiency and resistance to the livestock keepers will be created. The extent of the resistant bacteria to be transferred from animals to humans will be determined. The Government and Medical personnel will be informed on the extent of the problem after submitting research findings to the relevant organs.

**Confidentiality**
The data that will be collected and their findings will be treated confidentially. Names of the participants will not be mentioned any where to keep the secrecy of the personnel that will be voluntarily agreed to participate in this research.

**Incentives to livestock keepers**
Free of charge consultations about on farm problems and interventions of problems within the study capacity.

**Participation rights**
Livestock keepers will be free to decide on whether to participate or not (voluntarily participation). Livestock keepers will also have rights to withdraw from the research.

**Whom to contact**
Principle investigator (Dr. Abdul Katakweba) will be the one to be contacted through his phone number and E-Mail address (0754 972356/0787972356; Katakweba@suanet.ac.tz/selemani.abdul@yahoo.com).

**Certificate of consent**
I have read the foregoing information, or it has been read to me. I have had the opportunity to ask question/questions about the research and question/questions that I have asked have been answered to my satisfaction. I consent voluntarily that I will participate in this study.

Name: Date and signature:

_____ _____
/ / (*dd/mm/yy*)

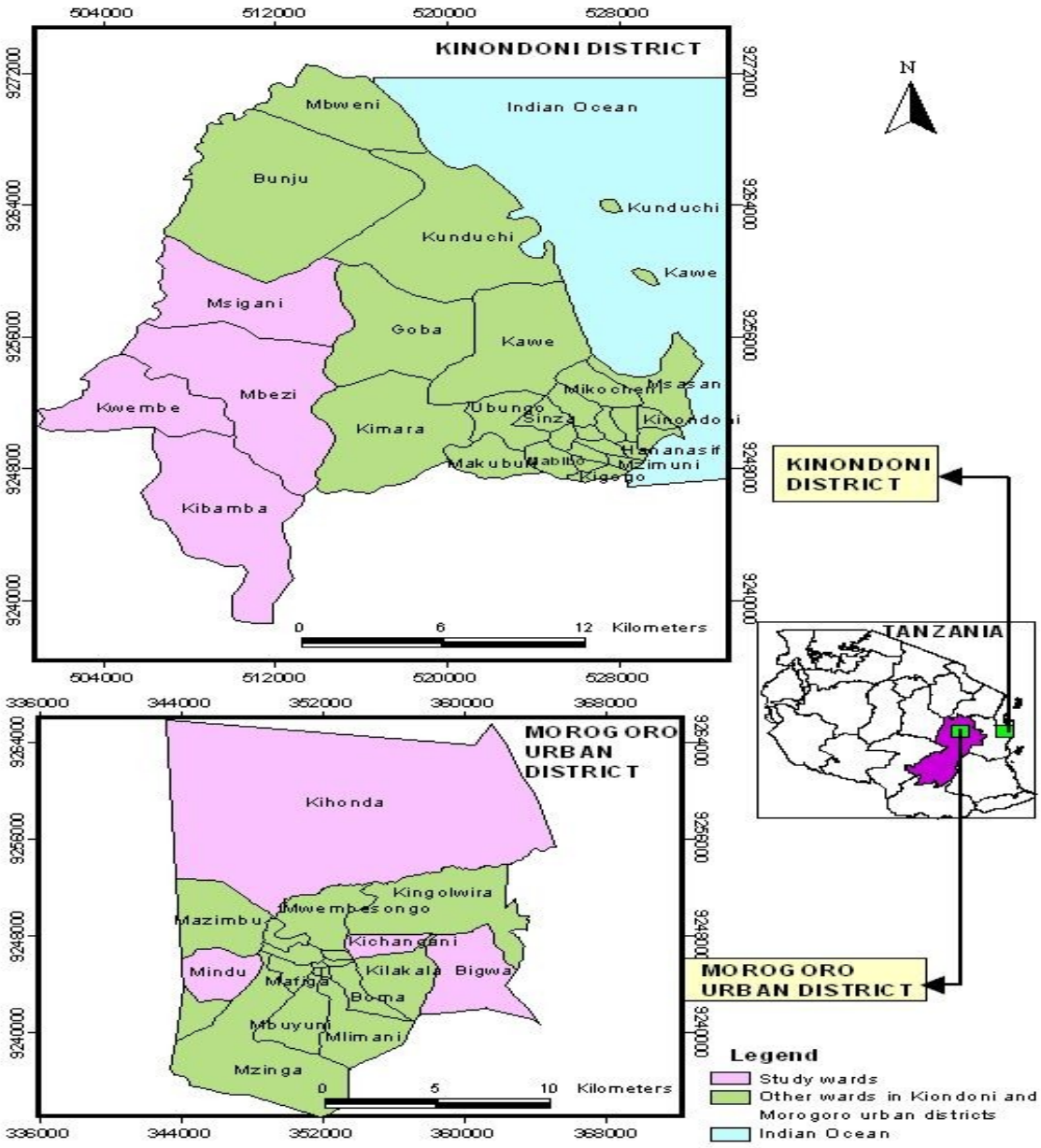
**If illiterate**
Name of independent literate witness: Date and signature of witness:

_____ _____
/ / (*dd/mm/yy*)

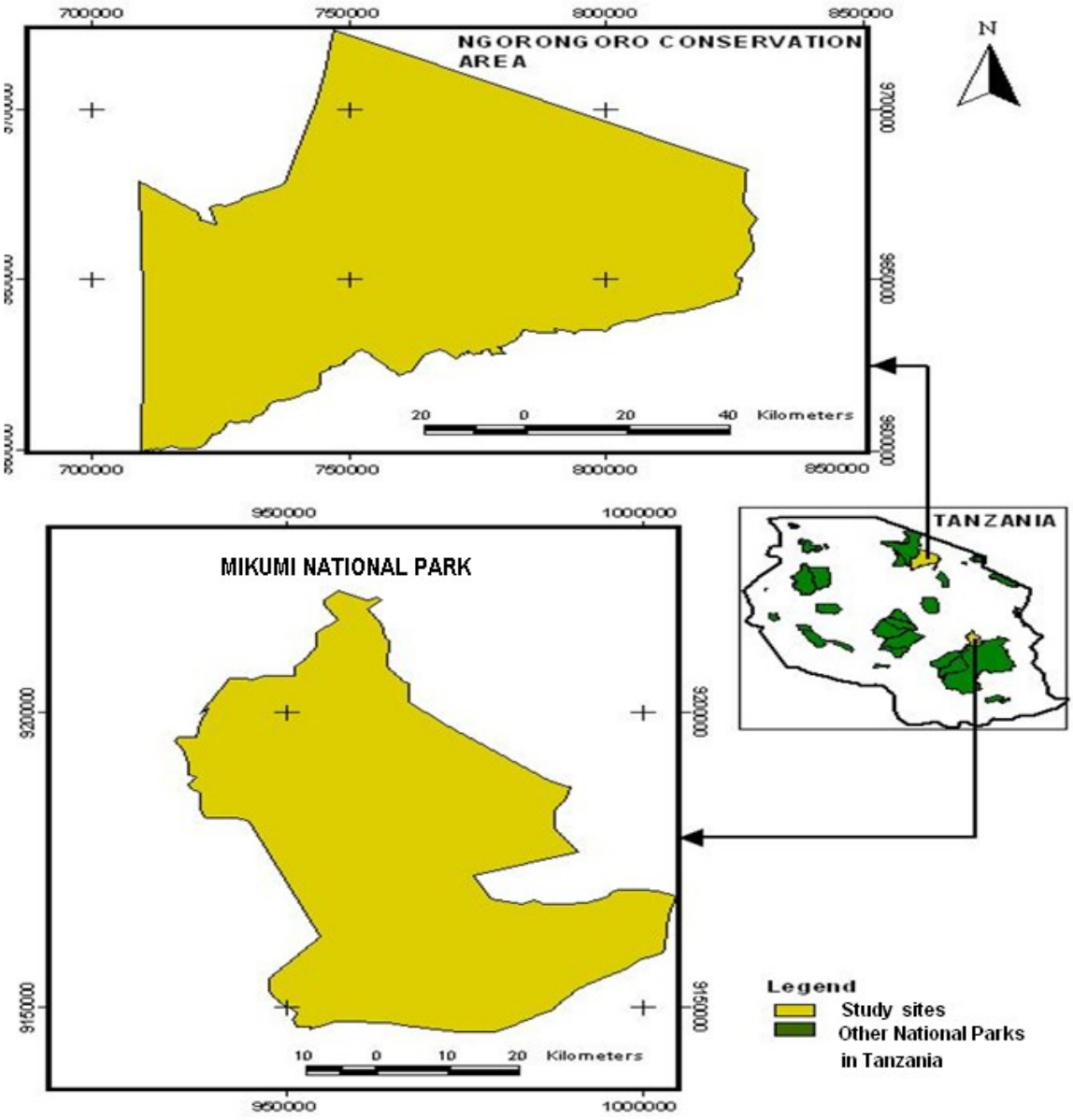
Name of researcher: Date and Signature of Researcher:

_____
Dr Abdul A.S. Katakweba / / (*dd/mm/yy*)

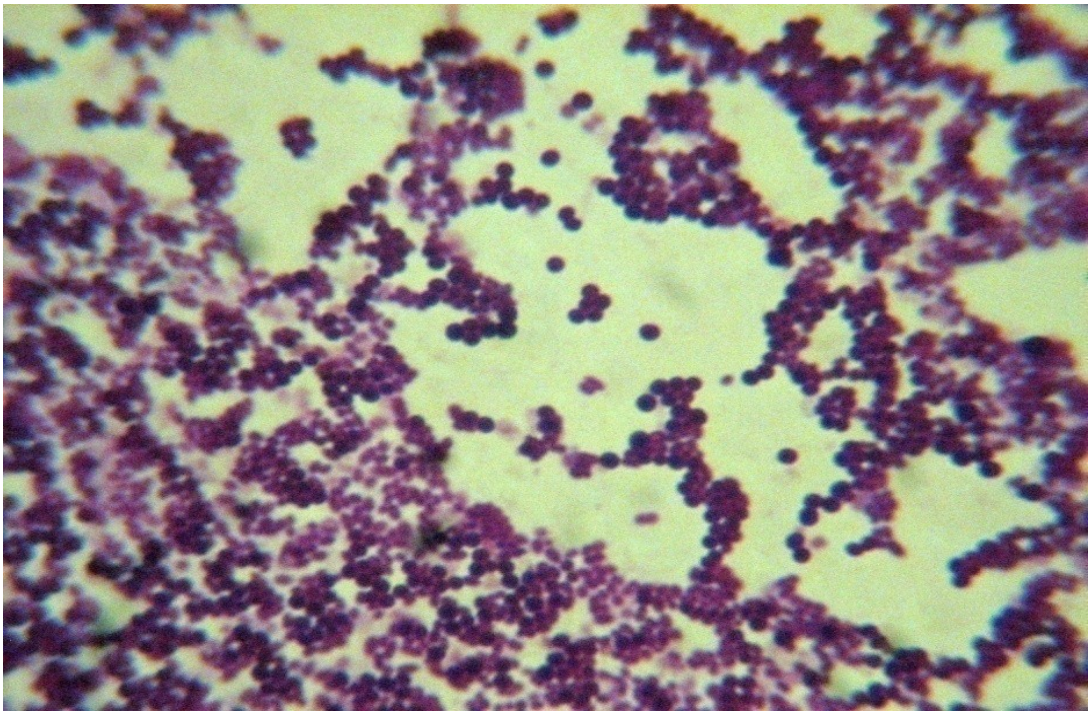
## Appendix 3: Study area in objective one and manuscript one



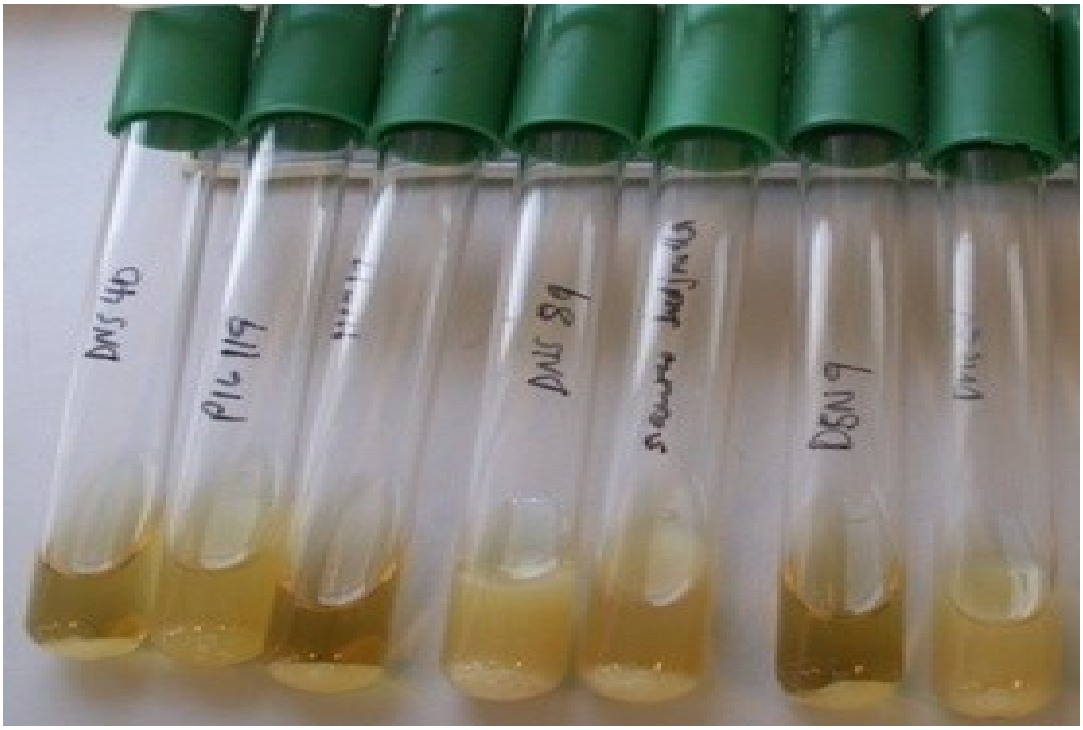
**Appendix 4: Study area in objective four and manuscript four**



**Appendix 5: Gram stain of *S. aureus***

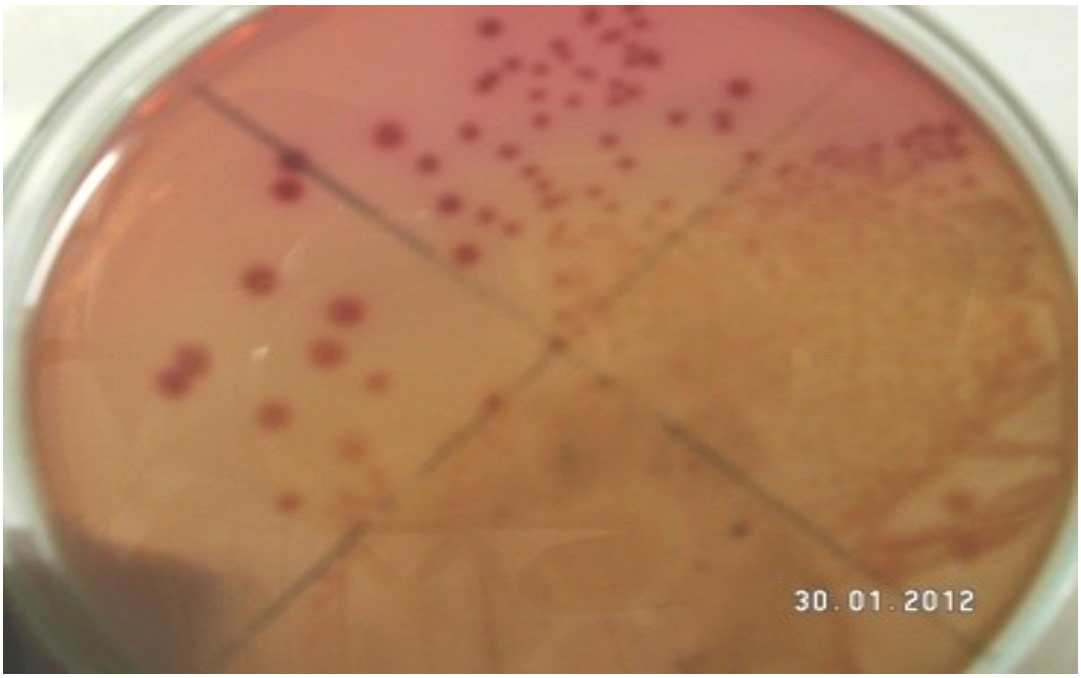


**Appendix 6: Coagulase test of *S. aureus***

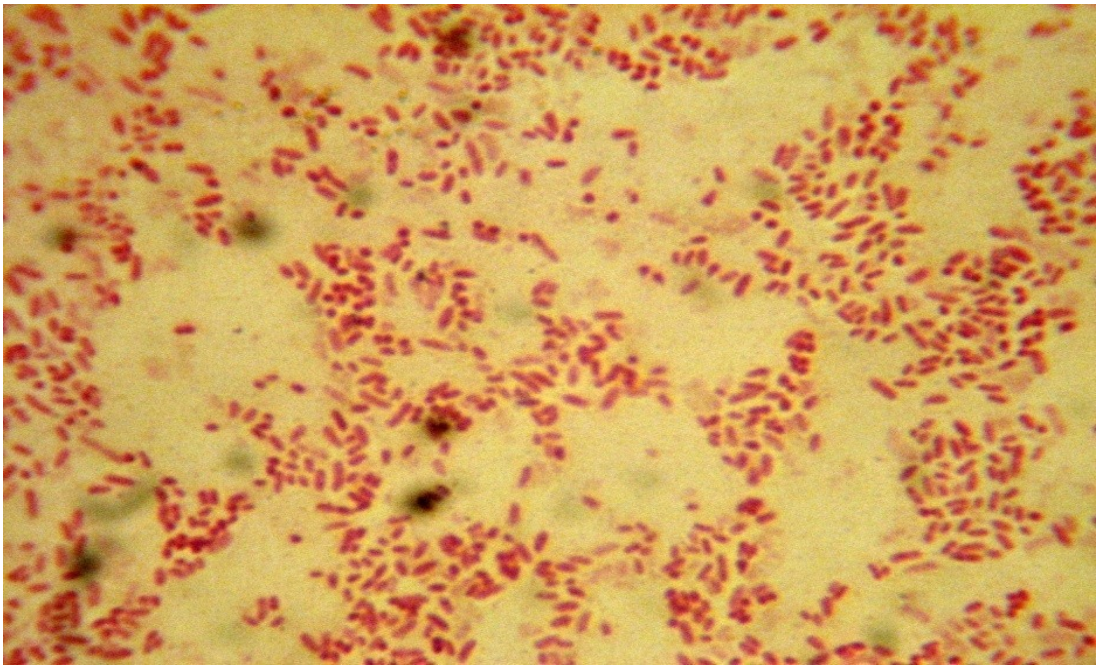


## Appendix 7: Antimicrobial used in the media

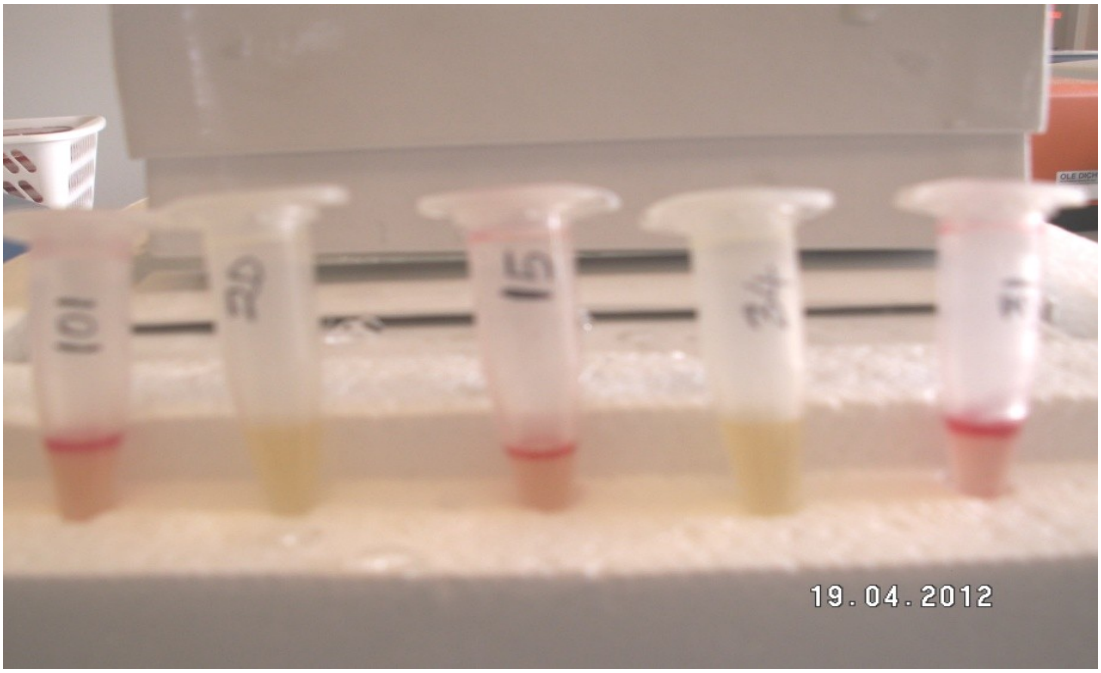

## Appendix 8: Colonies of *E. coli*



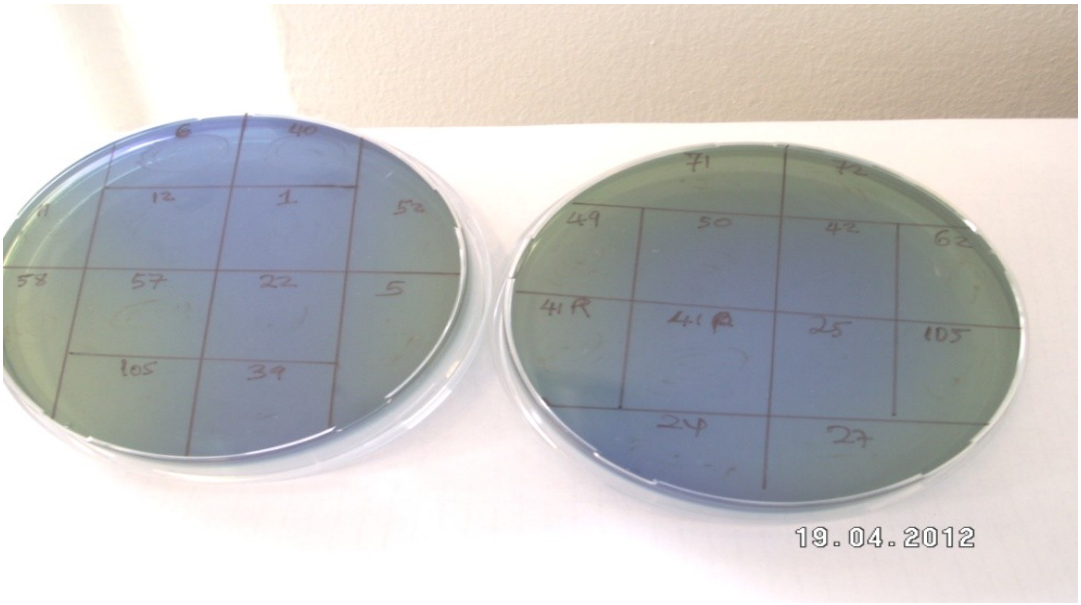
**Appendix 9: Gram stain of *E. coli***



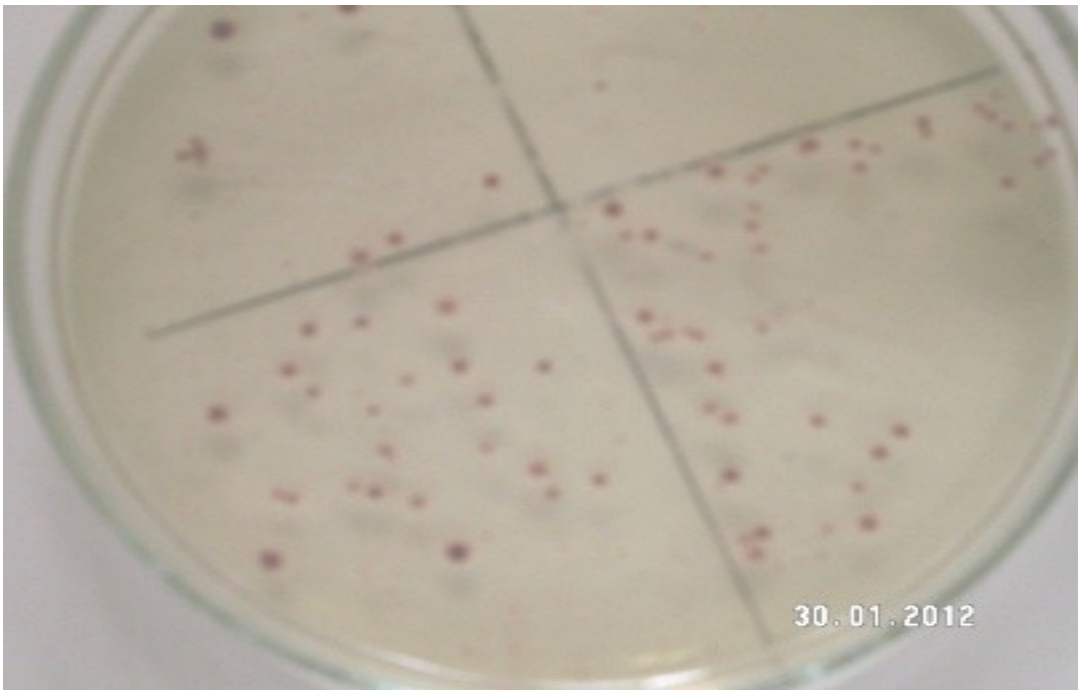
**Appendix 10: Indole test results of *E. coli***



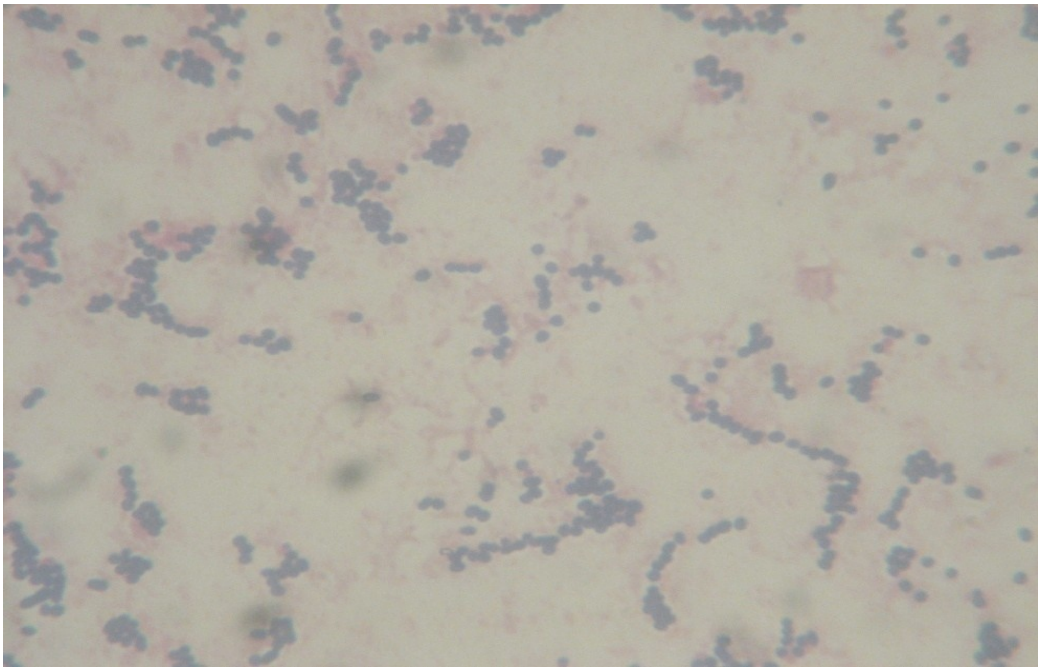
**Appendix 11: Citrate test results of *E. coli***



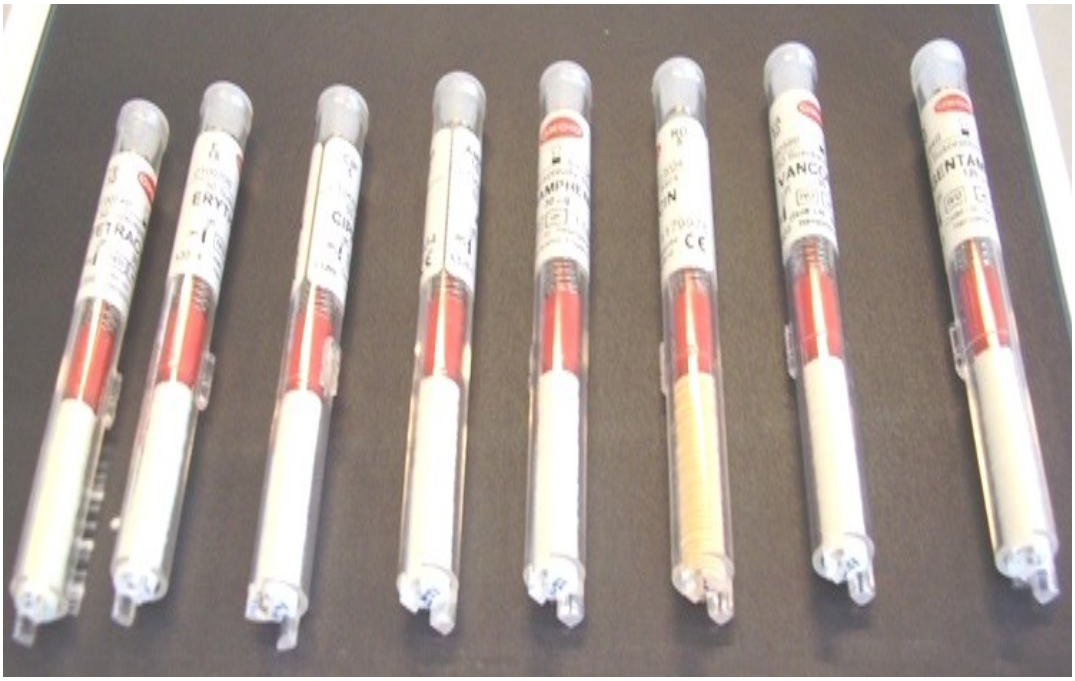

**Appendix 12: Colonies of *Enterococci spp***



**Appendix 13: Gram stain of *Enterococci spp***



**Appendix 14: Antibibic discs used in the study**



**Appendix 15: Antibibic discs used in the study**