PREVALENCE, ANTIMICROBIAL USAGE AND RESISTANCE PROFILES OF CAMPYLOBACTER IN COMMERCIAL BROILER PRODUCTION SYSTEMS IN KENYA

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this thesis to my parents Margaret and George Muhia whose life-long keen interest in furtherance of my education has been unwavering. To my dear wife Elizabeth, my sons George, Michael and Kamau Kariuki for their love, understanding and encouragement during the whole period of this research work.

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LIST OF ABBREVIATIONS/ ACRONYMS

AMAN	-	Acute motor axonal neuropathy
AMR	-	Antimicrobial resistance
BHI	-	Brain-heart infusion
bps	-	Base pairs
CCDA	-	Charcoal cefoperazone dextrose agar
CDC	-	Centers for Disease Control
CFU	-	Colony Forming Units
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxynucleotides
EFSA	-	European Food Safety Authority
EUCAST	-	The European Committee on Antimicrobial Susceptibility Testing
FAO	-	Food and Agriculture Organization
GARP	-	Global Antibiotic Resistance Partnership
GBS	-	Guillain-Barre syndrome
GoK	-	Government of Kenya
MDR TB	-	Multi-drug resistant tuberculosis
MIC	-	Mean inhibitory concentration

MPCR - Multiplex polymerase chain reaction

MRSA - Methicillin Resistant *Staphylococcus aureas*

OIE - World Organization for Animal Health

PBW - Peptone Buffered Water

PHPT - Department of Public Health Pharmacology and Toxicology, University of Nairobi

ReA - Reactive arthritis

SPSS - Statistical Package for Social Scientists

TDE - Tris Borate EDTA buffer

UK - United Kingdom

UKFSA - United Kingdom Food Standards Agency

WHO - World Health Organization

XDR TB - Extensively drug-resistant tuberculosis

XDA - Xylose Deoxycholate Agar

XLDA - Xylose lysine deoxycholate agar

Operation terms/Definitions

Slaughter house: These are premises legalized to receive livestock for purposes of butchering them

Hatchery : This is a premise where eggs are incubated for purposes of yielding day old chicks

Processing plant: This is an area within a building where slaughtered livestock are cut up to develop by-products

Commercial broiler production: This refers to the broiler rearing farms with good animal husbandry practices and with high management levels required to supply an export standard abattoir

ABSTRACT

Antimicrobial resistance (AMR) is the ability of a microbe to resist the effects of medication previously used to treat them. This phenomenon has emerged as a public health concern globally.

Campylobacter is one of the four key global causes of diarrhea diseases and it is considered to be the most common bacterial cause of human gastroenteritis in the world. The high incidence of Campylobacter diarrhoea, as well as its duration and possible complications, makes it highly important from a socio-economic perspective. This bacterium is commonly found as a contaminant of foods of animal origin particularly poultry products. Humans get infected by consumption of contaminated meat and meat products. Studies in Kenya and elsewhere indicate that poultry are the major source of Campylobacter infections in humans.

Poultry production is an important economic activity in Kenya both in large scale (commercial) and small holder set ups. However, the situation in commercial broiler production systems in Kenya with regard to Campylobacter bacteria has not been previously evaluated. The purpose of this study was to assess the prevalence and antimicrobial resistance profiles of Campylobacter in intensively managed commercial broiler production systems in Kenya to three commonly used antibiotics namely ciprofloxacin, tetracycline and erythromycin, and genetic characterization of the various species of Campylobacter occurring in the broilers.

A cross-sectional study with the specific objectives being to determine the prevalence levels, genetic characterization of the various species of Campylobacter and antimicrobial resistance profiles to commonly used antibiotics in Kenya, was carried out between February 2015 and October 2016. Cloacal swabs were collected from a total of randomly selected 600 day-old-chicks at the hatchery over a period of four consecutive months at a rate of 150 samples per month.

Likewise, 300 (n=300) cloacal samples were collected from randomly selected broilers that were ready for slaughter for purposes of consumption. Fifty samples were collected from each farm.

These broilers were from farms located in six counties of Kenya, namely Kajiado, Machakos,

Murang'a, Kiambu, Nairobi and Nakuru. The sample collection was done at a point just before the carcasses entered into the scalding tank. The sterile swabs used for cloacal swabbing were immediately immersed into Stuart® transport media and stored in a cooler box and transported to the laboratory at the Faculty of Veterinary Medicine, Department of Public Health Pharmacology and Toxicology for microbiological evaluation. The cloacal swabs from day old and 33-day old market - ready broilers were streaked on charcoal cefoperazone dextrose agar (CCDA), and anaerobically cultured at 42^{o}C for 48 hours and morphological characteristics described. The Kirby-Baeur disk diffusion method of antimicrobial sensitivity determination was used and zones of inhibition diameters were compared with the EUCAST v 7.1 clinical break-point standards for interpretation. The raw data collected were entered into MS Excel v.2016, with positive samples recorded as numeral 1 (one) and negatives as 0 (zero). The same was applied to antimicrobial sensitivity where 1 represented sensitivity and 0 represented non-sensitivity. These data were then imported into SPSS v.20 statistical software for analysis to determine the prevalence, proportions, means and analysis of variance (ANOVA). A structured questionnaire was administered to supervisor and manager of each farm to gather information on day to day management of the broiler farms and knowledge levels regarding antimicrobial usage.

No Campylobacter growth was reported from all the samples from the hatchery while bacterial growth was reported from samples from market ready birds from all the counties with the general prevalence rate of 92.3% (range of 80 to 100%), highest in Murang'a and lowest in Kajiado counties. The species identified included *C. jejuni* (66%), *C. lari*,(10%), *C. coli* (5%), *C. fetus*

(1.7%), *C. upsaliensis* (1.7%) and *C. hyoinstestinalis*(0.6%). *C. jejuni* was the most prevalent and occurred in all counties while *C. hyointestinalis* had low prevalence and was only reported from Machakos county. The Campylobacter species were found to be resistant to ciproflaxacin, tetracycline and erythromycin which are commonly used in poultry production. Two counties (Kajiado and Nakuru) had prevalence rates of 82% and 88% for *C. jejuni* respectively, while the rest of the counties had over 90%. On average 98.5% of the isolates showed resistance to ciprofloxacin while 100% were resistant to both tetracycline and erythromycin. This study found that the types of antimicrobials used in the commercial broiler farms were phosphomycin/ tylosin and phenol. None of the farms reported using ciprofloxacin or erythromycin but one had used tetracycline at one time only. On the contrary these antimicrobials are commonly used by small scale broiler farmers, humans and other animals. This may attribute to the high resistance rates observed. The study concluded that there was prudent use of indicated antimicrobials and Campylobacter species were present in market - ready broilers from commercial large-scale production systems in Kenya at high prevalence rates with *C. jejuni* as the most prevalent. The isolates also exhibited high resistance levels to antimicrobials commonly used in both veterinary and human medicine. It is recommended from this study that the farm management should engage managers and supervisors with a good level of knowledge on antimicrobials. Ineffective antimicrobials should be avoided or withdrawn. Further studies should be done to establish at what age broilers become infected and also if the critical control points in processing plants are effective in the control of Campylobacter contamination.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Antimicrobial resistant-microbes are found in people, animals, food, and the environment. In many places, antibiotics are overused and misused in both people and animals, and often given without professional oversight. Examples of misuse include when they are taken by people with viral infections like colds and flu, and when they are given as growth promoters in animals and fish and in addition poor infection control, inadequate sanitary conditions and inappropriate food-handling encourage the spread of antimicrobial resistant organisms (WHO, 2015). Microorganisms that develop antimicrobial resistance are sometimes referred to as "superbugs". As a result, the medicines become ineffective and infections may persist in the human body, increasing the risk of spread to others. Antimicrobial resistance (AMR) threatens the effective prevention and treatment of an ever-increasing range of infections caused by microorganisms and is therefore an increasingly serious threat to global public health (WHO 2014.).

Campylobacter infections are generally mild but can be fatal among children under five years of age, the elderly and immune-suppressed individuals (CDC, 2013). The bacteria normally inhabit the intestinal tract of warm-blooded animals such as poultry and cattle and are frequently detected in foods derived from these animals. In developing countries, Campylobacter infections in children under the age of five years are especially frequent, sometimes resulting in death (Nachamkin *et al*., 2008). Campylobacter genus is a microaerophilic Gram-negative bacteria frequently found in raw meat, particularly chicken, and a significant cause of food poisoning

following handling raw or undercooked meat. *C. jejuni* is now recognized as one of the main causes of bacterial foodborne disease in many developed countries (Moore *et al*., 2005). Over a dozen species of Campylobacter have been implicated in the human disease, with *C. jejuni* and *C. coli* the most common (Ryan *et al.*, 2004). *C. fetus* is the common cause of spontaneous abortions in cattle and sheep, as well as an opportunistic pathogen in humans (Perez-Perez *et al*., 1996). Poultry are recognized as a primary source of food-related transmission of Campylobacter species to humans (Saenz *et al*., 2000). Campylobacter species are found in abundance on poultry farms and their surrounding environment, including the soil, water sources, dust, building surfaces, and the air (Iversen *et al*., 2012). Poultry are also an important reservoir of other

Campylobacter species, such as *C. lari, C. upsaliensis and C. concises* (Kaakoush *et al.*, 2014). Domesticated broiler chicken and imported chicken both contribute to the overall burden of Campylobacter infections (Boysen *et al.*, 2014). It has been estimated that 71% of human Campylobacteriosis cases in Switzerland between 2001 and 2012 were attributed to chicken (Wei *et al*., 2014). Given that *C. jejuni* strains survive in chicken faeces for up to six days after excretion, chicken faeces may also be a potential source of transmission to the environment or humans when poultry manure is used as a fertilizer (Ahmed *et al*., 2013). The United Kingdom Food Standards Agency (UKFSA) reported preliminary findings showing that 72.9% of fresh whole retail chicken surveyed during 2014 to 2015 were infected with Campylobacter, with 18.9% of these harboring a level of >10,000 CFU/g, which is considered highly contaminated (UKFSA, 2014). In a study conducted in

Denmark among a flock of 162 chickens, it was found that Campylobacter species were isolated from 100% of organic broiler flocks, 36·7% of conventional broiler flocks and 49·2% of extensive indoor broiler flocks. Six of 62 Campylobacter isolates were resistant to one or more of the

antimicrobials tested (Heuer *et al*., 2008). Data from Canada also supports the finding that broiler chickens are a major source of Campylobacter species.

1.2 Broiler production in Kenya

Chicken in Kenya is a popular source of protein, and broiler production is a major economic activity. Kenya has an estimated 37.3 million birds, comprising of free ranging indigenous birds 31.4million (84.1 %), 3.1 million layers (8.4%) and 2.1 million broilers (5.7 %), while the other poultry account for 0.7 million (1.8%) and in addition poultry contributes about
55% to the livestock sector and 30% of agricultural Gross Domestic Product (Republic of Kenya, 2010). Broilers (like layers) in Kenya are reared mainly in urban areas and **t**he commercial poultry sector is estimated to produce over one million chicks per week**.** The features of the commercial broiler market are a growing urban population and growing retail sector such as fast food and supermarket branches and restaurants. The demand for commercial chicken (whole, half, parts, grilled and fried chicken) and eggs is high and growing (MoALD, 2012). The main broiler abattoir in Kenya has a throughput of over seven million broilers annually

1.3 Antimicrobial resistance

The rapid emergence of resistant bacteria occurring worldwide is endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives. Many decades after the first patients were treated with antibiotics, bacterial infections have again become a threat (Spellberg and Gilbert, 2014). The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements (Wright,

2014). Coordinated efforts to implement new policies, renew research efforts, and pursue steps to manage the crisis are greatly needed (Spellberg and Gilbert, 2014). Care should be taken to slow down development of antibacterial resistance to organisms causing common diseases including Campylobacteriosis (WHO, 2015). Antimicrobial resistance (AMR) involving last line oral antimicrobials such as cephalosporins and ciprofloxacins, would eventually lead to increased illness and death. This implies reversing the gains made in control of such infections. In the absence of effective antibiotics, the success of major surgeries and cancer chemotherapy would be compromised, thus raising the cost of health care for patients with resistant infections due to longer duration of illness, additional tests and use of more expensive drugs (WHO, 2016).

The growth of global trade and ease of travel around the world means resistant microorganisms may be spread to distant countries and continents within short periods of time. The emergence of mechanisms by ordinary pathogens such as production of enzymes to counter powerful last generation antibiotics, especially among gram-negative bacilli, has rapidly spread to many countries of the world, rendering the often-last line of defense drugs ineffective (CDC, 2014)

1.4 Campylobacteriosis

In the evaluation of global burden of disease, Campylobacter was considered to be the most common cause of bacterial gastroenteritis worldwide and was associated with seven and a half million disability-adjusted life years (DALYs) more than Shigella and enterotoxigenic *E. coli* **(**Murray *et al*., 2012). The epidemiology of Campylobacter infection in developed countries is notably different from that which is observed in the developing world with the infections being sporadic, of low prevalence and with a marked seasonality in the former (Nicholas *et al*., 2011). Infections are typically linked directly to contaminated food, including poultry (Nicholas *et al.*,

2011). Studies with improved diagnostics remain limited in number and epidemiologic context, but it is increasingly clear that current estimates significantly underestimate disease burden (WHO, 2012). In view of the above facts on AMR, coordinated actions by all stakeholders including farmers, researchers, pharmacists and policy makers must be encouraged. Creating policy guidance, support for surveillance, technical assistance, knowledge generation and partnerships among all involved as well as fostering innovation, research and development, in all parts of the world is imperative in order to combat antimicrobial resistance. This study will shed more light on the current situation in Kenya and hopefully, encourage stakeholders to device ways and means to join the global war on antimicrobial resistance.

1.5 Problem statement

Campylobacter is a common contaminant of poultry and poultry products. This microorganism commonly causes disease in humans especially children below five years of age. Work done in Kenyan studies have reported occurrence of Campylobacter in poultry and poultry products from indigenous chicken (Mageto, 2017; Ngethe, 2015). Commercial broiler production is an important economic enterprise in Kenya and antimicrobials are commonly used. However, the prevalence and antimicrobial resistant profiles of Campylobacter species have not been analyzed in large scale commercial broiler production systems in Kenya.

Campylobacter causes diarrhoea (often bloody), fever, and abdominal cramps (CDC, 2017). Serious complications such as temporary paralysis can also occur and physicians rely on ciprofloxacin and azithromycin for treating patients with severe disease. However,

Campylobacter has been reported to show resistance to these antibiotics (CDC, 2016*).* Improper use of antibiotics, such as sub-lethal doses in feeds to promote growth in livestock especially in

poultry and pigs, self-medication with failure to complete the full dose in humans, as well the unnecessary use of antibiotics for such conditions as colds or flu have immensely contributed to the rising resistance to antibiotics observed among several bacteria hitherto very susceptible to the particular drugs specified for their treatment (Aaestrup, 2005). Multidrug resistant Campylobacter, and several other bacteria, are now a major global concern (CDC, 2016). Poor control of the use of antibiotics in underresourced countries coupled by weak antimicrobial resistance surveillance makes mapping of resistance to antibiotics rather difficult (PHAS, 2014). The use of antibiotics in wellresourced countries is tightly monitored and this may have contributed to deceleration of observed resistance (WHO, 2014). AMR threatens a return to the pre-antibiotic historic times and many infectious diseases such as tuberculosis, (especially multidrug resistant TB), salmonellosis, meningitis and other drug-resistant threats, including methicillinresistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), extended spectrum beta-lactamase (ESBL), vancomycin-resistant Enterococcus (VRE), multidrug-resistant *Acinetobacter baumannii* (MRAB) all with a real risk of becoming untreatable (CDC, 2016; WHO, 2014). This is likely to derail the progress made towards reaching the targets of the health-related United Nation Development Goals. These targets were to improve maternal health, combat HIV/AIDS, malaria, and other diseases as well as to ensure environmental sustainability (UN, 2013). More expensive therapies, prolonged duration of illnesses and treatment will inevitably increase the health care costs thus raising the economic burdens to families and society (WHO, 2014). AMR puts at risk the achievements made by modern medicine regarding control and management of such conditions as cancers, organ transplants and surgery.

1.6 Justification

Information on prevalence and antimicrobial resistance profiles of Campylobacter in commercial broilers in Kenya was scanty. In a similar study on small scale and backyard chicken high resistance rates for ciprofloxacin, nalidixic acid and tetracycline were recorded, with more than 70 % resistance levels observed (Tuan *et al*., 2016), This was in contrast to those of Brooks et al. (2006), who reported resistance rates for Campylobacter recovered from humans with diarrhoea in Western Kenya for ciprofloxacin, nalidixic acid and tetracycline with 6, 26 and 18%, respectively. The general high rates of resistance in the chicken isolates may be caused by availability and uncontrolled use of antibiotics by small farmers (CDDEP, 2015). Recent studies however suggest that more work needs to be done in order to establish the exact situation. Rapidly emerging resistant bacteria threaten the extraordinary health benefits that have been achieved with antibiotics (Piddock, 2012). Despite the alarming and increasing threat posed by emerging antibiotic-resistant bacteria worldwide, the implementation of recommended steps, new policies to manage the crisis, and renewed research efforts to find novel agents and approaches to treating bacterial infections could dramatically reduce these risks (Lushniak, 2014). Health care providers, researchers, policy-makers, and representatives of the pharmaceutical industries need to work together to fight the antibiotic resistance crisis. Although success will require a considerable investment of human and financial resources, the cost of not acting would likely be much greater. This study will shed more light on current situation on prevalence and antimicrobial resistance profiles of Campylobacter isolated from commercial broiler farms. The need to control indiscriminate use of antimicrobials, enhancement of research and development in the field of

antimicrobial resistance by Government of Kenya will also be hereby elucidated and thus contribute to the global war on AMR.

1.7 Study Hypothesis

1.7.1 Null hypothesis

Campylobacter species isolates from the gut of commercially reared broilers in Kenya do not show resistance to commonly used antibiotics.

There is no correlation between prevalence of Campylobacter *spp* in day-old chicks and market–ready broiler chicken

1.8 Study objectives

1.8.1 General objectives

The general objective of this research was to assess the prevalence, antimicrobial usage and resistance profiles of Campylobacter bacteria in commercial broiler production systems in Kenya.

1.8.2 Specific objectives

The specific objectives of the study were:

To identify types and levels of antimicrobials used by the commercial broiler farmers during production.

To determine the prevalence of Campylobacter in day-old-chicks and mature

broilers at point of slaughter.

To establish antimicrobial usage and antimicrobial susceptibility profiles of the Campylobacter isolates from commercial broilers.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background to literature review

Information on the Kenyan situation regarding intensively produced large-scale commercial broilers was scanty and this study will shed more light. However, a study on free-range indigenous chicken from some locations of Makueni County of Kenya, (Ngethe, 2015), and another (Mageto, 2017) on live chickens in peri-urban Nairobi, found prevalence rates of the genus Campylobacter at 50.87% and 70.6%, respectively. Lower rates have been reported in Tanzania (Mdegela *et al*., 2006), in Iran

(Ansari-Lari *et al.,* 2011) as well as in Malaysian broiler farms (Yap *et al*., 2005).. Studies on prevalence and antimicrobial resistance of Campylobacter in intensive and highly managed broilers in Kenya were lacking.

The emergence of fluoroquinolone-resistant Campylobacter species, with poultry as an important source, has been documented in the United States (Gupta *et al.,* 2004). Further, Ge et al. (2003), found that in the USA, Campylobacter isolates from conventional broilers showed significant resistance levels to tetracycline, erythromycin, nalidixic acid and ciprofloxacin. Nguyen et al. (2016) reported high resistance levels by Campylobacter isolates from small scale and backyard chickens in Kenya, to both ciprofloxacin and tetracycline. In addition, Brooks et al. (2003) in Western Kenya found resistance levels to ciprofloxacin and tetracycline for Campylobacter isolates from human diarrhoeal cases. In

Europe, Dohne et al. (2012) reported resistance to ciprofloxacin and tetracycline in Northern Germany among Campylobacter isolates from pigs, while in Spain, GonzálezHein et al. (2013) reported resistance of Campylobacter isolates found in chicken meat to ciprofloxacin and erythromycin. In Turkey, Abay et al. (2014) found the resistance of Campylobacter isolates from chickens to nalidixic acid and tetracycline to be high. The use of antimicrobials in the farms involved in this study was strictly controlled and there was no routine use of drugs in sub-lethal doses for either prophylaxis or growth promotion. According to this study, all the broiler farms sampled reported having used Phosfomycintylosin combination (Fosbac®) routinely but Oxytetetracycline and Quinolone several years before commencement of this study, thus there was no correlation between drugs used in the farms and AMR observed. Antimicrobial use was largely due to advice from the technical teams.

Campylobacter (meaning "curved bacteria") is a genus of Gram-negative bacteria and typically appear comma or s-shaped, are motile and generally appear curved or commashaped, and are able to move via unipolar or bipolar flagella (Vandamme *et al*., 2006). They generally survive in environments with low oxygen and when exposed to atmospheric oxygen, *C. jejuni* is able to change into a coccal form, and like many Gram-negative, spiral curved rods such as *Helicobacter pylori* and *Vibrio cholerae,* are oxidase and catalase positive (Crushell *et al.,* 2004).

The catalase test facilitates the detection of the enzyme catalase in bacteria. It is valuable in speciation of gram-negative organisms such as *C. fetus*, *C jejuni*, and *C. coli* (all positive) from other Campylobacter species (MacFaddin *et al*., 2000; Mahon *et al*., 2011). Campylobacter are nonfermentative (Vandamme *et al*., 2006) and are best cultured at 42^{o}C

(Crushell *et al*., 2004). Survival at room temperature is poor, but they can survive for a short time at refrigeration temperatures – up to 15 times longer at 2°C than at 20^{o}C. The bacteria die out

slowly at freezing temperatures and are heat sensitive, and are destroyed at temperatures above 48°C (Ivić -Kolevska *et al*., 2017). Most Campylobacter species can cause disease and can infect humans and other animals. The bacterium's main reservoir is poultry (CDC, 2016). Humans can contract the disease from eating food contaminated with Campylobacter species. Another source of infection is contact with infected animals, which often carry the bacteria asymptomatically and at least a dozen species of Campylobacter have been implicated in human disease, with *C. jejuni* and *C. coli* being the most common (Ryan *et al*., 2004). *C. jejuni* is now recognized as one of the main causes of bacterial food borne disease in many developed countries (Moore *et al*., 2005). *C. jejuni* infection can also spread to the blood in individuals with AIDS, while *C. lari* is a known cause of recurrent diarrhoea in children (Mahmud, 2017). The incubation period in humans is 24–72 hours after infection (Zilbaue *et al*., 2007) and the infection is characterized by inflammatory, sometimes bloody diarrhoea or dysentery syndrome, mostly including cramps, fever, and pain (Humphrey *et al*., 2007). *C. fetus* is a cause of spontaneous abortions in cattle and sheep, as well as an opportunistic pathogen in humans (Sauerwein *et al*., 1993). This genus has been found to be part of the salivary microbiome (Wang *et al*., 2016). An important sequelae of *C. jejuni* infection in humans is the Guillain-Barré syndrome (GBS), specifically the acute motor axonal neuropathy (AMAN) variant (Kuwabara & Yuki, 2013), Inflammatory Bowel Disease (IBD) and reactive arthritis (ReA). In poultry *C. jejuni, C. coli* and *C. lari* are most frequently encountered (OIE, 2014).

Studies have estimated that up to 98% in United States and 60% - 80% in Europe of retail chicken meat was contaminated with *C.jejuni* with the giblets and skin particularly having high concentrations of Campylobacter (Bull *et al*., 2006).

In this study biochemical tests were carried out to identify oxidase and catalase characteristics of the various species of Campylobacter isolated from commercial broilers. Molecular characterization was done using multiplex PCR and the study enumerated the prevalence rates of the genus in commercial broilers as well as resistance profiles to commonly used antimicrobials.

2.2 Large scale commercial broiler farming in Kenya

Kenya is estimated to have about 37.3 million birds, 2.1 million (5.7%) of which are broilers (Republic of Kenya, 2008). Farmers contracted by export class abattoirs incur much heavier investments costs and tend to observe strict compliance to antimicrobial use in order to avoid losses associated with management oversights, and also due to closer supervision by government officers at meat inspection. Large scale commercial broiler farms are located in the six counties identified, namely Kajiado, Machakos, Murang'a, Kiambu, Nairobi ad Nakuru. Their location was also governed by a contractual requirement that they be not more than 100 -150 kilometers radius away from the processing plant, in order to avoid prolonged vehicular transportation. It is also a requirement that feeds be withdrawn 3 hours before collection and any severe starvation would compromise carcass quality. Once contractual agreements are met the farmer is supplied with dayold chicks and feeds for the placement to cater for the rearing period of 33-36 days. The farmers are required to have the capacity to rear at least 12000 birds of different ages at any one time. Technical services such as poultry house construction measurements, feeding and drinking equipment as well as flock management are free for contracted farmers, but vaccines and medication costs have to be met by the farmer. The farmer was also responsible for filling in a health card details of daily feed intake, weight gains, mortalities, vaccinations and medications (if any). They also have the benefit of free veterinary professional advice provided by the abattoir

management. This is unlike small scale broiler farmers who avoid cost implications associated with such services, by obtaining and administering veterinary drugs with minimal professional supervision. The initial total investment cost is estimated at $50,000 and includes $10,000 refundable deposit. These costs were high considering that the per capita income of Kenya is about $1587 (World Bank, 2015). Each of the entities is required to have a minimum of 12000 broilers of various ages at any one time. The abattoir management then collects the market ready birds for immediate slaughter and processing. The Directorate of Veterinary Services under the national Government of Kenya, offers meat inspection as well as abattoir hygiene management services through its officers (The Meat Control Act Cap. 356, Laws of Kenya). The sales workforce employed by management is responsible for sales and marketing of chicken and processed chicken products. Marketing uses mass media such as newspapers, brochures, point of sale materials as well as posters. A production department mandated to yield different cuts of chicken meat driven by market demand as well as company innovation is in place.

2.3 Antimicrobial agents used in large-scale commercial broiler farms

Studies have shown that tylosin-phosphomycin combination, tetracycline, (OTC®) and quionolone are the abtibiotics of choice

during the broiler rearing periods in large-scale and small scale commercial farms in peri- urban Nairobi (Muthuma, 2015). Table 2.1 shows some of the antimicrobials available in the Kenyan market for use by poultry farmers as reported by owners of shops trading in agricultural and veterinary products. However, the large-scale commercial farmers have a common source of day-old chicks, feeds and professional veterinary personnel, unlike the small-scale farmers who access

a wider range of veterinary therapeutics and often lacking the professional control due to costs. Notably, and according to CDC the number of new antibiotics developed and approved has decreased steadily over the past three decades (although four new drugs were approved in 2014), leaving fewer options to treat resistant bacteria (Table 2.2). This means the range of drugs available for therapy has narrowed over the same time (CDC, 2013).

Table 2.1: Some antimicrobials used in poultry production in Kenya

Trade name	Active ingredients
Agraryl	Streptomycin, Erythromycin, Oxytetracycline, Colistin
Aliseryl	Oxytetracycline, Erythromycin, Streptomycin, Colistin, vitamins A, D_3, E, B_1,B_2,B_3,B_6, B_{12} and K_3
Ampidox	Sulfamethazine
Amprocox	Sulfadimidine
Anticox	Sodium sulfadimidine
Biosol	Sulfamethoxazole and Trimethoprim
Biotrim	Sulfamethoxazole and Trimethoprim
Esb3	Sulfachloropyrazine
FosBac	Tylosin sulfate, Calcium fostomycin
Limovit	Chlorohydrateoxytetracycline
Miramid	Erythromycin, Sulfamethazine and Trimethoprim
S-Dime	Sulfadimidine
Trimovet	Sulfamethoxazole and Trimethoprim
Tylo Doxy	Tylosin and Doxycycline
Vet trim	Sulfamethoxazole, Trimethoprim
Vetoxy	Oxytetracycline

Source: Agro-veterinary products outlet in Nairobi, Kenya, 2016

Table 2.2: Number of new drug applications and approvals since 1980

Year	Number of drugs approved
1980 - 1984	19
1985 – 1989	11
1990 – 1994	11
1995 – 1999	11
2000 – 2004	4
2005 – 2009	3
2010 – 2014	6

Source: Centers for Disease Control and Prevention, Office of Infectious Disease. Antibiotic resistance threats in the United States, 2013.Antibiotic resistance threats in the United States, 2013 (*http://www.cdc.gov/drugresistance/threat-report*2013*).*

The development of new antibiotics by the pharmaceutical industry, a strategy that had been effective at combating resistant bacteria in the past, had essentially stalled due to economic and regulatory obstacles. Out of the 18 largest pharmaceutical companies, 15 abandoned the antibiotic field (Bartlett *et al*., 2013). Mergers between pharmaceutical companies have also substantially reduced the number and diversity of research teams (Piddock, 2012). Antibiotic research conducted in academia has been scaled back as a result of funding cuts due to the economic crisis (Piddock, 2012). Because antibiotics are used for relatively short periods and are often curative, they are not as profitable as drugs that treat chronic conditions, such as diabetes, psychiatric disorders, asthma, or gastroesophageal reflux (Bartlett *et al*., 2013). A cost– benefit analysis by the Office of Health Economics in London calculated that the net present value (NPV) of a new antibiotic is only about $50 million, compared to approximately $1 billion for a drug used to treat a neuromuscular disease (Bartlett *et al*., 2013). Because medicines for chronic conditions are more profitable, pharmaceutical companies prefer to invest in them (Gould *et al*., 2013). Another factor that causes antibiotic development to lack economic appeal is the relatively low cost of antibiotics. Newer antibiotics are generally priced at a maximum of $1,000 to $3,000 per course compared with cancer chemotherapy that costs tens of thousands of dollars (Bartlett *et al*., 2013). The availability, ease of use, and generally low cost of antibiotics has also led to a perception of low value among payers and the public and, microbiologists and infectious-disease specialists have advised restraint regarding antibiotic use (Piddock, 2012). Therefore, once a new antibiotic is marketed, physicians, rather than prescribing it immediately, often hold this new agent in reserve for only the worst cases due to fear of promoting drug resistance, and they continue to prescribe older agents that have shown comparable efficacy (Gould *et al*., 2013). Therefore, new antibiotics are often treated as "last-line" drugs to combat serious illnesses (Golkar *et al*., 2014). This practice

leads to the reduced use of new antibiotics and a diminished return on investment (Bartlett *et al*., 2013).

When new agents are eventually used, the emergence of resistance is nearly inevitable (Gould *et al*., 2013). However, since bacterial evolution is uncertain, the timeline for the development of resistance is unpredictable. A manufacturer that invests large sums of money into antibiotic development may therefore discover that profits are prematurely curtailed when resistance develops to a new antibiotic while economic uncertainty related to the recession has also had a restraining effect on the end users of antibiotics (Golkar *et al*., 2014). Developed countries with well-funded health care systems have applied austerity measures, while developing countries such as China and India still have a large cohort of population that cannot afford expensive new medicines. As an additional complication, most antibiotics are currently off-patent and are supplied by manufacturers of generic drugs. The result has been accessing cheap and generally effective drugs, which is good for the public; however, the downside is that many players expect all antibiotics to be priced similarly, even new agents that target multidrug-resistant (MDR) pathogens (Wright, 2014).

2.3.1 Phosphomycin and Tylosin (Fosbac®)

Phosphomycin has broad antibacterial activity against both Gram-positive and Gramnegative pathogens (Grif *et al.,* 2001*).* It is bactericidal and inhibits bacterial cell wall biogenesis by inactivating the enzyme UDP-N-acetylglucosamine-3enolpyruvyltransferase, also known as MurA which catalyzes the committed step in peptidoglycan biosynthesis, namely the ligation of phosphoenolpyruvate (PEP) to the 3'hydroxyl group of UDP-N-acetylglucosamine. This pyruvate moiety provides the linker that bridges the glycan and peptide portion of peptidoglycan.

Fosfomycin is a PEP analog and enters the bacterial cell through the glycerophosphate transporter (Santoro *et al.*, 2011). The global problem of advancing antimicrobial resistance has led to a renewed interest in its use more recently (Falagas *et al.,* 2008). Mutations that inactivate the nonessential glycerophosphate transporter render bacteria resistant to phosphomycin (Castañeda-García *et al.,* 2013).

2.3.2 Tylosin

Tylosin is used in veterinary medicine to treat bacterial infections in a wide range of species and has a high margin of safety. It is a macrolide that has a bacteriostatic effect on susceptible organisms and whose mechanism of action is inhibition of protein synthesis through binding to the 50S subunit of the bacterial ribosome. Tylosin has a wide spectrum of activity against Gram-positive bacteria but a much narrower Gram-negative spectrum of activity and has been shown to be active against *C. coli*, and certain spirochaetes. In poultry, the treatment should be administered for one to five days depending upon severity of infection. Treated chicken must consume enough medicated water to provide 50 mg per pound of body weight per day. Only medicated water should be available to the birds

(drugs.com, 2016).

2.3.3 Tetracycline

Tetracycline is a broad spectrum antibiotic used orally in chicken rearing. Its mode of action is by blocking the bacteria's ability to synthesize protein and is bacteriostatic. Tetracycline was patented in 1953 and came into commercial use in 1978 (Fischer *et al*., 2006). It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system. Tetracycline is available as a generic medication (WHO, 2015). It is first-line

therapy for Rickettsia, Coxiella, Chlamydia, Mycoplasma among other micro-organisms (Chow *et al.,* 1975). However, acquired (as opposed to inherent) resistance has proliferated in many pathogenic organisms and greatly eroded the formerly vast versatility of this group of antibiotics. Tetracyclines remain especially useful in the management of infections by certain obligatory intracellular bacterial pathogens such as Chlamydia, Mycoplasma and Rickettsia (Papich, 2016). They are also of value in spirochaetal infections, leptospirosis as well as exotic infections, including anthrax, plague and brucellosis (Harvery *et al*., 2009). These agents also have activity against certain eukaryotic parasites, including those responsible for diseases such as malaria and balantidiasis. Bacteria usually acquire resistance to tetracycline from horizontal transfer of a gene that either encodes an efflux pump or a ribosomal protection protein. Efflux pumps actively eject tetracycline from the cell, preventing the build-up of an inhibitory concentration of tetracycline in the cytoplasm (Chopra and Roberts, 2001). Ribosomal protection proteins interact with the ribosome and dislodge tetracycline from the ribosome, allowing for translation to continue (Connell *et al*., 2003).

2.3.4 Quinolones

The quinolones are a family of synthetic broad-spectrum antibiotic drugs (Ivanov *et al*., 2006). Quinolones, and their derivatives, have been isolated from natural sources such as plants, animals and bacteria and can act as natural antimicrobials and/or signalling molecules (Heeb *et al.*, 2011). Quinolones exert their antibacterial effect by preventing bacterial DNA from unwinding and duplicating. Fluoroquinolones are mostly in clinical use and have a fluorine atom attached to the central ring system, typically at the 6-position or C-7 position. The basic pharmacophore, or active structure, of the fluoroquinolone class is based upon the quinoline ring system (Schaumann & Rodloff, 2007). The addition of the fluorine atom at C6 distinguishes the successive-generation

fluoroquinolones from the first-generation of quinolones (Chang *et al*., 1997). First and second-generation fluoroquinolones selectively inhibit the topoisomerase II ligase domain, leaving the two nuclease domains intact (Elsea *et al*., 1992). This modification, coupled with the constant action of the topoisomerase II in the bacterial cell, leads to DNA fragmentation via the nucleonic activity of the intact enzyme domains. Third and fourth generation fluoroquinolones are more selective for the topoisomerase IV ligase domain, and thus have enhanced gram-positive coverage. Fluoroquinolones enter cells easily via porins and, therefore, are often used to treat intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*. For many gram-negative bacteria, DNA gyrase is the target, whereas topoisomerase IV is the target for many gram-positive bacteria. Some compounds in this class have been shown to inhibit the synthesis of mitochondrial DNA. Resistance to quinolones can evolve rapidly, even during the course of a treatment. Numerous pathogens and widespread veterinary usage of quinolones, (particularly in Europe), has been implicated (Nelson *et al*., 2007).

Three mechanisms of resistance are known (Robicsek *et al*., 2006). In gram-negative bacteria, plasmid-mediated resistance genes produce proteins that can bind to DNA gyrase, protecting it from the action of quinolones (Xiong *et al*., 2011). Mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drugs' effectiveness (McDermott *et al*., 2005).

2.3.5 Phenol (Farmguard®)

This is a concentrated phenolic disinfectant whose active ingredient is high boiling tar cresylic acid (C_7H_8O) and is used at a dilution rate of 1:100 – 200 Liters (Bio Agri Mix, 2002). Phenolic type antimicrobial agents have long been used for their disinfectant properties. It has been known for many years that, although they have often been referred to as “general protoplasmic poisons,”

they have membrane-active properties which also contribute to their overall activity (Goddard & McCue, 2001). Phenol induces progressive leakage of intracellular constituents, including the release of K^+, the first index of membrane damage, and of radioactivity from C-labeled *E. coli,* (Prindle, 1983). The phenolics also possess antifungal and antiviral properties. Their antifungal action probably involves damage to the plasma membrane resulting in leakage of intracellular constituents

(Lippincott *et al*., 2001).

2.4 Antimicrobial resistance overview

Antimicrobial resistance (AMR) is the ability of a microbe to resist the effects of medication previously used to treat them (CDC, 2015). Resistance arises through one of three ways: natural resistance in certain types of bacteria, genetic mutation, or by one species acquiring resistance from another (www.tufts.edu., 2015). Resistance can appear spontaneously because of random mutations or more commonly following gradual buildup over time, and may be due to misuse of antibiotics or antimicrobials (CDC, 2015). Resistant microbes are increasingly difficult to treat, requiring alternative medications or higher doses, both of which may be more expensive or more toxic (CDC, 2015). Multidrug resistant (MDR) microbes are sometimes referred to as superbugs (CDC, 2009).

Antimicrobial resistance is on the rise with millions of deaths every year (WHO, 2014). Antibiotics should only be used when needed as prescribed by health professionals. The prescriber should closely adhere to the five rights of drug administration i.e.: the right patient, the right drug, the right dose, the right route, and the right time with preference to narrow over broad-spectrum antibiotics when possible, as effectively and accurately, targeting specific organisms is less likely to cause resistance (NPSA, 2013). Specimens for laboratory culturing should be taken before

treatment when indicated, and treatment potentially changed based on the susceptibility report (Leekha *et al*., 2011). Rising drug resistance is caused mainly by improper use of antimicrobials in humans as well as in animals and spread of resistant strains between the two (CDC, 2015). Antibiotics increase selective pressure in bacterial populations, causing vulnerable bacteria to die which increases the percentage of resistant bacteria which continue growing (Ali *et al*., 2015).

With resistance to antibiotics becoming more common, there is greater need for alternative treatments. Calls for new antibiotic therapies have been issued, but new drug development is becoming rarer (Cassir *et al*., 2014). A World Health Organization (WHO) report released in April 2014 stated, "this serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country (WHO, 2015). Antibiotic resistance is when bacteria change so that antibiotics no longer work in people who need them to treat infections and is now a major threat to public health." Increasing public calls for global collective action to address the threat include proposals for international treaties on antimicrobial resistance (Hoffman, 2015). Worldwide antibiotic resistance is not fully mapped, but poorer countries with weak healthcare systems are more affected (PHAS, 2014). The WHO concluded that inappropriate use of antibiotics in animal husbandry is an underlying contributor to the emergence and spread of antibiotic-resistant germs, and that the use of antibiotics as growth promoters in animal feeds should be restricted (Mathew *et al*., 2007). The OIE has added to the Terrestrial Animal Health Code (TAHC) a series of guidelines with recommendations to its members for the creation and harmonization of national antimicrobial resistance surveillance and monitoring programs, monitoring of the quantities of antibiotics used in animal husbandry and recommendations to ensure the proper and prudent use of antibiotic

substances. Another guideline is to implement methodologies that help to establish associated risk factors and assess the risk of antibiotic resistance (OIE, 2015).

2.4.1 Mechanisms of development of antimicrobial resistance

There are four mechanisms by which bacteria exhibit resistance to antimicrobials

1. **Drug inactivation or modification** by production of enzymes by the bacterial cell wall. These enzymes will add an acetyl or phosphate group to a specific site on the antibiotic, which will reduce its ability to bind to the bacterial ribosomes and disrupt protein synthesis (Criswell, 2014).
2. **Alteration of target- or binding site** as well as ribosomal protection proteins which protect the bacterial cell from antibiotics that target the cell's ribosomes to inhibit protein synthesis. The mechanism involves the binding of the ribosomal protection proteins to the ribosomes of the bacterial cell, which in turn changes its conformational shape. This allows the ribosomes to continue synthesizing proteins essential to the cell while preventing antibiotics from binding to the ribosome to inhibit protein synthesis (Michael *et al*., 2010).
3. **Alteration of metabolic pathway**: such as ability of bacteria to use preformed folic acid to synthesize nucleic acids , by-passing the need for para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by some antimicrobials (Harold *et al*., 1996).
4. **Reduced drug accumulation**: by decreasing drug permeability or increasing active efflux (pumping out) of the drugs across the cell surface (Li and Nikaido, 2009). These pumps within the cellular membrane of certain bacterial species are used to pump antibiotics out of the cell before they are able to do any damage. They are often activated by a specific substrate associated with an antibiotic. as in fluoroquinolone resistance (Aminov and

Mackie, 2007, Morita *et al*., 1998). Figure2.1 is a pictorial summary of the mechanisms by which bacteria acquire antimicrobial resistance.

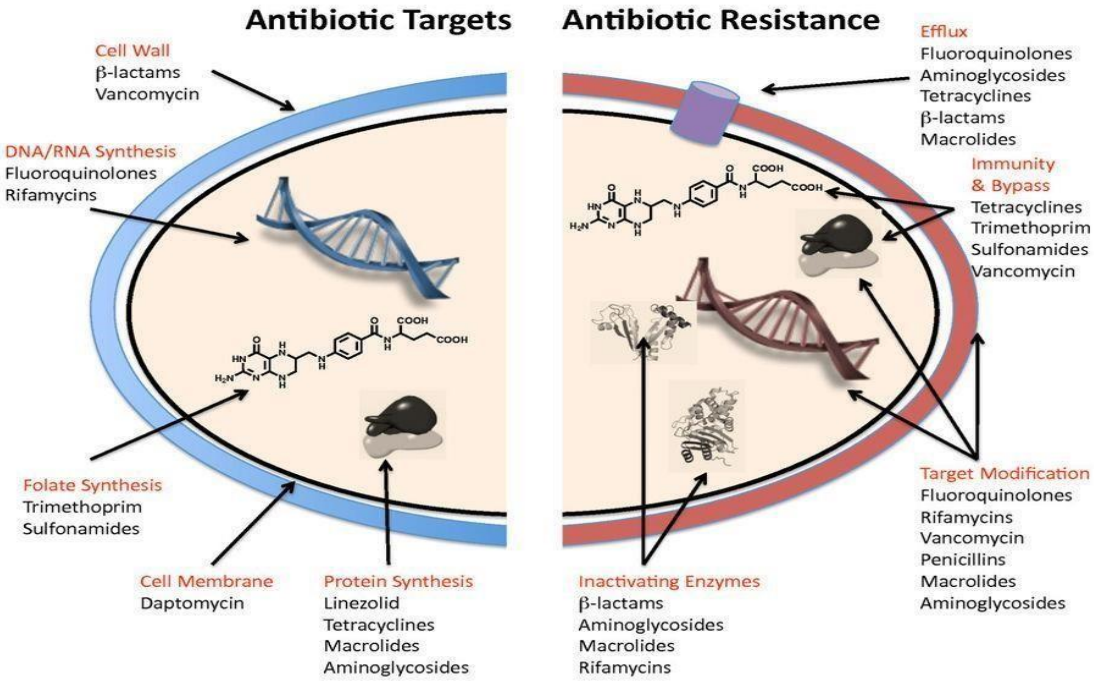


Figure 2.1 Pictorial illustration of antibiotic targets and mechanisms of resistance

Source: Wright, 2010

2.5 Antimicrobial Resistance/Antimicrobial Sensitivity Testing

Various methods are currently available for antimicrobial sensitivity testing and these include dilution, agar dilution, broth dilution, agar disk diffusion, gradient diffusion among others. For this study, the agar disk diffusion method was preferred due to its simplicity and cost effectiveness. Among these available tests, the two most commonly used methods in veterinary laboratories are the agar disk diffusion and the broth micro dilution.

2.5.1 Dilution methods

The Broth dilution method involves subjecting the isolate to a series of concentrations of antimicrobial agents in a broth environment (Turnidge and Peterson, 2007). Microdilution testing uses about 0.05 to 0.1 ml total broth volume and can be conveniently performed in a microtiter format. Micro dilution testing uses broth volumes at about 1.0 ml in standard test tubes. For both of these broth dilution methods, the lowest concentration at which the isolate is completely inhibited, (as evidenced by the absence of visible bacterial growth) is recorded as the minimal inhibitory concentration (MIC). The MIC is thus the minimum concentration of the antibiotic that will inhibit this particular isolate. The test is only valid if the positive control shows growth and the negative control shows no growth (Watts and Lindeman, 2006). A procedure similar to broth dilution is agar dilution. Agar dilution method follows the principle of establishing the lowest concentration of the serially diluted antibiotic concentration at which bacterial growth is still inhibited (Watts and Lindeman, 2006).

2.5.2 Disk diffusion method

A growth medium, usually Mueller-Hinton agar, is first evenly seeded throughout the plate with the isolate of interest that has been diluted at a standard concentration (approximately 1 to 2 x 108 colony forming units per ml). Commercially prepared disks each of which are pre-impregnated with a standard concentration of a particular antibiotic, evenly dispensed and lightly pressed onto the agar surface. The test antibiotic immediately begins to diffuse outward from the disks, creating a gradient of antibiotic concentration in the agar such that the highest concentration is found close to the disk with decreasing concentrations further away from the disk. After an overnight incubation, the bacterial growth around each disc is observed. If the test isolate is susceptible to a

particular antibiotic, a clear area of "no growth" will be observed around that particular disk. The zone around an antibiotic disk that has no growth is referred to as the zone of inhibition since this approximates the minimum antibiotic concentration sufficient to prevent growth of the test isolate. This zone is then measured in millimetres and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible or resistant. MIC measurement cannot be determined with this qualitative test which simply classifies the isolates as susceptible, intermediate or resistant. Because of convenience, efficiency and cost, the disk diffusion method is probably the most widely used method for determining antimicrobial resistance in private veterinary clinics (White *et al*., 2003).

2.6 Resistance of Campylobacter to various antimicrobials

Campylobacter has been shown to develop resistance to common antimicrobials in use both for human and animal treatment.

2.6.1 Resistance of Campylobacter to Quinolones

The targets of quinolones are two large bacterial enzymes, DNA gyrase and topoisomerase IV. These enzymes act mutually in bacterial DNA replication, transcription, recombination, and repairing of DNA (Jacoby, 2005). The gyrase and topoisomerase gene products are large enzymatic quaternary structures consisting of two pairs of subunits - GyrA and GyrB (i.e. DNA gyrase), and ParC and ParE (i.e., topoisomerase IV), respectively (Payot *et al*., 2004). There are several different single GyrA modifications reported to be associated with fluoroquinolone resistance in Campylobacter species: Thr86Ile, Asp90Asn, Thr86Lys, Thr86Ala, Thr86Val, and Asp90Tyr. However, the most frequently observed mutation in quinolone resistant Campylobacter is the C257T change in the GyrA gene, which leads to the Thr86Ile substitution in

the gyrase and confers the high-level resistance to this group of antimicrobials (Payot *et al*., 2006). Other reported resistance-associated mutations include

T86 K, A70T, and D90N, which are less common and do not play an important role in quinolone resistance as high as that observed for the Thr86Ile mutation (Payot *et al*., 2006). Fluoroquinolone resistance mechanisms appear to be mainly due to mutations in the GyrA gene encoding part of the GyrA (Aarestrup and Engberg, 2001). It was found that a highlevel resistance to ciprofloxacin was conferred by the point mutation Thr86Ile in the GyrA gene, which is homologous to Ser83Leu in *Escherichia coli* (Ge *et al*., 2005). Other reported mutations of the GyrA region in *C. jejuni* include Thr86Ala which is responsible for a high-level resistance to nalidixic acid and low-level resistance to ciprofloxacin and double point mutations of the GyrA gene together with Asp85Tyr, or Asp90Asn, or Pro104Ser have also been reported (Ge *et al*., 2005). In *C. jejuni* and *C. coli*, the absence of a secondary target for fluoroquinolones infers a situation whereby a unique modification in the GyrA subunit is sufficient to confer a fluoroquinolone-resistant phenotype (Payot *et al*., 2006). The CmeABC multidrug efflux pump has been described as the major efflux mechanism causing antimicrobial resistance to several antimicrobials including the fluoroquinolones and macrolide (Pumbwe and Piddock, 2002). CmeABC is coded by an operon consisting of three genes, cmeA, cmeB, and cmeC, which code for a periplasmic fusion protein, an inner membrane drug transporter, and an outer membrane protein, respectively (Pumbwe and Piddock, 2002). The CmeABC multidrug efflux pump is the most common efflux system in Campylobacter and works in synergy with GyrA mutations in causing fluoroquinolone resistance (Luo *et al*., 2003). Inactivation of the CmeABC efflux pump by insertional inactivation of CmeB or with efflux pump inhibitors leads to increased susceptibility to different antibiotics, including those to which Campylobacter are intrinsically resistant, showing

that CmeABC plays a key role in both intrinsic and acquired resistance of Campylobacter (Akiba *et al*., 2006). Furthermore, it was found that when the efflux pump is blocked, the minimum inhibitory concentration (MIC) values for ciprofloxacin are reduced to the level of susceptible strains even with mutations in the GyrA (Luo *et al*., 2003). Several studies have linked the use of antimicrobials, including fluoroquinolones, as the growth promoters in food animals and therapeutically in veterinary medicine, with the emergence and spread of resistance among Campylobacter strains, with potentially serious influence on food safety as well as veterinary and human health and the selective pressure of therapeutic fluoroquinolone administration in poultry flocks has been demonstrated to select for ciprofloxacin-resistant Campylobacters in poultry that enters the food chain (Humphrey *et al*., 2005). It was also found that the resistance was not as a result of the spread of a single resistant clone, but that several Campylobacter clones were selected by fluoroquinolone treatment (Humphrey *et al*., 2005).

2.6.2 Resistance of Campylobacter to Tetracycline

Resistance to tetracyclines in Campylobacter is conferred by the *Tet(O)* gene, which is widely present in both *C. jejuni* and *C. coli* (Connell *et al*., 2003). Tetracycline binds to Mg2+ cations in order to pass through outer membrane porins and then, in the periplasmic space, dissociates from magnesium and moves passively into the cytoplasm to bind to discrete sites on the ribosomal 30S subunit (Chopra and Roberts, 2001). Its primary antimicrobial effect takes place by direct steric hindrance by binding to the A site in the

30S subunit, thus hindering the movement of transfer RNA and inhibits peptide elongation (Harms *et al*., 2003). The *Tet(O)* gene, which encodes ribosomal protection proteins

(RPPs), is located on a self-transmissible plasmid of a molecular size from 45 to 58 kb (Taylor and Courvalin, 1988). The *Tet(O)* gene has been shown to confer extremely highlevels (512 mg/L) of tetracycline resistance (Gibreel *et al*., 2002). Recent study

demonstrates that this protein recognizes an open A site on the bacterial ribosome and binds it in such a manner that it induces a conformational change that results in the release of the bound tetracycline molecule (Connell *et al*., 2003). Furthermore, the conformational change persists for an extended period of time, thus allowing for continued protein elongation in an efficient manner (Connell *et al*., 2003). Tetracyclines, which are the subject of RPP mediated resistance, including *Tet(O),* bind to the ribosome and inhibit accommodation of the aminoacyl tRNA (aa-tRNA) into the ribosomal A site and, therefore, prevent the elongation phase of protein synthesis

(Chopra, 1985). Location of the *Tet(O)* gene on the chromosome has also been reported in

33–76% of tetracycline-resistant *C. jejuni* isolates lacking plasmids in Canada and Australia, respectively (Pratt and Korolik, 2005). The presence of an insertion element IS607, similar to IS607 found on the chromosome of *Helicobacter pylori*, has been reported on *Tet(O)*-carrying plasmids (Gibreel *et al*., 2004) and therefore, it is possible that mobile genetic elements other than transmissible plasmids may be involved in the acquisition and dissemination of *Tet (O).* Based on the G-C content, sequence homology, codon usage, and hybridization analysis, it seems that the Campylobacter *tet(O)* gene was probably acquired by horizontal gene transfer from either Streptomyces, Streptococcus, or Enterococcus species (Batchelor *et al.,* 2004). The *tet(O)* genes showed 75–76% homology sequence with the *tet(M)* gene of *Streptococcus pneumoniae* and have a G to C ratio of 40% (Taylor and Courvalin, 1998).

2.6.3 Resistance of Campylobacter to Erythromycin

Erythromycin (macrolides antimicrobial), interrupts protein synthesis in bacterial ribosome by targeting the 50S subunit and inhibit bacterial RNA-dependent protein synthesis (Poehlsgaard and Douthwaite, 2005). The binding of the macrolide antimicrobial leads to conformational changes in the ribosome and subsequent termination of the elongation of the peptide chain (Pfister *et al.*, 2004). The chromosome of Campylobacter contains three copies of the 23S rRNA gene (Fouts *et al*., 2005). In erythromycin-resistant strains, generally all copies carry macrolide resistance associated mutations, but the co-existence of wild-type alleles does not seem to affect the resistance level (Gibreel *et al*., 2004). Macrolide resistance in Campylobacteriosis is as a result of modification of the ribosome target binding site by mutation of the 23S rRNA or changes in resulting proteins at the site rather than target methylation or enzymatic drug modification seen in other bacterial species (Batchelor *et al*., 2004). Base substitutions at positions 2074 and 2075 of the adenine residues in all three copies of the 23S rRNA gene (rrnB operon) in Campylobacter are the most common mutations conveying erythromycin resistance (Jeon *et al*., 2008). The A2074C, A2074G, and A2075G mutations are found to confer a high-level resistance to macrolide antibiotics (erythromycin MIC > 128 mg/L) in *C. jejuni* and *C. coli*. Resistance to erythromycin tends to correspond with cross-resistance to other macrolides (e.g. azithromycin and clarithromycin) as well as related drugs of the lincosamide (e.g. clindamycin) and streptogramin groups (Avrain *et al*., 2004). One of the efflux mechanisms that confer resistance to macrolides is CmeABC multidrug efflux pump that works in synergy with specific mutations, even in the absence of any other factor affecting resistance (Cagliero *et al*., 2006). There is data suggesting that interplay between efflux activity and mutations in the 23S rRNA gene contribute to high-level macrolide resistance in some Campylobacter isolates (Corcoran *et al.*, 2006). It was found that

even in the highly resistant Campylobacter strains with the A2074G or A2075G mutation, inactivation of CmeABC also significantly reduced the resistance level to macrolides, suggesting that this efflux system functions synergistically with target mutations (Lin *et al*., 2007). In isolates with low level of erythromycin resistance (MICs 8–16 mg/L), no mutations have been detected in the target gene (Payot *et al*., 2004), and in these isolates the inactivation of

CmeABC leads to restored susceptibility to erythromycin, suggesting the involvement of CmeABC in the intrinsic resistance of Campylobacter (Lin *et al*., 2007). In strains with a high erythromycin resistance level

(MIC > 128 mg/L), the resistance is associated with a mutation in the 23S rRNA gene (Payot *et al*., 2004). In these isolates, the inactivation of CmeABC leads to 2- to 4-fold decrease in erythromycin resistance, implying synergistic action with the target mutations in achieving acquired macrolide resistance (Lin *et al*., 2007). Additionally, the synergy between the CmeABC efflux pump and mutations in the ribosomal proteins L4 (G74D) and L22 (insertions at position 86 or 98) was also shown to confer macrolide resistance in *C. jejuni* and *C. coli* (Caldwell *et al*., 2008).

2.7 Summary of Literature review

Large scale commercial farmers have access to professional veterinary personnel, unlike the smallscale farmers who access a wider range of veterinary therapeutics from agro veterinary outlets and often lacking the professional control due to costs. Studies have shown that tylosin-phosphomycin combination, tetracycline, (OTC®) and quinolones are the antibiotics of choice during the broiler rearing periods in large-scale and small-scale commercial farms in peri-urban Nairobi. Further studies on free-range indigenous chicken from some locations of Makueni County

of Kenya and on live chickens in peri-urban Nairobi, found prevalence rates of the genus Campylobacter at 50.9% and 70.6%,

respectively

High resistance levels by Campylobacter isolates have been reported from small scale and backyard chickens in Kenya, to both ciprofloxacin and tetracycline. In addition, in Western Kenya, resistance levels to ciprofloxacin and tetracycline for Campylobacter isolates from human diarrhoeal cases has been reported. However, information on the Kenyan situation regarding intensively produced large-scale commercial broilers in terms of Campylobacter prevalence and antimicrobial resistant profiles was scanty. This study was designed to fill this gap, in order to inform the broiler industry and enlighten on development of resistant strains which may pose a threat to public health.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The study was done in broilers from six commercial farms located in each of the six counties of Kenya namely Kajiado, Machakos, Murang'a, Kiambu Nairobi, and Nakuru as shown in map of study area (Figure 3.1). The identified farms in each county were similar in relation to source of day-old chicks, management practices and were the main suppliers of broilers to one major abattoir. The management of the farms had a high level of management practices with accurate records such as a health card with details of the number and date of placement, feed intake, mortality, daily weight gain, vaccinations, medication as well as culling. All farms were supported by veterinary technical field staff hired by the hatchery and abattoir management. All the farms had contractual agreement with the abattoir detailing among other issues expected hygienic practices, level of placements at any one time, type of poultry rearing equipment and water quality. All the farmers obtained their day-old chicks from the same hatchery.

The study was carried out between February 2015 and December 2016 and involved random sampling of day-old-chicks as well as 33-day market-ready broilers. The farms were coded per county as follows: Nairobi - NRB, Murang'a - MRG, Kiambu - KBU, Nakuru - NKU, Machakos - MKS, and Kajiado – KJD. Except for Nakuru, all other farms were within 100 km radius of the processing plant and no other counties had contracted farms. Notably, studies on Campylobacter

in highly managed and intensively reared broiler chickens in Kenya were lacking, but at least two studies on small scale farms had been documented.

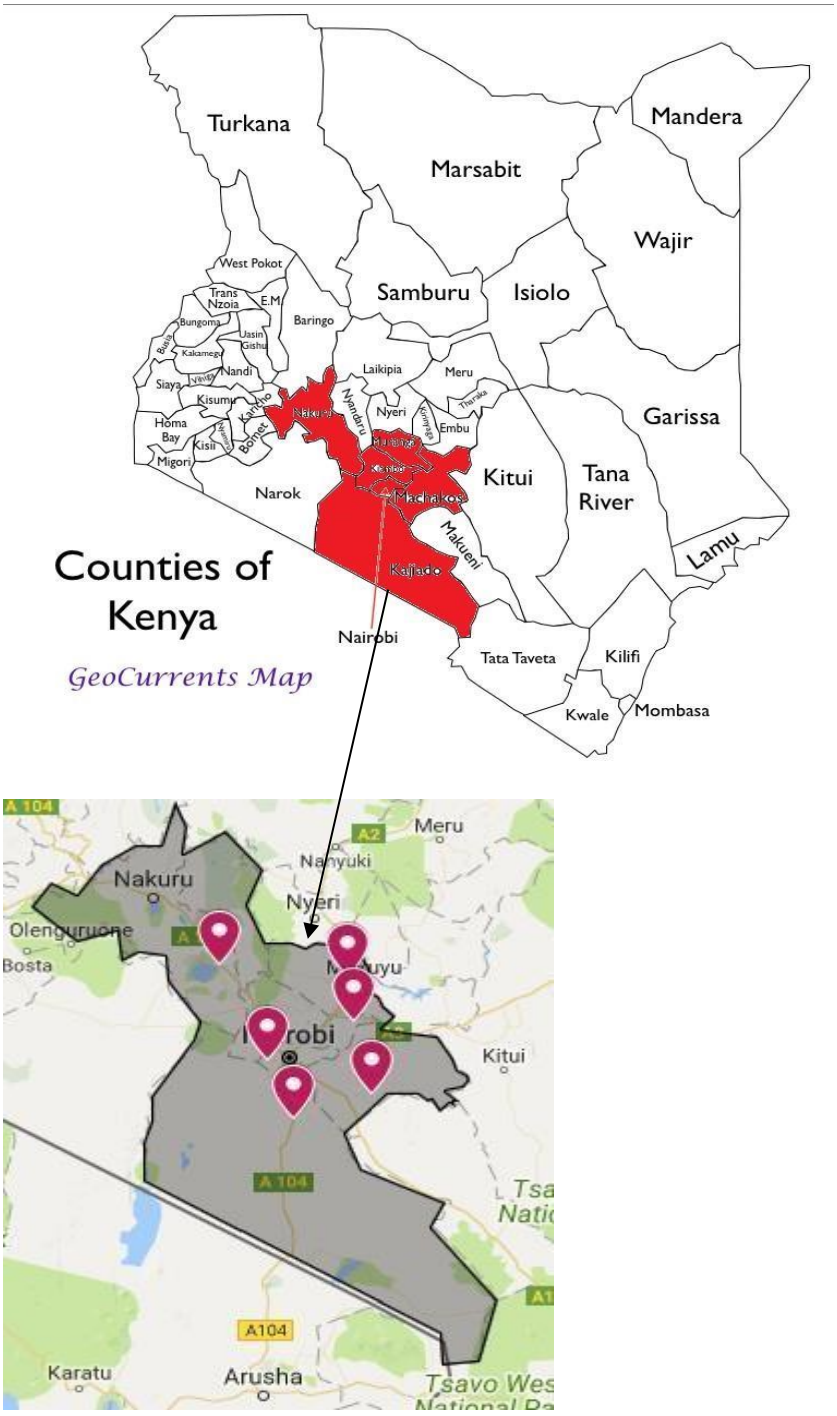


Figure 3.1: Map of study area showing the six counties of Kajiado Machakos, Murang'a,

Kiambu, Nairobi and Nakuru where the farms were located.

Source: google maps

3.2 Study design

The study design was cross-sectional and involved random collection of cloacal swabs from dayold-chicks at the hatchery at different times over a period of three months and again randomly from market ready broilers of age 33 – 36 days from six counties of Kenya.

3.3 Sampling framework

In collaboration with the hatchery management permission was first sought and then hatching dates confirmed. The technical assistants used to collect and handle the samples were recruited from the University of Nairobi laboratories, based on knowledge, expertise and competence. Sampling was done in the morning among available batches of day-old chicks hatched during the night, just before dispatch to various farms or outlets. The batches were counted and a representative number of chicks from each batch were then sampled. Six farms contracted to rear chickens for one major abattoir were previously identified and found to be located in six counties of Kenya were used in this study. In the case of Murang'a and Machakos there was only one farm while in Nakuru the only farm contracted was split into 6 units.

Sampling was done depending on the readiness of the birds at the time of conducting the study. For counties with more than one farm, these were listed and the first farm to deliver slaughter birds was used for sampling of the market ready broilers.

3.4 Sample size determination

The sample size determination was informed by similar studies by other researchers in a similar study carried out in North-Western Italy (Robino *et al*., 2010), the prevalence of Campylobacter was found to be 78.4%. (n=102, p< 0.0001). In another study carried out in CanTho province of Vietnam (Schwan, 2010) the prevalence rates for Campylobacter spp. in poultry ranged from 76% to 95%. The true rate of infection was considered to be higher than the number of reported cases (from 7.6 to 100 times higher) (Samuel *et al*., 2004). The formula used to calculate the sample size was as described by Martin et al. (1987).

$$\Pi = \frac{4PQ}{L_2}$$

Where n is the minimum sample size

P = 0.5 and with an expected survey level within 6% allowable error or required precision of the true level at 95% confidence level:

P = prevalence rate

$Q = 1-P$

On substituting the values:

n = (4 x 0.5 x 0.5)/ 0.0036 = 278

At least 278 sample swabs were required (46.3 from each farm), plus a 10% rounded off figure to add up to a total of 300 (50 from each farm) samples, were collected to cater for any unforeseen losses in the samples.

3.5 Sample collection and questionnaire administration

3.5.1 Sampling at hatchery

Hatchery visits were done early morning following the night after hatching. Strict hygiene and biosecurity measures were adhered to including taking a complete shower and dressing up with sterile clothing provided by the hatchery management. Day-old chicks were selected randomly from batches hatched on predetermined sampling days. Following advice from the lead supervisor, and to raise the level of certainty on prevalence findings, on each sampling day 50 birds were sampled until a total of 600 samples were collected. A sterile swab was inserted and rotated in a circular motion 3-4 times along the inner circumference of the cloaca. The end of the swab was then dipped into Stuart® transport media (Oxoid, UK), and the wooden splint cut off at a point to allow total immersion of the whole swab and tight closure of the vial. A pair of scissors dipped in 70% Ethanol was used. The sample vial was then labelled by date, of collection, county code and sample number and stored in a cooler box for transportation to the laboratory at the Faculty of Veterinary Medicine, Department of Public Health, Pharmacology and Toxicology (PHPT) at The University of Nairobi. The samples collected were recorded on a work sheet noting the sample number, date.

3.5.2 Sampling of market-ready broilers

Sampling preceded prior establishment that the broilers were about to be slaughtered and that they came from any of the six counties under investigation, and where the questionnaire had already been administered. The birds were sampled while shackled at a point immediately after incision of the jugular and carotid blood vessels and prior to entry into the scalding tank. A sterile swab

was inserted into the cloaca, swirled twice or thrice excess faecal material shaken off and the swab dipped into the prepared and pre-labeled vials containing Stuart® transport media. Information on the labels included the code of the county, the farm, sample number and date of collection. The vials were then stored in a cooler box, ready to be transported to the laboratory for further analysis. Fifty samples were randomly collected from individual birds in a batch from selected farms in each of the six counties. Sterile swabs were used and cloacal sampling for slaughter birds was done as described for the day-old-chicks. A total of 300 market – ready broiler birds were sampled

3.5.3 Questionnaire administration

Prior to administration of the questionnaire, the persons responsible for managing the targeted farms were contacted on phone and the objectives explained clearly to them. Upon granting of the permission a date for questionnaire administration was agreed on. The semi structured questionnaire was administered aimed at establishing the general knowledge of drug use, withdrawal periods as well as the types of drugs used in the farms (Appendix I). Experience in rearing of broilers among the managers was also sought. Questionnaires were administered by the researcher and research assistant using face to face method with the hands-on manager, supervisor or proprietor of the farm. Two people were interviewed per farm giving a total of twelve interviewees.

3.6 Laboratory culturing and analysis

3.6.1 Culturing and isolation

In the laboratory, the swab was retrieved from the transport media and streaked (single-line streak inoculation) onto petri-dishes containing modified cefoperazone charcoal agar

(mCCDA). These plates were then placed in a large glass jar with a candle at the bottom. The candle was then lit and the jar covered till the candle flame was extinguished. This method creates a micro-aerophilic environment suitable for growth of Campylobacter spp (Davis & DiRita, 2008). The jar was then placed in the incubator at 42^{o}C for 48 hours, conditions ideal for growth of Campylobacter. The plates were then examined for growth of grey white colonies characteristic of Campylobacter spp. Characteristic grey white colonies were then harvested and placed in skimmed milk contained in micro-titre tubes, vortexed and stored at -20^{o}C until the DNA characterization process.

3.6.2 Biochemical tests

3.6.2.1 Oxidase reaction

The oxidase test was used to determine if a bacterium produces certain cytochrome c oxidases. The test system uses disks impregnated with a reagent such as *N, N, N′, N′*-tetramethyl*p*phenylenediamine (TMPD) or *N*, *N*-dimethyl-*p*-phenylenediamine (DMPD), which is also a redox indicator. The reagent is a dark-blue to maroon color when oxidized, and colourless when reduced. Oxidase-positive bacteria possess cytochrome oxidase or indophenol oxidase - an iron-containing hemoprotein (Isenberg, 2004). Cytochrome oxidase and indophenol oxidase catalyse the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The test reagent, TMP dihydrochloride acts as an artificial electron donor for the enzyme oxidase. The oxidized reagent forms the coloured compound indophenol blue. The cytochrome system is usually only present in aerobic organisms that are capable of using oxygen as the terminal electron acceptor. The end product of this metabolism is either water or hydrogen peroxide as a result of breakdown by catalase (MacFaddin *et al*., 2000).

Oxidase positive (OX+) normally means the bacterium contains cytochrome c oxidase and can therefore use oxygen for energy production by converting oxygen to hydrogen peroxide or water with an electron transfer chain. The test procedure is to wet each disk with about four inoculating loops of deionized water, then using a loop, aseptically transfer a large mass of pure bacteria to the disk. Observe the disk for up to three minutes. If the area of inoculation turns dark-blue to maroon to almost black, then the result is positive. If a color change does not occur within three minutes, the result is negative.

3.6.2.2 Catalase reaction

The slide (drop) method was used in our case and this involved placing a microscope slide inside a petri dish while keeping the petri dish cover available (Johnson, 2008). Using a sterile inoculating loop or wooden applicator stick, a small amount of organism from a well-isolated colony was collected and placed onto the microscope slide. Using a dropper or Pasteur pipette, one drop of 3% hydrogen peroxide (H_2O_2) was placed onto the organism on the microscope slide, without mixing and immediately covered with a lid in order to limit aerosols and observing for immediate bubble formation (O_2 + water = bubbles). Positive reactions were evident by immediate effervescence (bubble formation). Weak positive reactions could be observed by placing the microscope slide over a dark background and using a magnifying glass or microscope. When using a microscope, a cover slip was placed over the slide and viewed under 40x magnification. Lack of bubble formation implied that there was no catalase enzyme to hydrolyse the hydrogen peroxide and represented a catalase-negative reaction. Quality control was performed by using organisms known to be positive and negative controls.

3.6.3 Disk diffusion method of antimicrobial sensitivity testing

The disk diffusion method used was as described by Kirby – Baeur (1966). Mueller-Hinton agar at pH level of 7.2 -7.4 was first sterilized and then cooled to 40^{o}C – 50^{o}C to which 5% horse blood was added. An amount of 20 ml of this mixture was then dispensed into Petri dishes and was used for inoculation. A sterile wire loop was used to transfer an inoculum containing about 150 cells per milliner onto the Mueller-Hinton agar for streaking. In order to ensure uniform growth, the plate was rotated at 90^{o} for a repeat streaking. This process was repeated thrice. The plates were allowed to dry for 5 minutes.

A sterile loop was used to collect several morphologically similar bacterial colonies from Mueller-Hinton agar plates which were then suspended in sterile saline (0.85% NaCl w/v in water) in a test tube to a turbidity of 0.5 McFarland standard. The density of the suspension was compared visually to a 0.5 McFarland turbidity standard. The density of the suspension was adjusted to McFarland 0.5 by addition of saline or more organisms.

Inoculum suspensions were optimally used within 15- 60 minutes of preparation. A sterile cotton swab was then dipped into the inoculum suspension and the excess fluid removed by turning the swab against the inside of the tube to avoid over inoculation of plates, spread evenly over the entire surface of the agar plate by swabbing in three directions. Antimicrobial disks containing ciprofloxacin, tetracycline and erythromycin were dispensed on to the agar using an antibiotic disk dispenser, within 15 minutes of inoculation of the plates The number of disks on a plate were limited so that unacceptable overlapping of zones was avoided. In this study nine disks were used per petri dish A flame - sterilized forceps was then used to gently press each disk onto the agar to ensure attachment. The plates were then incubated or 24 hours at 37^{o}C. The resultant zones of inhibition diameters, as visualized by the unaided eye read from the front with the lid removed

and with reflected light when the plate was held about 30 cm away, were measured using calipers, to the nearest millimetre, and simultaneously recorded.

These diameters were then interpreted using the European Union Committee on Antimicrobial Sensitivity Testing (EUCAST) standard to classify the organisms as either sensitive, intermediate or resistant. (Appendix I).

Gel Electrophoresis is a process where an electric current is applied to DNA samples creating fragments that can be used for comparison between DNA samples. The DNA migrates towards the anode (due to the negative charge it possesses) through the gel pores with the largest size migrating the least. DNA was extracted, isolated and amplified using a thermocycler whose settings were adjusted to 95^{o}C for 30 seconds, 58^{o}C for 90 seconds and 72^{o}C for 60 seconds and run for 3 hours. A working volume of 12.5µl comprising 0.2µl forward and 0.2µl reverse primers for each species of Campylobacter, 6.25µl of PCR Mastermix and topped up with 1.95µl distilled water. 2µl ethidium bromide (loading dye) was added onto the prepared 2% agarose (Appendix III), which was in TBE buffer (Appendix IV). Using microliter tubes the samples were then added onto the gel parafilm wells in the TBE buffer solution on the electrophoresis apparatus and an electric current applied. The DNA bands were then visualized via a digital camera (Cannon, Japan).

3.7 Polymerase chain reaction amplification assay

The polymerase chain reaction (PCR) is the molecular biological tool that was used for amplifying DNA from pure colonies of Campylobacter. Various techniques for PCR optimization have been developed to improve PCR performance and minimize failure. Multiplex PCR assay was the technique used in this study. The technique allows amplification of multiple targets in one PCR

experiment. using multiple primer pairs in a reaction mixture. It is effective and also saves time and effort. The primers used for amplification of DNA for multiplex PCR assay in this study were as referenced in Table 3.1, (procured from InqabaBiotec East Africa Ltd). The PCR conditions for amplification for Campylobacter species isolated from cloacal swabs of market ready broiler chickens were as set out in Table 3.2. The PCR thermal cycling parameters used for the same purpose, were as demonstrated in Table 3.3.

3.7.1 Optimization of primers

Optimization involved the use of the single primers specific for each Campylobacter species and varying the dilution rates until the gel electrophoresis patterns showed confluence with the molecular marker base pairs. A 12.5µl working volume containing 6.5 µl of master mix, 0.25µl forward primer, 0.25µl reverse primer and 3µl of PCR distilled water was initially prepared as per the manufacturers' guideline, but a dilution of 0.2µl in lieu of 0.25µl eventually produced the desired confluence and was used throughout the study. A total of ten PCR tubes hitherto sterilized using a pressure cooker at 121^{o}C for 15 minutes were used for generation of DNA in the thermocycler which was automatically programmed at 95^{o}C for 30 seconds, 58^{o}C for 15 minutes, 72^{o}C for 7 minutes and then ran for 3 hours. The PCR products were then subjected to gel electrophoresis and visualized against the molecular ladder through a digital camera. Figure 4.1 shows an example of visualization of a positive sample.

3.7.2 Contamination and PCR

Due to the extreme sensitive nature of the PCR method, only a few DNA molecules are required in a single reaction for amplification across several orders of magnitude. Adequate measures to avoid contamination from any DNA present in the lab environment such as bacteria, viruses, or

human sources are imperative. Products from previous PCR amplifications are a common source of contamination and dividing the lab into separate areas of operations is necessary (Balin *et al.,* 1998). One laboratory area was dedicated to preparation and handling of pre-PCR reagents and the set-up of the PCR reaction, and another area to post-PCR processing, such as electrophoresis for PCR product purification. Standard operating procedures involved using pipettes with filter tips and wearing fresh laboratory gloves. PCR was assessed against a known positive set up identical to the experimental PCR using Kilo base pairs (Kbps) specific for each of the various Campylobacter species.

Table 3.1: Primers used in the multiplex PCR assay for Campylobacter genes (procured from InqabaBiotec East Africa Ltd)

Species	Amplicon size (bp)	Oligonuclotides	References
C. coli	502	5'-GGTATGATTTCTACAAAGCGAG-3' 5-ATAAAAGACTATCGTCGCGTG-3	Linton et al (1997)
C. fetus	359	MG3F 5-GGTAGCCGCAGCTGCTAAGAT-3' CF359R 5'- GCCAGTAACGCATATTATAGTAG-3	Hum et al (1997) Inglis &Kalischuk (2003)
C. lari	251	CLF 5''TAGAGAGATAGCAAAAGAGA – 3' CLR 5'- TACACATAATAATCCCACCC – 3	Weng et al (2002)
C/ jejuni	161	C1 5'- CAAATAAAGTTAGAGGTAGAATGT – 3' C3 5'- CCATAAGCACTAGCTAGCTGAT – 3;'	Weng et al (1992)
C.upsaliencis	85	CU61F 5'- CGATGATGTGCAAATTGAAGC -3' CU146R 5'- TTCTAGCCCCTTGCTTGATG – 3	Yanazaki et al (2007)
' *C.hyointestinalis*	611	HYO1FATAATCTAGGTGAGAATCCTAG -3' HYOFET23SR 5'GCTTCGCATAGCTAACAT -3	Inglis& Kalischuk (2003a)
Genus Campylobacter	816	HYO1F 5'- ATAATCTAGGTGAGAATCCTAG – 3' HYOFET23SR 5'- GCTTCGCATAGCTAACAT – 3	Linton et al (1996)

Table 3.2: PCR cycling conditions for amplification of Campylobacter species specific genes at UoN- PHPT, Nairobi in 2016

Sno.	Component	µl	Final composition
1	Distilled water	37.5	1.45
2	10X PCR Buffer	5.0	1.25
3	dATP, dCTP, dGTP, dTTP mix	3.0	0.2
4	$MgCl_{2;}$ 25 Mm	1.5	1.5
5	Primer 1	2.0	0.2
6	Primer 2	2.0	0.2
7	AmpliTaq Polymerase	5	5
8	Template DNA	2	
	Total	58	12.5

Table 3.3: PCR thermal cycling parameters used for the amplification for Campylobacter Spp isolated from cloacal swabs of market ready broiler chicken studied at UoN- PHPT, Nairobi in 2016

Sno	Step	Temperature ^{o}C	Duration
1	Initial activation/ Denaturation: 1 cycle	95	5 minutes
2	Amplification: 35 cycles	94	30 seconds
3	Annealing	52	30 seconds
4	Extension	68	60 Seconds
5	Final Extension: 1 cycle	72	5 minutes
6	Hold	4	

3.8 Data management and analysis

Demographic information obtained was entered into the open-ended questionnaire and was checked for accuracy and corroboration. The level of education of the persons involved in day to day management of the farm was extracted during the interview and tabulated. Data necessary for statistical analysis from laboratory work was first tabulated in MS Excel v.2016. and then imported into SPSS statistical package for analysis of variance, statistical significance and prevalence rates of the genus Campylobacter isolated from the six counties. Similarly, catalase and oxidase positivity prevalence were analysed using the same software and results likewise illustrated by bar graph. Data on the six species of Campylobacter identified by MPCR method was also tabulated in MS Excel v.2016 and prevalence per country illustrated by bar graphs generated. Antimicrobial resistance profiles were recorded in MS Excel work sheet as zones of inhibition diameters in millimetres and these were compared with the EUCAST clinical antimicrobial sensitivity standards for interpretation. This standard interprets sensitivity for the antibiotics ciprofloxacin, tetracycline and erythromycin. Interpretation for tetracycline is also used for doxycycline. The zonal diameters were statistically analysed.

3.9 Ethical views and logical considerations

Permission to conduct this study was sought from the Biosafety Animal Use and Ethics Committee of the Faculty of Veterinary Medicine, The University of Nairobi (Permit no.

Appendix V).

The study participants were persons directly involved in day to day hands-on management of the broiler flocks who were purposively selected to include the manager and supervisor in each broiler

farm per county. Informed consent was obtained from each participant through telephone communication and a uniquely identifying code assigned which corresponded to each farm selected. Confidentiality of the data was assured by use of codes on the questionnaires which were safely kept by the researcher under lock and key. Aspects of confidentiality were discussed and mutually agreed on between the research assistants and farm managers. There were no direct benefits to the study participants, but a research dissemination seminar was held to give feedback on the findings and advise on prudent use of antimicrobial agents to slow down development of resistant strains.

The collection of cloacal samples from day old chicks at the hatchery was gently and carefully done, minimizing any adverse stress effects. Other animal welfare issues observed by the factory included the use of blue lighting to calm pre-slaughter birds, provision of high speed cooler fans to the live birds at resisting prior to shacking, as well as using an electric current to stun the birds to render them unconscious before severing the neck blood vessels. Samples from market- ready broilers were collected from the cloaca of birds whose jugular and carotid vessels had just been severed which is one of the recommended humane slaughter methods for chickens hence taking care of animal welfare concerns. Safety for all persons collecting samples was ensured and they adhered to mandatory use of personal protective equipment (PPEs) which comprised of head cover, face mask, hand gloves, dust coat and knee –high gum boots.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic data

Demographic data of persons involved in the management of the commercial broiler farms surveyed from the six counties in the study on assessment of Campylobacter species antimicrobial profiles in commercial broiler production systems in Kenya, was as determined by the questionnaire administration (Appendix VI).

The farms were located within 100 kilometers radius from the processing plant in the different counties. The mean number of years of broiler rearing experience among the managers of the farms surveyed during the study was 12.5 years with a range of 4-25 years. (Appendix VII), with flock sizes ranging from 12000 – 18000 (μ=16788) birds in batches of various ages. Of interest to the study was the 33-day-old market-ready, broilers. The study found that the training level among supervisors was college of animal health (level 4) while the managers, who for one farm was the proprietor, had tertiary level of education (level 3). The average number of workers directly involved in rearing of the flocks was 4 males (range 4 – 6) and 1 female (range 0 – 2) as shown in Table 4.1. 1.

Table 4.1.1: Demography and characteristics of the farm workers directly involved in rearing of the broiler flocks per County, Kenya 2015

County	No. of farms sampled	Flock size (0-33days)	Level of training of supervisor	Level of training of manager	Years of experience	No. of male workers	No. of female workers
Nairobi	1	12000	4	4	9	4	0
Kajiado	1	14000	3	4	7	6	1
Nakuru	1	18000	4	4	25	4	1
Machakos	1	12000	3	3	7	3	0
Kiambu	1	12000	3	3	23	3	2
Murang'a	1	12000	4	4	4	4	1
Sum	N=6				75		

Key: Level 1 – Primary school

Level 2 –Secondary

Level 3 – Tertiary

Level 4 – Animal health training

4.1.1 Antimicrobial use in the broiler production systems studied

Phosphomycin-tylosin combination and phenol were reported to have been used in all the farms during broiler production with Kiambu reporting the use of tetracyclines also (Table 4.1.2).

Table 4.1.2: Large scale commercial farms and drugs used during production in the study counties, Kenya, 2015

County/ Farm	Drugs
Nairobi	phosphomycin, tylosin, phenol
Kajiado	phosphomycin, tylosin, phenol
Kiambu	phosphomycin, tylosin, tetracycline phenol
Murang'a	phosphomycin, tylosin, phenol
Machakos	phosphomycin, tylosin, phenol
Nakuru	phosphomycin, tylosin, phenol

4.2 Prevalence and identification of Campylobacter

4.2.1 Growth and cultural characteristics of Campylobacter

All the cloacal samples totaling 600 (100%) from day-old chicks showed no growth on CCDA, following incubation at 42^{o}C for 48 hours under anaerobic conditions which indicates that the chicks at the hatchery had no Campylobacter infections. For these samples no further tests were carried out.

Out of 300 samples from 33-day-old market-ready broilers, 277 (92.3%) showed growth of grey-white colonies characteristic of Campylobacter *spp* while the remaining 23 (7.7%) had no growth. All samples (100%) collected from Murang'a county showed growth on culturing while 82% which was the lowest prevalence, was observed from Kajiado county.

4.2.2 Biochemical characteristics of the Isolates

The findings of the biochemical tests on the bacterial isolates indicated that the bacterium present was capable of producing Cytochrome C oxidase and catalyse enzyme. The findings indicate high reaction rates which mirror the presence of Campylobacter and on the average oxidase and catalase positivity was almost equivalent among the counties at 91.6% and 91.3% respectively (Table 4.2.1)

Table 4.2.1: Prevalence rates, catalase and oxidase positivity of genus Campylobacter per County

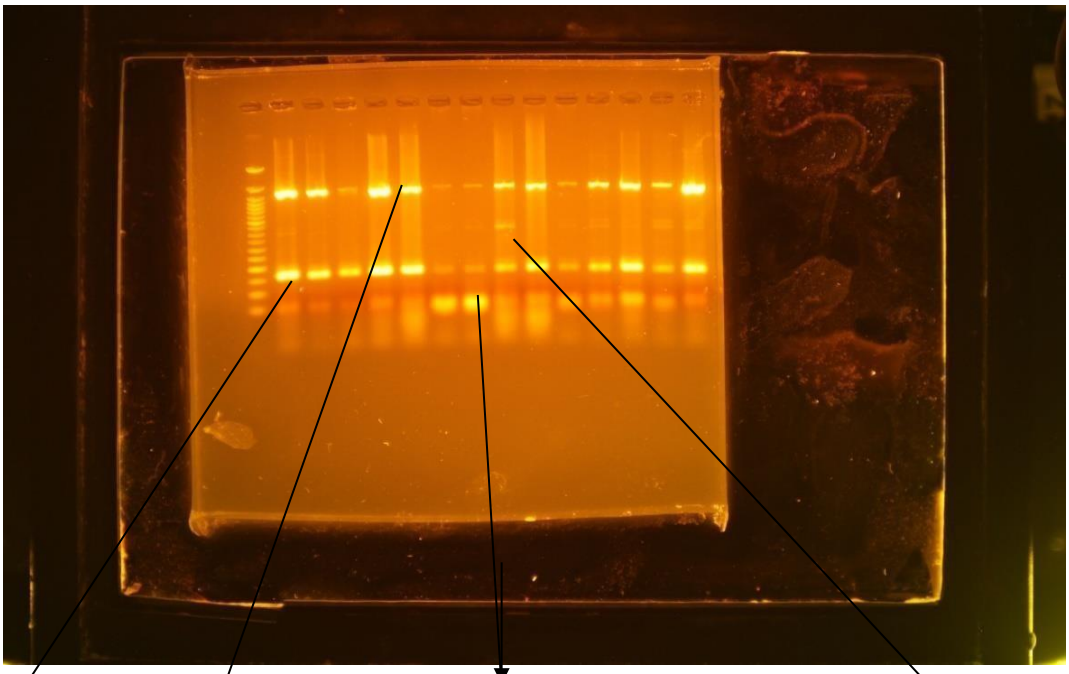
County	Number sampled	Number Campylobacter +ve (n, %)	Catalase +ve (n, %)	Oxidase +ve (n, %)
Nairobi	50	47 (94.0)	47 (94.0)	47 (94.0)
Kajiado	50	41 (82.0)	39 (78.0)	40 (80.0)
Nakuru	50	44 (88.0)	43 (86.0)	43 (86.0)
Machakos	50	46 (92.0)	46 (92.0)	46 (92.0)
Kiambu	50	49 (98.0)	49 (98.0)	49 (98.0)
Murang'a	50	50 (100)	50 (100)	50 (100)
Total (N)	300	277		
%	100	92.3	92.0	92.0

Six species of the genus Campylobacter were isolated and identified in this study with varying prevalence rates in the counties. Using PCR amplification 92.3% of all the cloacal samples tested were positive for Campylobacter genus, with *C. jejuni* being the most commonly observed species. Murang'a County had the highest prevalence at of the genus 100% with Kajiado having the lowest prevalence at 86% (Table 4.2.2). The species identified were *C. jejuni, C. lari, C. coli, C. fetus, C. upsaliensis* and *C. hyointestinalis. C. jejuni* was isolated in all the counties with *C. hyointestinalis* only occurring in Machakos County.

Four counties namely Kiambu, Machakos, Murang'a and Nakuru had over 72% of isolates turning positive for *C. jejuni,* while the remaining two counties of Nairobi and Kajiado had 62% and 48% positivity respectively, for the same species. *C. upsaliensis* had the least occurrence in Nairobi and Murang'a at 4% and 6% respectively. Overall, *C. jejuni* was most prevalent of the species in all counties at 66% while *C. upsaliensis* together with *C.fetus* were least prevalent, each at 1.7% (Table 4.2.2)

Table 4.2.2: The prevalence and species of Campylobacter per County per farm identified by PCR from 300 cloacal swabs of market ready broilers studied in 2016

County/ Farm	**Campylo------- bacter Genus 816 bps n=300 (%)**	***C. jejuni* 191 bps n (%)**	***C. coli* 502 bps**	***C. hyointestinalis* 611bps**	***C. lari* 251 bps**	***C. fetus* 359bps**	***C. upsalien sis* 86 bps**
Kajiado	41 (86%)	24(48%)	2(4%)	0	0	0	0
Nairobi	47 (94%)	31(62%)	6(12%)	0	2(4%)	2(4%)	2(4%)
Nakuru	44 (88%)	36(72%)	6(12%)	0	0	0	0
Muranga	50 (100%)	46(92%)	0	0	2(4%)	3(6%)	3(6%)
Kiambu	49 (98%)	41(82%)	0	0	11(22%)	0	0
Machakos	46 (92%)	44(88%)	1(2%)	2(4%)	15(30%)	0	0
Total	277 (92.3%)	198 (66%)	15 (5%)	2 (0.67%)	30 (10%)	5 (1.7%)	5 (1.7%)



C. jejuni (161bps), *C. coli* (502bps), *C.upsaliensis* (86bps), *C. fetus* (359bps),

Figure 4.1 Visualization of PCR Electrophoresis of Campylobacter positive samples isolated in various counties, Kenya, 2016

Multiplex PCR electrophoresis digital photo of a positive sample as seen at PHPT laboratory, Kabete, the bright bands corresponding to the base pairs of the respective Campylobacter species. The extreme left column is the molecular marker at intervals of 50 bps.

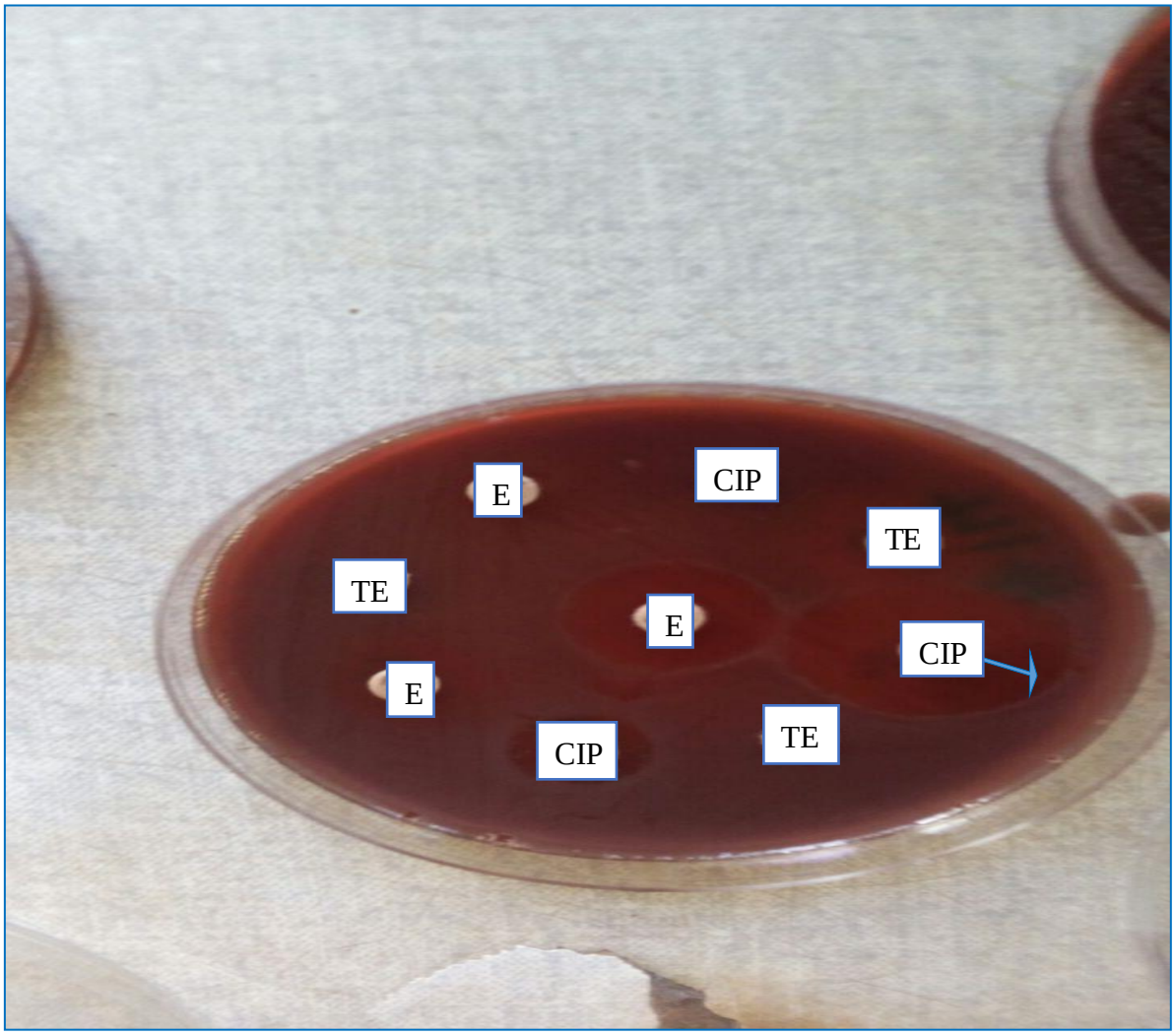
4.3 Antimicrobial sensitivity

4.3.1 Microbiological cultures characteristics

Evaluation of pure colonies of Campylobacter streaked on the agar, with antibiotic impregnated disks and incubated at 42^{o}C for 48 hours showed clear zones of inhibition for effective antibiotics while in non-effective antibiotics there were no zones of inhibition (Figure 4.2). The EUCAST clinical break-points of 26mm, 24mm and 30mm being minimum diameters for classification of

Campylobacter as sensitive to ciprofloxacin, tetracycline and erythromycin, respectively. In addition, according to EUCAST, the findings of diameters for tetracycline are also used to interpret for doxycycline. The implication here is that the organism was also resistant to doxycycline. The results are as shown in in Tables: 4.3.1, 4.3.2 and 4.3.3).

4.4 Microbiological cultures characteristics



Key:

CIP- Ciproflaxacin, TE- Tetracycline and E- Erythromycin

Figure 4.2 Zones of inhibition by Ciprofloxacin, Tetracycline and Erythromycin against Campylobacter-inoculated Muller-Hinton Agar at PHPT Laboratory, 2016

4.4.1 Antimicrobial resistance of Campylobacter to ciprofloxacin

For ciprofloxacin there was significant difference in zone diameters across the counties and following separation of the means into two groups ("a" with means that were not significantly different and "b" whose means showed significant differences), Nakuru appeared in both groups. (Table 4.3.1) only 2.2%, 2.4% and 5.4% of samples from Kajiado, Kiambu and Nairobi respectively showed sensitivity to ciprofloxacin, or 98.47% of the isolates were resistant to ciprofloxacin

As indicated, the inhibition zone diameters had an average of 9.92 mm for 261 isolates with a standard deviation of 0.327 (Table 4.3.1). However, the diameters were significantly different (Fvalue = 5.007; df = 5, 255; $p < 0.001$) across the counties. From the study findings, 1.5% met the clinical break off point based on EUCAST standard. The isolates in this category were from Nairobi, Kiambu and Kajiado. The source of the difference was determined by separation of the means by Tukey's Honestly Significant Difference (HSD) method and was due to Machakos which had the lowest mean and partly by Nakuru which appeared in both groups.

Table 4.4.1: A comparison of ciprofloxacin inhibition zones among various counties and p-values

County	*N*	Mean±SE	Min	Max	Percentage≥26 mm	F-statistic	P-value
Kajiado	45	10.78±0.86b	6	29	2.22		
Kiambu	42	10.48±0.83b	6	29	2.38		
Machakos	46	6.78±0.36a	6	18	0.00	5.007	< 0.001
Murang'a	46	11.70±0.79b	6	24	0.00		
Nairobi	37	10.16±1.01b	3	29	5.41		
Nakuru	45	9.76±0.71ab	6	22	0.00		
Total	261	9.92±0.33	6	29	1.53		

4.4.2 A comparison of tetracycline inhibition zones among the various counties and pvalues

The study showed that 100% of the isolates were resistant to tetracycline and there was no significant difference across the counties (Table 4.3.2). The highest inhibition zone diameter for tetracycline was 27 mm and fell short of 30 mm, which is the clinical breakpoint for the antimicrobial resistance. For tetracycline, the μ was 8.33 mm, N=261, SE=0.317 with no significant difference (F= 1.485; df = 5, 255 p- < 0.195 (Table 4.3.2).

Table 4.4.2: A comparison of tetracycline inhibition zone diameters among the various study counties

County	*N*	Mean±SE	Minimum	Maximum	Percentage≥30mm	F Statistic	p-value
Kajiado	45	9.40±0.95	6	26	0		
Kiambu	42	8.17±0.73	6	27	0		
Machakos	46	7.41±0.64	6	25	0		
Murang'a	46	8.28±0.72	6	26	0	1.485	0.195
Nairobi	37	9.59±1.05	6	27	0		
Nakuru	45	7.36±0.53	6	23	0		
Total	261	8.30±0.32	6	27	0		

4.4.3 A comparison of inhibition zones among the various study counties and p-values

The EUCAST zone diameter clinical break point for antimicrobial sensitivity to erythromycin is 24mm and the result of this study indicate that 100% of the isolates showed resistance with the diameters ranging from 6mm to 12mm however, none of the isolates met the clinical break of point of 24 mm. There was significant difference in the occurrence of resistance shown to erythromycin across the six counties (Table 4.3.3). For erythromycin, the μ was 6.37, N = 261, SE = 0.065 ($F = 2.525$; $df = 5, 255$; $p < 0.030$).

Table 4.4.3: A comparison of inhibition zones among the various study counties and p-values

County	*N*	Mean±SE	Minimum	Maximum	Percentage≥24mm	F statistic	p-value
Kajiado	45	6.18±0.09ab	6	9	0		
Kiambu	42	6.38±0.16ab	6	10	0		
Machakos	46	6.37±0.14ab	6	10	0		
Murang'a	46	6.80±0.24b	6	12	0	2.525	0.03
Nairobi	37	6.08±0.06a	6	8	0		
Nakuru	45	6.36±0.15ab	6	10	0		
Total	261	6.37±0.07	6	12	0		

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The average number of workers directly involved in rearing of the flocks was 4 males and 1 female with a range of 4 – 6 for males and 0 – 2 for females. This is in contrast with a study done in peri urban Nairobi where women were found to be more involved in broiler rearing compared to men who were more involved in work outside their homesteads (Muthuma, 2015)..The demographic results in this study suggest high levels of knowledge and experience among the managers in animal husbandry practices as well as flock management. This in turn meant a better understanding among them on antimicrobial usage.

The antimicrobials used in the farms were Phosphomycin and phenol, and only one farm reported having used ciprofloxacin once and long before the study commenced. Fosbac® (phosphomycin and tylosin combination) was the antibiotic of choice and whenever it was used a multivitamin preparation was included. Farmguard ®, a phenolic compound was regularly used as a disinfectant in all the farms. These findings had no impact on the results of the study regarding antimicrobial resistance.

The finding that there was no growth of any microbes observed from samples collected from day-old chicks at the hatchery is significant in that it assures of Campylobacter free commercial chicks for the broiler producers and rules out a horizontal transmission. This is the expected standard of

a well-run hatchery with no contamination. It is important in that it assures farmers of clean stock from source.

According to this study, the prevalence rate of Campylobacter among market-ready broiler chicken from large-scale commercial farms, was very high and six species of the genus were genetically characterized using multiplex PCR method. This prevalence is much higher than that found in a study on free-range indigenous chicken from some locations of Makueni County of Kenya (Ngethe, 2015).

It is also higher than that found in another study on live chickens in peri-urban Nairobi (Mageto, 2017). In Tanzania, Mdegela et al. (2006) reported a lower prevalence rate as did Ansari-Lari et al. (2011) in Iran. Another study in Malaysian broiler farms Yap et al. (2005) observed a lower rate. The European Union flock prevalence rate (EFSA, 2010) is also lower than observed in this study (EFSA, 2010). The selected farms in this study were large scale commercial broiler production farms with high level of management and supervision, however, the high prevalence rate or the source of contamination was not explained nor determined. Studies on prevalence and antimicrobial resistance of Campylobacter in intensive and highly managed broilers in Kenya were lacking. The studies done in Kenya among broilers in Makueni county and peri-urban Nairobi involved smaller sample sizes and probably this, combined with the duration between the studies, could have contributed to the differences seen in prevalence rates, especially due to lack of any mitigation measures. As far as AMR is concerned, transmission of genetic materials or plasmids that confer resistance among bacteria to the antimicrobials has been documented in various studies. The ease of travel from one continent to another globally has made transmission of these gametic materials faster and more widespread, and this phenomenon may be a contributing factor to contamination of the environment, fomites or water.

In the current study, six species of Campylobacter were genetically characterized using the PCR process. On average *C.jejuni* had the highest prevalence rates while *C. lari* had the least. *C. jejuni* infection is one of the most commonly identified bacterial causes of acute gastroenteritis in humans worldwide. In the United States more than 99% of reported infections with *Campylobacter* are with *C. jejuni* (Friedman *et al.*, 2000). *C. coli* is involved in a majority of the other cases (Lansing *et al*., 2005) while *C.lari* has been involved in cases of enteritis (Simor and Wilcox, 1987). It is also associated with severe abdominal pain and terminal bacteremia in humans (Nachamkin, 1984). *C. hyointestinalis* is implicated as a pathogen in gastroenteritis and diarrhoea in humans (Edmonds *et al*., 1987). *C. fetus* is an opportunistic human pathogen and can cause bacteremia and thrombophlebitis (PerezPerez *et al*., 1996). Though rare, *C. fetus* can lead to fatal septicemia in newborns and immunocompromised individuals (Monno *et al*., 2004). *C. fetus*, along with *C. coli*, has been shown to cause septicemia (Kist *et al.*,1984). In humans bacteremia can lead to localized infections of the meninges in the brain, the respiratory pleural spaces or lungs, joints, the pericardial sac or the peritoneum (David *et al*., 2005). The high prevalence of these Campylobacter species in market ready broilers poses a risk to humans who may consume this meat and particularly immunocompromised individuals who could develop gastroenteritis and the complications caused by this bacterium.

In this study measurement of zones of inhibition diameters showed that 1.5% of the pure cultures of Campylobacter met the EUCAST standard of 26mm which is the clinical break

- point. The clinical break point is the minimum radius in millimetres away from the antimicrobial

- impregnated disk where no bacterial growth is observed in a growth medium. The emergence of fluoroquinolone-resistant Campylobacter species, with poultry as an important source, has been documented in the United States (Gupta *et al.,* 2004). In a study carried out in the United States,

resistance of Campylobacter isolates from conventional broilers showed considerable levels with respect to tetracycline 82%, erythromycin 54%, nalidixic acid 41%, and ciprofloxacin 35% (Ge *et al*., 2003). The findings on antimicrobial resistance in the current study are in agreement with the USA study. As reported in this study, antimicrobial resistance to commonly used antibiotics namely ciprofloxacin, tetracycline, doxycycline and erythromycin was as high. These results are in agreement with a similar study by Nguyen et al. (2016) on antimicrobial resistance of Campylobacter isolates from small scale and backyard chickens in

Kenya, which reported high resistance levels to both ciprofloxacin and tetracycline.

Resistance to erythromycin in this study was in agreement with another carried out in Vietnam by Nguyen et al. (2016).

In another study in Northern Germany among Campylobacter isolates from pigs, resistance to ciprofloxacin and tetracycline was also lower than that found in this study (Dohne *et al.,* 2012). In Spain, Campylobacter isolates found in chicken meat had slightly lower resistance levels to ciprofloxacin and erythromycin (González-Hein *et al.,* 2013). Similar studies in Turkey (Abay *et al*., 2014). found the resistance of Campylobacter isolates from chickens to nalidixic acid and tetracycline to be in agreement with this study undertaken in Kenya

Studies among humans with cases of diarrhoea, have been documented. Campylobacter infection is a major cause of fatalities in children under the age of five and among adults is a precursor to the Guillean-Barre syndrome, reactive arthritis (ReA) as well as traveller's diarrhoea, hence its public health importance. This however contrasted another study that showed much lower resistance levels to ciprofloxacin and tetracycline for Campylobacter of human diarrhoeal cases by Brooks et al. (2003) in Western Kenya. All these studies support the rising concern about development of antimicrobial resistance among bacteria of public health importance. It is clear

that with time the prevalence and antimicrobial resistance rates of Campylobacter are generally on the rise.

Several studies have suggested that the indiscriminate and unregulated use of antimicrobials in agriculture and livestock production has contributed significantly to the development of AMR among bacteria. The use of antimicrobials in the farms involved in this study was strictly controlled and there was no routine use of drugs in sub-lethal doses for either prophylaxis or growth promotion. According to this study, all the broiler farms sampled reported having used Phosfomycin-tylosin (Fosbac®),Oxytetetracycline and Quinolone at one time or the other whenever need arose and on advice from the technical teams. The use of these antibiotics was only if, and when infection occurred. The farms were found to enjoy free technical back-up from a common source upon signing of contractual agreements and had strict adherence to production protocols, with accurate and detailed record keeping. All farms used the same phenol-based compound for disinfection of poultry houses but Fosbac® was the antibiotic of choice. Loss of profits through condemnations at the abattoir was a major driving force in strict observation of drug withdrawal periods among all the farms. This is in contrast with another study done among small scale broiler farmers in peri-urban Nairobi (Muthuma, 2015) which showed that a good number of farmers failed to observe withdrawal periods, lacked free technical backup, and had limited knowledge of veterinary drugs, leading to residues in meat and likely contribution to antimicrobial resistance. The hands-on managers were fairly literate and well versed with issues surrounding usage of veterinary drugs and their withdrawal periods. Of great concern was the very high resistance shown to tetracycline, erythromycin and ciprofloxacin by the Campylobacter isolates in the study. According to a WHO report, selection for resistance in one part of the world affects health in other parts of the world, through international travel and trade and *invitro*

antimicrobial susceptibility testing is essential to provide guidance to physicians and veterinarians on appropriate treatment of infections and to generate data on the occurrence of acquired resistance in Campylobacter (WHO, 2012).

Contamination of chicken meat in the poultry processing plants is possible especially because the scalding tank can be a massive reservoir of faecal material. The handlers may also cross contaminate following handling of carcasses prior to entry into the spin washer. Evisceration machines can also be a source of dissemination of the bacteria especially due to splash-washing and handling. However, in this study the cloaca swabs were collected at the beginning of the slaughter process when the poultry were shackled and being bled. Hence the prevalence rates observed in the study cannot be attributed to the slaughter process. Furthermore, the day-old chicks from the hatchery were free of any Campylobacter species. Therefore, the most likely source of the Campylobacter may be the feed, water, environment, farm workers or fomites during the broiler growing period.'

5.2 CONCLUSIONS

There was prudent use of antimicrobials in the large-scale commercial broiler farms. Phenol (Farmguard®) was routinely used for disinfection of premises and formites while phosphomycin-tylosin (Fosbac®) was the antibiotic of choice whenever indicated. The levels of use were as guided by veterinarians.

Day-old chicks from the hatchery are free of Campylobacter bacteria, ruling out vertical transmission of the organism.

. The prevalence rate of Campylobacter in broilers ready for slaughter was 92.3%

At least six species of Campylobacter exist in the guts of broilers sampled from the various counties of Kenya.

The antimicrobial resistance levels showed that 98.5% of the isolates were resistant to ciprofloxacin, while 100% showed resistance to both tetracycline and erythromycin. This is despite the fact that the three antimicrobials were not being used by the farms studied, but are commonly used in small scale chicken farms.

5.3 RECOMMENDATIONS

Based on the study findings, the following recommendations were made:

The level of animal health training and rearing experience of the supervisors and managers may contribute to prudent use of antimicrobials in broiler production systems and hence slow down the development of antimicrobial resistance. Emphasis should be laid on good education levels in both small and large-scale broiler production systems, regardless of their flock sizes or location.

The three antibiotics tested are commonly used in both veterinary and human treatment, and the level of resistance shown by Campylobacter in chickens indicates an impending therapeutic difficulty if and when the organism is transmitted to humans. Therefore, the use of these antibiotics should be minimized or withdrawn.

There is need to establish the age at which broilers in commercial production systems in Kenya develop Campylobacter infection and the risk factors involved and source of infection.

Further investigations need to be carried out to evaluate the slaughter process in order to assess level of contamination of broiler meat to assure consumers of broiler meat safety.

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7.0 APPENDICES

Appendix I: EUCAST standard Campylobacter AST (disk diffusion) breakpoints.

1. Fluoroquinolones	Disk content (µg)	Zone diameter Breakpoint (mm)	
		S >	R <
Ciprofloxacin	5	26	26
2. Macrolides			
Erythromycin, (C, jejuni)	15	20	20
Erythromycin, (C. coli)	15	24	24
3. Tetracycline	30	30	30

Key: S – sensitive R - resistant

Method: Muller-Hinton + 5 % defibrinated horse blood and 20 mg/L β-NAD (MH-F). The MHF plates should be dried prior to inoculation to reduce the swarming (at 20 – 25^{o}C overnight or at 35^{o}C, with the lid removed for 15 minutes)

Inoculum: McFarland 0.5

Incubation: Microaerobic environment 41-42^{o}C for 24 hours. Isolates with insufficient growth are reincubated immediately and inhibition zones read after a total of 40-48 hours of incubation.

Reading: Read zone edge as the point showing no growth viewed from the front of the plate with the lid removed and with reflected light.

Quality control: Campylobacter *jejuni* ATCC 33560

Source: EUCAST clinical Breakpoint Tables v.6.0, valid from 01/01/2016

Appendix II: Biosafety, Animal Use and Ethics Committee of the Faculty of Veterinary Medicine, The University of Nairobi Permit (Ref: FVM BAUEC/2019/145).

UNIVERSITY OF NAIROBI

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Dr. John Kariuki
c/o Dept of PHP&T
REF: FVM BAUEC/2018/145

29/01/2018

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Assessment of antimicrobial resistance profiles in Campylobacter and Salmonella species in commercial broiler production systems in Kenya By Kariuki John (J56/67683/2013)

We refer to the above proposal that you submitted to our committee for review. We note that your study does not involve use of experimental animals or birds. Furthermore, it is observed that samples of biologicals will be collected from the poultry production units and transported to laboratories for analysis. We are satisfied that the poultry will be handled humanely and appropriate biosafety precautions will be used in the study. We hereby approve your work as per the proposal.

Rodi O. ojoo BVM M.sc Ph.D
Chairman, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine

Appendix III: Preparation of 10 X TBE Buffer (Tris Borate Ethylene Diamine Tetra Acetate)

The substances required are: EDTA disodium salt, Tris base, boric acid and deionized water. The equipment required include: pH meter, Calibration standards, 600- and 1500-ml beakers, graduated cylinders, Stir bars and plates. Make a concentrated (10x) stock solution of TBE by weighing 54 grams of Tris base and 55 grams of boric acid (FW = 61.83) and dissolving both in approximately 900 milliliters of deionized water. Then add 40 milliliters of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 liter. This solution can be stored at room temperature, but a precipitate will form in older solutions. Store the buffer in glass bottles and discard if a precipitate has formed (Brody and Kern, 2004)

Appendix IV: Preparation of 2% agarose in TBE

Two grams of agarose electrophoresis grade was added to 90 mls of distilled water in a 250 ml flask and then 10mls of 10|X TBE added. This mixture was swirled to evenly distribute the agarose in the liquid. This was then heated to dissolve, then cooled to 60^{o}C.A volume of 7.5µl of ethidium bromide (10gm/ml) added and the mixture shaken to mix thoroughly and left to stand at room temperature. (ThermoFisher Scientific 2017)

Appendix V: Study Questionnaire

Questionnaire on farm broiler production practices by contracted broiler farmers

Date administered ……..

Farm……..

Owner ……

Location:

District……County……

Broiler rearing experience :……

Medications used for treatment in the last 21 days (apart from vaccines)

Drug 1……Duration of medication……...

Drug 2…….

Drug 3……..

Did the birds recover to good health and after how long?

Person responsible for medication

Farm Manager/Vet/Supervisor/Farmhand:……

Drug Additives to feed……Yes/No……If Yes what drug…….

Do you as a regular farmer know about drug withdrawal periods? Yes/No

How do you know how long to withdraw? ……

Do you observe drug withdrawal periods Yes/No?

Appendix VI: Years of broiler rearing experience and compliance to observation of drug withdrawal periods by the farm managers

Appendix VI: Years of broiler rearing experience and compliance to observation of drug withdawal periods by the farm managers

Farm observed	Years	Withdrawal period observed
Nairobi	9	Yes
Kajiado	7	Yes
Kiambu	23	Yes
Murang'a	4	Yes
Machakos	7	Yes
Nakuru	25	Yes

Appendix VII: Reagents supplier and country of origin

Chemical reagent	Supplier, country of origin
Oligonucleotides	InqabaBiotec East Africa Ltd., Kenya
Taq polymerase master mix with standard buffer	New England Biolabs, United Kingdom
Tris base (Hydroxymethyl aminomethan)	Riedel – de Haen, Germany
EDTA	Unilab , Kenya
Boric acid	Laborama, Kenya
CCDA	Oxoid , UK
Tetrathionate	HiMedia, India
Hippurate	Merck KGaA, Germany
Indoxyl acetate	Merck KGaA, Germany
Stuart media	Oxoid, UK
XLD	HiMedia , India
Skimmed Milk	Oxoid, UK
Hydrogen Peroxide	BDH, Germany
Oxidase strips	Oxoid, Uk

Appendix VIII: Instruments supplier, country of origin

Instrument	supplier, countryoforigin
PCR microliter tubes	Treft plastics, Netherlands
Veriti 96 well Thermocycler (Model 9902)	Applied Biosystems, Singapore
UV transillumiator digital camera (Gemax 125 imager)	Cambridge, United Kingdom