PREVALENCE AND ANTIMICROBIAL RESISTANCE OF ZOONOTIC

*CAMPYLOBACTER* ISOLATED FROM LIVESTOCK AND RODENTS IN URBAN

INFORMAL SETTLEMENTS IN NAIROBI.

CHEPKWONY MAURINE CHEROTICH

Thesis submitted in partial fulfilment of Masters of Veterinary Public Health at the

University of Nairobi

Department of Public Health, Pharmacology and Toxicology

2016
DECLARATION

This thesis is my original work and has not been submitted for a degree award in any other University.

[Signature]

CHEPKWONY MAURINE CHEROTICH (J56/67719/2013) Date

11th Nov 2016

This thesis has been submitted to the University of Nairobi for examination with our approval as supervisors:

Prof. Erastus K. Kang’ethe (BVM, MSc, PhD)
Department of Public Health, Pharmacology and Toxicology
Signature [Signature] Date 1/11/16

Prof. Eric M. Fevre (BSc, MSc, PhD)
International Livestock research Institute (ILRI).
Signature [Signature] Date 1/11/16

Dr. Gabriel O. Aboge (BVM, MSc, PhD)
Department of Public Health, Pharmacology and Toxicology
Signature [Signature] Date 1/11/2016
DEDICATION

To my loving Parents Mr. and Mrs. Chepkwony, my brother Lenny Tanui and my best friend Dr. Dennis Makau. I thank God for you all for being by my side, cheering me on through this journey and for your undying support and prayers.
ACKNOWLEDGEMENT

My gratitude goes to the extensive Urbanzoo project team, without any of whom the exercise would not have been as efficient and a success. First, I thank God for his grace and favour in making the process a success. I acknowledge the University of Nairobi, Department of Public health, pharmacology and toxicology for the opportunity to enrol for my MSc degree which this thesis is in partial fulfilment of.

My sincere gratitude goes to my supervisors Prof. Erastus K. Kang’ethe, Prof. Eric M. Fevre and Dr. Gabriel A. Oluga for their consistent, patient guidance and supervision throughout the project phases. I appreciate the time they created to help me solve the hitches I encountered in the course of the research project and thesis writing and also for linking me up with the different people I consulted during my project.

I would like to thank Prof. Kang’ethe and Prof. Fevre for the opportunity to join the ESEI-Urban Zoonoses Project which has given me immense experience and growth in the research field and interpersonal skills. The opportunity has fed my interest in research and given me a wealth of skills.

I am exceptionally grateful to the University of Nairobi ESEI team: Prof Kang’ethe for his continuous support and mentorship and whose help contributed greatly to the successful accomplishment of this project task; Mr. Nduhiu Gitahi who I consulted on many occasions and who was involved and gave guidance in every aspect of my project work especially my methodologies and logistics. Thank you for always being available and willing to help me in the big as well as the little aspects I needed assistance in; Beatrice Wandia, Lucy Nyoroka, Caroline Gateri and Johnstone Masinde whom I have worked well with and who gave their skills and time including weekends in making the laboratory aspect successful and also Mr. Macharia, Mr. Masinga and Mr. Rono for their involvement and assistance whenever they could.
I acknowledge the ESEI team from Kemri; Prof. Kariuki for allowing me access to his laboratory and reagents; Dr. Kiru for his expert consultation and access to some of the Primers used in this study; Mr. Ngetich for patiently teaching me agar dilution and Mr. Samuel Njoroge for his assistance in learning how to perform PCR and how to analyse sequence data. I am also grateful to Mr. Tom Ouко, Ms. Purity and Ms. Hannah Njeri for their welcome and ready assistance.

Gratitude also goes to the International Livestock Research Institute ILRI - ESEI field team led by Dr. Vicky Kyallo and Mr. James Akoko who coordinated the field work, access to sampling and laboratory reagents and equipment with great efficiency and who also offered his assistance in the field on several occasions. I thank Mr. Amany and M/s Alumasa for their sacrifice in making sure the field work started early and back to the laboratory in good time. Other members of the team too not mentioned here for the different roles they played in making the project a success.

I also acknowledge the African Population Health Research Centre (APHRC) for providing access to accurate demographic data of Korogocho and Viwandani which served as the sampling frame for the project, for their field coordinator; Ms. Sophia and for organizing a security team on the ground. Their presence made the work swift and a great success.

I thank Dr. Gemma Wattret of the University of Liverpool for her willingness to give me her expert opinion and guidance from her experience working with Campylobacter species and who facilitated my access to the primers used for species identification in this project. Your assistance is greatly appreciated.

I thank my two delightful colleagues: Dr. Cianjoka Gichuyia and Dr. James Macharia. We have encouraged and supported each other toward mutual success and it has been a superb experience. You are both much appreciated.
I cannot forget to thank the Urban Zoonoses Project for facilitating my project and without whom my project would not have been possible and its funding Agencies the UK Medical Research Council, Biotechnology and Biological Science Research Council (UK), the Economic and Social Research Council (UK), the Natural Environment Research Council (UK), through the Environmental & Social Ecology of Human Infectious Diseases Initiative (ESEI), Grant Reference: G1100783/1. This work also received support from the CGIAR Research Program on Agriculture for Nutrition and Health (A4NH), led by the International Food Policy Research Institute (IFPRI). We also acknowledge the CGIAR Fund Donors (http://www.cgiar.org/who-we-are/cgiar-fund/fund-donors-2).

I thank my family and friends whose patience and constant cheer has been my source of encouragement.

It has been a blessing to work with each and every person in the course of the project. I am very grateful.
TABLE OF CONTENTS

DECLARATION .............................................................................................................. 2
DEDICATION .................................................................................................................. iii
ACKNOWLEDGEMENT ................................................................................................. iv
TABLE OF CONTENTS ................................................................................................. vi
LIST OF TABLES ............................................................................................................ ix
LIST OF FIGURES ......................................................................................................... x
LIST OF APPENDICES ................................................................................................. xii
CHAPTER 1.0 INTRODUCTION .................................................................................... 1
  1.1 Statement of the problem ....................................................................................... 2
  1.2 Justification of the study ...................................................................................... 2
  1.3 Hypothesis 3 ......................................................................................................... 3
  1.4 Overall objective ................................................................................................ 3
    1.4.1 Specific objectives ......................................................................................... 3
CHAPTER 2.0 LITERATURE REVIEW ......................................................................... 4
  2.1 General Information ............................................................................................ 4
    2.1.1. Urban Livestock keeping .......................................................................... 4
    2.1.2. Importance of livestock in drug resistance and pathogen transmission .... 4
    2.1.3. Situation in Nairobi informal settlements .................................................. 4
  2.2 About Campylobacter ........................................................................................ 5
  2.3 Epidemiology of Campylobacter ......................................................................... 5
    2.3.1. African perspective .................................................................................... 5
    2.3.2 Kenyan perspective ...................................................................................... 6
  2.4 Transmission of Campylobacter species ............................................................. 7
  2.5 Zoonotic perspective/ public health importance .................................................. 7
  2.6 Role of livestock in transmission of campylobacter to humans ......................... 8
  2.7 Role of rodents in transmission of Campylobacter ............................................ 8
  2.8 Clinical syndrome ............................................................................................... 9
    2.8.1. Animals .................................................................................................... 9
    2.8.2. Humans .................................................................................................... 9
2.9 Sequelae of *Campylobacter* infections ................................................................. 9

2.10 Antimicrobial resistance ......................................................................................... 10
   2.10.1. Mechanisms of drug resistance .................................................................. 10
   2.10.2. Antimicrobial Resistance Patterns ......................................................... 14

2.11 Diagnosis options ................................................................................................. 14
   2.11.1. Culture-based detection methods ............................................................... 15
   2.11.2. Immunological based identification methods ............................................ 17
   2.11.3. Nucleic acid based detection methods ...................................................... 18

2.12 Resistance testing methods .................................................................................. 18

CHAPTER 3.0 METHODOLOGY .................................................................................. 21
3.1 Study area ................................................................................................................ 21
3.2 Study design ............................................................................................................ 23
   3.2.1 Sample and data collection ........................................................................ 23
3.3 Sampling .................................................................................................................. 24
   3.3.1. Random selection process ........................................................................ 24
   3.3.2 Sample collection: ...................................................................................... 25
3.4 Determination of the prevalence of zoonotic *Campylobacter* ...................... 26
   3.4.1. Isolation by culture method ...................................................................... 27
   3.4.2. Identification by gram staining (morphology) ........................................... 27
   3.4.3. Identification by Biochemical tests ............................................................. 27
   3.4.4. Confirmation of *Campylobacter* species by Polymerase Chain Reaction (PCR) .... 28
   3.4.4.2. Identification by PCR .......................................................................... 28
3.5 Identification of risk factors associated with the prevalence of zoonotic *campylobacter* .31
3.6 Determination of antibiotic resistance by Campylobacter .....................................31
   3.6.1. Phenotypic Antibiotic sensitivity testing .................................................. 31
   3.6.2 ....................................................................................................................... 32
3.8. Data handling and analysis .................................................................................... 36

CHAPTER 4.0 RESULTS .......................................................................................... 37
4.1 Household characteristics ....................................................................................... 37
4.2 summary of livestock and rodents sampled ......................................................... 40
4.3 Prevalence of *campylobacter* in livestock and rodents. ...........................................41

4.4 Risk factors for prevalence of *campylobacter* ..........................................................42

4.5 Drug resistance patterns ..............................................................................................43

4.6 antimicrobial resistance genes detected in zoonotic *Campylobacter* isolates ..........46

4.6.1. Resistance to aminoglycosides and tetracycline ......................................................46

4.6.2. Resistance to betalactams .........................................................................................46

4.6.3. Resistance of the isolates to quinolones and macrolides ..........................................48

CHAPTER 5.0 DISCUSSION ..................................................................................................50

CHAPTER 6.0 CONCLUSION AND RECOMMENDATIONS ...........................................58

CHAPTER 8.0 REFERENCES ...............................................................................................60
LIST OF TABLES

Table 1: Basic phenotypic characteristics of selected thermophilic Campylobacter ........... 16
Table 2: The sampling frame showing the total number of households in each category and their proportions in Korogocho and Viwandani. ......................................................... 25
Table 3: Primers used for species identification ................................................................. 30
Table 4: Primers used to identify of resistance genes present in the isolates ...................... 34
Table 5: A summary of household characteristics including gender roles in cleaning livestock premises and feeding the livestock; livestock species kept; sources of feed and water .......................................................................................................................... 37
Table 6: A summary of household characteristics showing the level of education of the respondents; training attained; level of awareness/ knowledge of the respondents ........................................................................................................ 39
Table 7: The proportion of people who carry out livestock treatment and who the sources are for the veterinary drugs used in the different farms .................................................... 40
Table 8: Zoonotic Campylobacter species isolated from the different livestock sampled. ..... 42
Table 9: Results of the model selected from the backward fitted multivariate analysis. ......... 43
Table 10: Summary of antimicrobial resistance results and the resistance patterns .......... 45
Table 11: A summary of the PCR results showing the percentage of positive isolates containing the resistance genes tested in the study ......................................................... 46
LIST OF FIGURES

Figure 1: A map of the study area showing the two sites within Nairobi. The legend shows the villages sampled within the study area. ................................................................. 22

Figure 3: Pie chart showing the distribution of livestock species sampled in the study in numbers. .............................................................................................................. 41

Figure 4: A Heat map showing the isolates with multi-resistant genes and Class 1 integrons. ......................................................................................................................... 47

Figure 5: DNA Gyrase B amino acid sequences showing deletion of the amino acid Q at position 407.............................................................................................................. 48

Figure 6: Nucleic acid sequences of the 23 SRNA genes showing the C2268T substitution in sample 507 similar to that of sequence ENAU09611 from genbank. ................. 49

Figure 7: Protein l4 aligned sequences showing the T362C substitution in sample 507 .... 49
LIST OF APPENDICES

APPENDIX 1: PROTOCOL FOR CAMPYLOBACTER ISOLATION ...........................................69

APPENDIX 2: GLOBAL SALM SURV ANTIMICROBIAL SUSCEPTIBILITY TESTING
           BY AGAR DILUTION PROTOCOL USED IN THIS STUDY............................81

APPENDIX 3: NARMS ANTIMICROBIAL SUSCEPTIBILITY BREAKPOINTS..103

APPENDIX 4: SEQUENCE BLAST RESULTS.................................................................104

APPENDIX 5: DETAILS OF PHENOTYPIC DRUG RESISTANCE TEST RESULTS....107
ABSTRACT

*Campylobacter* is a pathogenic zoonotic bacterium that causes mild to fatal illnesses in livestock while in humans, *Campylobacter* symptoms may range from transient watery diarrhoea to bloody diarrhoea and may lead to development of serious chronic effects. Campylobacteriosis is the highest aetiology of human enteric diseases in industrialized countries. However, there is limited knowledge on whether livestock in developing countries like Kenya are important reservoirs of zoonotic Campylobacter and also limited information concerning the drug resistance patterns and resistance genes present in the zoonotic species in Kenya.

Thus, the study sought to describe the epidemiology and antimicrobial resistance associated with zoonotic *Campylobacter* species in informal settlements in Nairobi. This study covered Korogocho and Viwandani informal settlements in Nairobi, Kenya, representing the densely populated urban settlements. Livestock samples (cloacal swabs from poultry, and faecal samples from rabbits, cattle, pigs and goats) were collected and the prevalence of Campylobacter identified by culture, biochemical characterization and Polymerase Chain Reaction. A questionnaire was administered to each household head or spouse to gather information on the associated risk factors. Agar dilution technique was employed to test for antimicrobial susceptibility in confirmed isolated *Campylobacter* and genes responsible for resistance to the tested drugs identified using primer specific PCR. Representative samples were then sequenced using the ABI 3130XL genetic analyzer to investigate presence of mutations that could result in quinolone and macrolide resistance. The summary statistics, chi square test as well as logistic regression were used in the analysis and the data was interpreted at 95% confidence interval.

Eight hundred two (802) livestock and 16 rodent samples were collected from (203) households.

The overall prevalence of *Campylobacter* from livestock and rodents was 21% with Prevalence in livestock being 170 (21.2%) and that of rodents being 12.5% (2/16). Two zoonotic *Campylobacter* species were identified and confirmed from the livestock samples: Campylobacter *jejuni* (16%)
and *Campylobacter coli* (0.6%). Bands specific for a third zoonotic species; *Campylobacter hyointestinalis*, were identified in five isolates (3% prevalence). Upon sequencing however, these were found not to be *C. hyointestinalis*. The two *Campylobacter* isolates from rodents did not belong to any of the identified zoonotic species.

Results from the questionnaire administered were used to identify associated factors and the presence of livestock faecal matter in the drinking water of the livestock was a risk factor for *Campylobacter* infections in livestock. On the other hand, livestock drinking water from road surfaces, awareness on antibiotic resistance and zoonoses were identified as protective factors that contributed to reduced *Campylobacter* infections in the livestock.

The prevalence of resistance to the tested drugs (gentamycin, erythromycin, tetracycline, chloramphenicol, ciprofloxacin and nalidixic acid) shows a worrying trend. Resistance genes for tetracycline (tet O gene), betalactams (TEM and OXA genes) and aminoglycosides (aac 6 lb cr gene). The risk posed by the potential transmission of these zoonotic isolates is that of possible transmission of the identified resistance genes to humans. The aac6 lb cr gene mediates resistance to aminoglycosides as well as quinolones in other bacteria, but has not yet been studied in *Campylobacter*.

Several missense mutations were seen and have been reported from the DNA gyrase genes and the 23S RNA gene that are responsible for resistance to quinolones and macrolides respectively. However, mutations linked with quinolone resistance were absent in the five isolates sequenced suggesting that the resistance may be mediated by other means. Nevertheless, a larger sample size needs to be studied to draw a more conclusive picture concerning mutations in these genes.

In conclusion *Campylobacter* has been shown to be an important pathogen in livestock in densely populated urban settling. The potential risk of transmission of these pathogenic strains and their drug resistance genes to humans and the environment is of great concern. This study has
demonstrated that awareness in the society on zoonoses and antimicrobial resistance is important in trying to reduce or control infections. The study recommends creation of public awareness and further studies to be undertaken to identify i) The other species of *Campylobacter* not identified and their importance; ii) The prevalence of *Campylobacter* in humans in densely populated informal settlements and iii) The significance of the role played by the aac6 lb cr gene in *Campylobacter* isolates and lastly this study recommends education on prudent use of antimicrobials by farmers.
CHAPTER 1.0 INTRODUCTION

1.1 Background information.

Campylobacters are bacteria of great public health importance as an emerging zoonosis. It is a gastro intestinal commensal in chicken (Newell and Fearnley, 2003) and in animals it is reported to cause abortions in ruminants (Milnes et al., 2009). In humans, however, Campylobacter causes majority of bacterial human gastroenteritis universally (Wilson et al., 2008). Acute infections with the bacterium have serious sequelae such as Miller Fisher syndrome, peripheral neuropathies and Guillain–Barré syndrome (World Health Organization, 2013). Recently in Kenya several deaths of children less than 5 years in hospital were confirmed to be associated with Campylobacter infection (O’Reilly et al., 2012).

Wilson et al, (2008) concluded that the chief route through which humans contract Campylobacter is the food chain. The pathogen contamination accumulates along the food chain from the farm level to edible food products (Lupindu et al., 2012). Studies by Wilson et al., (2008) in England, and Turkson et al., (1988) in Kenya identified livestock as key sources of human Campylobacter infections. Campylobacter jejuni is principally linked to poultry (Moran et al., 2011), however, it is found in sheep, cattle, goats, dogs and cats (OIE., 2012). C. coli is associated with porcine,( Jensen et al., 2006) but can be found in sheep, cattle and chicken too(World Health Organization, 2013). A study done in Scotland showed that various strains of Campylobacter were present among clinical, food and environmental isolates at a single point source of infection (Food Standards Agency, 2009).

Drug resistant cases have been documented in different countries. Studies on antimicrobial resistance in Campylobacter show high resistance to tetracyclines and fluoroquinolones with resistance being lower in C.jejuni than in C.coli (Food and Authority, 2014). This phenomenon can be attributed to treatment of chicken with these drugs (World Health Organization, 2013). In the USA, Campylobacter resistant to fluoroquinolone in humans was related to eating poultry
meat (World Health Organization, 2013). Resistance prevalence data are a great start to monitoring antimicrobial resistance risk (World Health Organization, 2013).

1.1 Statement of the problem.

_Campylobacter_ causes more human diarrhoeal cases compared to Salmonella, Shigella and E.coli 0157:H7 (Acheson and Allos, 2001). O’Reilly _et al._, (2012) showed _Campylobacter_ to be important in the ever increasing case fatality rates in children less than 5 years. This result was shared by (Shapiro _et al._, 2001) from Kenya and also by (Mitike _et al._, 2009) in Ethiopia. Most African countries, Kenya included do not know the prevalence of _Campylobacter_ in their countries well. Few studies have been done on Campylobacter in Kenya with Kabiru (2014) reporting _Campylobacter_ in humans, Osano and Arimi, (1999) reporting campylobacter in animal meat and Turkson _et al_ (1988) in live animals. Turkson _et al_ (1988) reported the highest isolation rate of _Campylobacters_ from livestock sampled at Nairobi slaughter houses as well as a few households. There have been changes in the ecosystem and environment since 1985 to date and therefore _Campylobacter_ prevalence in Livestock in Kenya today is not really known and the role of livestock in Kenya as reservoirs of the common zoonotic _Campylobacter_ species is also unknown.

1.2 Justification of the study

This study serves to inform on the importance of livestock farmed in urban Nairobi, Kenya as sources of zoonotic _Campylobacter_ and the risk of antimicrobial resistance that is carried by the pathogens. This information will benefit policy makers, and stakeholders in the public health sector in trying to control zoonotic transmission of _Campylobacter_. The results on the risk factors provide possible areas to guide the public health stakeholders when instituting control measures for reducing and preventing zoonotic transmission of the pathogen _Campylobacter_.

2
1.3 **Hypothesis**

Antimicrobial resistant zoonotic Campylobacter phenotypes are prevalent in livestock and rodents found in peri-urban Nairobi.

1.4 **Overall objective**

To describe the epidemiology and antimicrobial resistance associated with zoonotic *Campylobacter* species in Korogocho and Viwandani informal settlements in Nairobi.

1.4.1 **Specific objectives**

1. To determine the prevalence of zoonotic *Campylobacter* isolated from livestock and rodents in informal settlements of Nairobi.

2. To investigate factors associated with prevalence of zoonotic *Campylobacter* in livestock and rodents kept in informal settlements in Nairobi.

3. To determine phenotypic antimicrobial resistance patterns of zoonotic *Campylobacter* isolated from livestock and rodents in informal settlements.

4. To determine the presence of genes that code for antimicrobial resistance phenotypes in zoonotic *Campylobacter* species prevalent in livestock and rodents in informal settlements in Nairobi.
CHAPTER 2.0 LITERATURE REVIEW

2.1 General Information

2.1.1. Urban Livestock keeping

Urban livestock rearing is an essential source of proteins to the urban inhabitants (Smith and Olaloku 1998). Almost ninety percent of the households in 1985 kept livestock for subsistence and the rest as an asset. Since then, informal settlements have doubled in size as has, in all likelihood, the number of livestock has increased (Guendel, 2002).

Urban agriculture has been proposed as a solution to food insecurity in low income urban households in Kenya (Korir., 2015). According to Guendel (2002) the urban poor farm livestock as a coping strategy to food insecurity. Different studies show that vulnerable groups keep livestock for social security (Guendel, 2002).

2.1.2. Importance of livestock in drug resistance and pathogen transmission.

Despite the significance of urban livestock to the farmers, their role as a source of zoonotic diseases cannot be underestimated (Guendel, 2002). Consumption of animal source foods produced under poor quality control measures such as happens in poor urban communities is a concern (Guendel, 2002).

2.1.3. Situation in Nairobi informal settlements

Informal settlement settings lack sanitation; many of the households with livestock lack latrines (“New Agriculturist_ Making more of livestock part 2,” May 2006). Half of the livestock keepers in the informal settlement dispose animal waste into the drains, open sewers and dump sites (“New Agriculturist_ Making more of livestock part 2,” May 2006). Sheep, pigs, poultry, dogs and cats are often found roaming and scavenging for feed in the open spaces and dumpsites, where children often play. The informal settlement dwellers have very limited awareness of public health concerns associated with the close proximity to livestock and
specifically zoonotic diseases (Guendel, 2002). Many are new to livestock keeping and are dependent on more informed neighbours for animal husbandry and animal health advice.

2.2 About Campylobacter

*Campylobacter* belongs to the family *Campylobacteraceae* which comprises of curved or S-shaped Gram negative rods; occasionally round shapes appear in below ideal conditions. They grow as grey-white or creamy-grey and moist colonies growing under microaerophilic conditions on selective media. They are motile and oxidase positive that don’t oxidize or ferment carbohydrates. A challenge however, is lack of effective discriminating tests (Milnes *et al.*, 2009). The species most frequently linked with human diarrhoea are thermophilic (Public Health England, 2014).

2.3 Epidemiology of *Campylobacter*

The real figure of number of cases of *Campylobacter* infections is not understood (World Health Organization., 2013). Children and young adults are the ones more diagnosed with *Campylobacter* from developed countries whereas in developing countries, most affected are children less than two years (World Health Organization., 2013).

In the tropics *Campylobacter* is hyper endemic in young (Acheson and Allos, 2001), community-based research approximate occurrences of *Campylobacter* infection for under 5 years of age to range at 40 000 and 60 000 notifications/100 000 population (Coker *et al* 2002). *Campylobacter* still is an important bacterial cause of diarrhoea. (Alfredson and Korolik, 2007).

2.3.1. African perspective

Bacterial diarrhoea has been reported to cause deaths in rural Africa (Brooks *et al*., 2006), majority of the people live in rural areas with characterestic challenges in water quality and unsanitary human waste disposal among several other threats for frequent exposure to bacterial causes of diarrhoea. Generally, in developing countries, (African countries included,) where
surveillance platforms targeting *Campylobacteriosis* are nonexistence; there are no case reports from the population (Coker, *et al* 2002).

*Campylobacter* has however been identified to occur in both humans and animals in the Central African Republic (Georges-Courbot, *et al* 1987), Senegal (Cardinale *et al*., 2006), Malawi (Mason *et al*., 2013), South Africa (Jonker and Picard, 2010) and Ethiopia (Mitike., 2009). These studies stress the importance of *Campylobacter*.

### 2.3.2 Kenyan perspective

Studies have identified *Campylobacter* in humans (Kabiru, 2014), animal meat (Osano and Arimi, 1999) and live animals. (Turkson *et al* 1988) reported the highest isolation of *Campylobacters*: from pigs with diarrhoea (55.1%), chicken (51.5%), dogs with diarrhoea (47.2%), pigs (44.0%), ducks (29.4%), goats (6.3%), cattle (5.8%) and humans with diarrhoea (3.1%), and sheep (2.0%) respectively. Out of 317 isolates in Turkson’s study, 51.4% of them were *C. jejuni* and 40.1% *C. coli*. The results indicate that domestic animals could serve as reservoirs potentially being of epidemiological significance in human Campylobacter cases (Turkson *et al*., 1988).

In a study to identify *Campylobacter* species isolated from human in Nairobi, (Kabiru., 2014), 96% were *Campylobacter jejuni* and the rest *C.coli*. Among patients with diarrhoea in Western Kenya, *Shigella* was isolated most frequently, followed by *Campylobacter* species and *V. cholerae*. Among <5 years old children, *Campylobacter* was isolated from the majority (Shapiro *et al*., 2001). This was in agreement with a rural western Kenya study (Brooks *et al*., 2006) and another done in Ethiopia (Mitike *et al*., 2009), which reported *Campylobacter* isolation rates twice that of salmonella and shigella species from under 15 year old children.
2.4 Transmission of *Campylobacter* species

*Campylobacter* spp are common commensals in the gastrointestinal tract of poultry; thus, transmission of infections to humans occurs frequently (Acheson and Allos, 2001). *Campylobacter* infection is transmitted faecal-orally, and spread by either direct or indirect contact with contaminated fomites, food or water. *Campylobacter* species could stay viable in faeces for 9 days, in milk for 3 days, and in water for 2 to 5 days (The Center for food security and public health, 2013). Humans may be infected after consuming improperly cooked poultry and other meats, unboiled milk, uncooked clams, contaminated foodstuffs or un-chlorinated water, and contact with infected pets or livestock (The Center for food security and public health, 2013). Environmental water could lead to infection in humans by consumption of unclean potted water or recreational water (Sails, *et al* 2002).

2.5 Zoonotic perspective/ public health importance

Campylobacteriosis is a zoonosis. The causative bacteria is commonly found in food animals and in pets (World Health Organization., 2013). This makes contact with any infected member of these groups of animals a potential risk for transmitting the pathogen to humans.

*Campylobacter* gastroenteritis instigated by *Campylobacter jejuni* and *Campylobacter coli* is of major public health importance among all infections caused by Campylobacter (World Health Organization., 2013). Growing antimicrobial resistance observed in *Campylobacter*, in both medicine and agriculture is acknowledged by many global experts as an important emerging community health concern (Moore *et al*., 2006).

Poor hygiene, sanitation and interaction with animals in growing economies leads to frequent contracting of enteric pathogens leading to sporadic cases in these countries (World Health Organization., 2013). Persons working with farm animals, laboratory technicians/ personnel and those handling human excreta have increased risk of contracting *Campylobacter* enteritis.
(Jocelyn., 2006), as well as homosexual men are at risk (Loue, 2007). The risk of transmission is however reduced in lesbians (William, 1981) the reasons for this are however not given in the study.

2.6 Role of livestock in transmission of campylobacter to humans

More recent studies using Multi Locus Sequence Testing(MLST) technique compared wild and farmed animal Campylobacter genotypes with human isolates and attributed animals as a source of human Campylobacteriosis (Wimalarathna et al., 2013). Sheppard et al., (2009) linked chicken isolates to human disease causing isolates. The infection is mainly transmitted from animals through eating undercooked meat from diseased livestock, raw milk from infected lactating animals (The Center for food security and public health, 2013), contact with infected animals (World Health Organization., 2013) and handling of contaminated manure with bare hands and failure to clean hands properly afterwards (Lupindu et al., 2012).

2.7 Role of rodents in Campylobacter transmission

Rodents are known to harbor and transmit several disease causing (such as Leptospira spp., Campylobacter spp., Salmonella spp., Trichinella spp.,and Toxoplasma spp (Meerburg and Kijlstra, 2007).

Rodents acquire the infections from infected animal faeces, other wild animals such as birds or from other rodents they come in contact with (Meerburg and Kijlstra, 2007). The close living nature of rodents facilitates circulation of resident infections in a rodent population without showing any disease symptoms (Meerburg and Kijlstra, 2007). Infected rodents can therefore, transmit pathogens in the farm environment to animals meant for consumption ( Newell and Fearnley, 2003).

Several studies have been done to elucidate ow rodents transmit Campylobacter and in one study, the risk of broiler houses having Campylobacter was heightened by presence of rats on farm (Newell and Fearnley, 2003).
Wild rodents are not problematic since they rarely interact with food animals but in farm environment, rodents can be vectors of bacteria and increase environmental bacterial load thus rodent populations residing on a farm pose a hazard for re-occurring infections (Meerburg and Kijlstra, 2007).

2.8 Clinical syndrome

2.8.1. Animals
Infected animals may not have symptoms. *Campylobacter jejuni/coli* colonization in livestock is usually without symptoms but they can lead to abortions in sheep and cattle (Milnes et al., 2009). Other nonspecific clinical signs include enteritis, hepatitis, diarrhoea and sometimes death in young ostriches (OIE, 2012).

2.8.2. Humans
In humans *Campylobacter* affects the intestinal tract resulting in diarrhoeas with blood and mucus, abdominal pain, nausea and vomiting, fever and general malaise that present for about 2-5 days and may occasionally relapse in adults. However, majority of the population becomes asymptomatic (WHO, 2013).

2.9 Sequelae of *Campylobacter* infections
In some people, reactive arthritis which is a painful inflammation of the joints, nervous illnesses like meningitis and Guillian-Barre syndrome or rarely seizures as a result of high fever may occur. Death is the extreme sequelae which although rare, happens immune challenged individuals (World Health Organization, 2013). Universally, *Campylobacter* has been linked to roughly a third of Guillian-Barre syndrome (Poropatich, et al, 2010) and reactive arthritis happening in between 1-5% of *Campylobacter* infections (World Health Organization, 2013).
Deaths have been reported to range between <0.01% (Werber et al., 2012) and 8.8% (O’Reilly et al., 2012) in different populations and also varying with method used and period of records with some of the deaths being caused by sequelae (World Health Organization., 2013).

### 2.10 Antimicrobial resistance

Resistance to antimicrobials is of heightened public health worry globally (Nachamkin et al., 2002). Antimicrobials used in agriculture (veterinary included) have been linked with occurrence and distribution of *Campylobacter* resistance hence being a threat to food safety (Humphrey et al., 2005).

Macrolides together with fluoroquinolones are usually used in *Campylobacter* treatment (Coker, et al 2002). *C. jejuni* that is not susceptible to Fluoroquinolones was identified in Europe in the late 1980s, with scientists claiming that this resistance was obtained from animals (Engberg et al, 2001).

Systemic infections with *Campylobacter* are usually treated using aminoglycosides (Engberg et al., 2001 and Wimalarathna et al., 2013). The resistance in Kenya may be due to the common practice of purchasing drugs on the open counter and private pharmacies without doctors’ prescriptions (Kabiru, 2014).

#### 2.10.1. Mechanisms of drug resistance.

##### 2.10.1.1. Resistance to Quinolones.

The acting mechanism of quinolones is through hindrance of bacterial DNA synthesis causing cell death. Quinolones exert their action by targeting the enzymes topoisomerase IV and DNA gyrase found in bacteria which are involved in DNA duplication, transcription, repair and recombination (Jacoby, 2005). The products of the enzymes are large structures which have two sub units each i.e. ParC and ParE, GyrA and GyrB respectively (Wieczorek and Osek, 2013a).
Resistance mainly occurs through replacement of amino acids in a segment known as the quinolone resistance-determining region (QRDR) within DNA attachment area on the enzymes. In *Campylobacter*, resistance to fluoroquinolones has been reported to be mainly a result of gyrA gene mutations (Engberg *et al*., 2001). A Thr86Ile point mutation in the gyrA gene is reported to be responsible for high resistance to ciprofloxacin. This mutation is similar to Ser83Leu mutation in *Escherichia coli* (Ge, McDermott, White, and Meng, 2005). There exist other mutations affecting the gyrA gene of *C. jejuni* that are attributed to increased resistance to nalidixic acid and the inverse for ciprofloxacin (Beckmann *et al*., 2004). More than one point mutation can also occur (Ge and McDermott, 2005).

Since *C. jejuni* and *C. coli* lack an alternative area that can be responsible for quinolone resistance, a unique alteration of the Gyr A is thus enough to result in resistance to fluoroquinolones (Engberg *et al*., 2001). The cmeABC efflux system responsible for multiple antimicrobial resistance also works in tandem with the gyrA mutations resulting in resistance (Lin, *et al*, 2002).

### 2.10.1.2. Resistance to Tetracycline.

Tetracyclines act by attaching to ribosomes and hampering elongation of protein production (Gibreel *et al*., 2004). They use their attachment to Mg+2 cations to go through outer membrane porins (Chopra and Roberts, 2001).

Ribosomal protection proteins such as the *tetO* and the *tetM* genes, facilitate tetracycline resistance (Connell *et al*., 2003). The *tetO* is liable for tetracycline resistance in *Campylobacter* (Connell, *et al*, 2003). *Tet M* is the only other gene that has been identified in *Campylobacter* isolates (Abdi-Hachesoo *et al*., 2014).

The *tetO* gene, is plasmid mediated and is associated with very elevated tetracycline resistance levels (Gibreel *et al*., 2004). It has however been reported to be found on the chromosome in
some isolates (Gibreel et al., 2004). It is likely that other mobile extra chromosomal genetic elements may be involved in the attainment and distribution of \textit{tetO}. (Wieczorek and Osek, 2013a). Examples of these could be integrons and transposons. Studies show the likelihood of \textit{Campylobacter tetO} having been obtained from \textit{Streptomyces}, \textit{Streptococcus}, or \textit{Enterococcus species} through horizontal genetic transmission (Batchelor, \textit{et al} 2004).

\textbf{2.10.1.3. Resistance to Macrolides}

Macrolides act by targetin the 50S subunit and interrupting production of proteins (Wieczorek and Osek, 2013a). Studies show the 23S rRNA nucleotides 2058 and 2059 to be of key importance in the attachment of macrolides. Changes in the attachment area of macrolides on the ribosome are what mediate their resistance (Batchelor \textit{et al}., 2004). Replacement of nucleotides at positions 2074 and 2075 of the adenine residues in the 23S rRNA gene in \textit{Campylobacter} frequently occur in erythromycin resistance (Luangtongkum \textit{et al}., 2009). The A2074C, A2074G, and A2075G mutations result in increased macrolide resistance in \textit{C. jejuni} and \textit{C. coli}, with erythromycin resistance corresponding with resistance to all other macrolides, lincosamides and streptogramin antimicrobials (Avrain, \textit{et al} 2004).

Other mechanisms include:

- L4 and L22 protein modification could result in low resistance levels. However, the precise of these alterations is still not clear (Cagliero, \textit{et al}, 2005).

- There are approximately eight efflux systems recognized with the CmeABC multiple drug efflux pump which combined with target mutations works to facilitate resistance (Cagliero \textit{et al}., 2005). This is an energy dependent efflux pump which is chromosomally encoded by three genes cmeA, cmeB and cmeC. These three genes are a “periplasmic protein, an inner membrane drug transporter, an outer membrane protein respectively” (Lin \textit{et al}, 2003). These work together to remove antimicrobials among other substances from a \textit{Campylobacter} cell (Lin \textit{et al}., 2003).
2.10.1.4. Resistance to Aminoglycosides.

Aminoglycosides act through the 30S ribosomal subunit, preventing precise codon-anticodon identification and in disturbance of protein longation by impeding the movement of Trna from the A-site to the P-site (Jana and Deb, 2006).

Enzyme changes which weaken the aminoglycoside attachment to the rRNA are what effect their resistance (Llano-Sotelo, et al. 2002). These aminoglycoside deactivating enzymes are classified as: “aminoglycoside adenyltransferases, acetyltransferases and phosphotransferases”, all with their own specific alteration areas and products (Wieczorek and Osek, 2013). They act by compromising attachment of aminoglycosides to their targets through shifting a substrate functional group to the antimicrobial (Toth et al., 2010). The acetyltransferases use acetyl-coA to acetylate the amino groups of these antibiotics, the adenylyltransferases, modify hydroxyl groups of aminoglycosides by transferring the nucleoside moiety and the phosphotransferases modify the antibiotics by phosphorylation of their hydroxyl groups (Toth et al., 2010).

A gene: apha-3 responsible for kanamycin-resistance among others, are recognized as part of a resistance cluster in C. jejuni plasmid (Gibreel et al., 2004) This gene is suggested to have been transferred to Campylobacter from gram positive bacteria. The apha-3 gene is also present on plasmids mediating tetracycline resistance in Campylobacter genus (Gibreel et al., 2004).

2.10.1.5. Resistance to Other Antimicrobial Agents.

Betalactam resistance in Campylobacter poorly defined (Stones, 2010). Most C. jejuni and C. coli isolates can produce betalactamases, rendering the betalactam particle inactive (Stones, 2010). Efflux pumps are also involved in this resistance too (Lin et al., 2002).

Chloramphenicol acts by inhibition of protein elongation in bacteria (Wieczorek and Osek, 2013a). resistance to chloramphenicol is via an acetyltransferase encoding gene that is plasmid
mediated (Wieczorek and Osek, 2013a) this has been shown in *C. coli* although this resistance is rarely seen phenotypically (Wieczorek and Osek, 2013a).

Sulphonamide resistance in *C. jejuni* is a chromosome mutation with substitutions of various amino acids in the dihydropteroate synthetase (DHPS. Competition for DHPS between sulphonamides and PABA (4-aminobenzoic acid) prevents the latter from assimilation into folic acid (Engberg *et al.*, 2001).

Another mechanism by which *Campylobacter* has been reported to develop resistance to multiple drugs is the CmeABC multidrug efflux pump (Pumbwe, *et al*, 2004). The three fragments of the pump i.e. membrane fusion proteins inner drug transporter and outer membrane protein act to enable the transportation of substrates from outside the cell into the cell matrix (Krishnamoorthy, *et al* 2008).

Of the three genes, the cmeB is reported to be the best target in detecting the efflux system by polymerase chain reaction (PCR) (Olah, *et al*, 2006)

### 2.10.2. Antimicrobial Resistance Patterns

Since the late 1980’s incidences of antimicrobial resistance has increased in cases of human *Campylobacteriosis* (Wieczorek and Osek, 2013a). Resistance is mostly attributed to lack of prudent use of antimicrobial agents, however, the gap in this argument exists in the numerous self-limiting cases of human and animal diseases that are not treated using any antimicrobials (Wimalarathna *et al.*, 2013).

### 2.11 Diagnosis options

In animals infections are associated with the various clinical signs but due to the largely asymptomatic manifestation in adult animals, laboratory analysis is indicated for confirmation. Samples collected include faeces, rectal swabs and caeca contents. There are various detection
methods described by different scientists for the isolation of *Campylobacter* organisms that can be grouped into three according to Isaacson (2003). These are:

### 2.11.1. Culture-based detection methods

Culturing *Campylobacter* species from faeces can be cumbersome and needs special media to culture and specific conditions to grow. Usually selective media is required to obtain *Campylobacter* from field samples (Martin, *et al*., 2002). Selective media include: Skirrow, Karmali, Preston, modified Charcoal Cefoperazone Deoxycholate agar among others (Martin *et al*., 2002). However, these media have different growth rates of thermophillic *Campylobacter* depending on the source (Martin *et al*., 2002). Both solid and liquid media have different combinations of antibiotics to which thermophillic *Campylobacter* are resistant. These antibiotic combinations function to retard growth of other bacteria present in feces thus enhancing recovery of the fastidious *Campylobacter* species (Isaacson ., 2003). These antimicrobials may include polymixin, rifampicin, trimethoprim, vancomycin, cefoperazone, cephalothin, colistin and cycloheximide (Isaacson ., 2003).

For animal fecal samples, fecal swabs, direct plating has been shown to be better than enrichment before plating (Madden *et al*., 2000). The same study showed the use of modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) to be better than preston in *Campylobacter* isolation from animal fecal material. Therefore the best technique in this case would be to do direct plating of the fecal samples onto mCCDA. Usually colonies are visible after 48 hours but may take longer for some slow growing strains (Isaacson ., 2003). Morphology may be typical grey with irregular edges and spreading thinly in moist plates or show atypically; round, convex, shiny morphology if plates are not moist (Isaacson ., 2003). Further identification is then done using, culture morphology, gram stain morphology, and biochemical tests; table 1 below.
Table 1: Basic phenotypic characteristics of selected thermophilic *Campylobacter*

<table>
<thead>
<tr>
<th></th>
<th><em>c. jejuni</em></th>
<th><em>C. coli</em></th>
<th><em>C. lari</em></th>
<th><em>C. hyointestinalis</em></th>
<th><em>C. fetus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram stain morphology</strong></td>
<td>Gram negative curved rods</td>
<td>Gram negative curved rods</td>
<td>Gram negative curved rods</td>
<td>Gram negative curved rods</td>
<td>Gram negative curved rods</td>
</tr>
<tr>
<td>Test for Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Test for Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hippurate hydrolysis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indoxyl acetate hydrolysis</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

There have been reports of hippurate negative *Campylobacter* jejuni isolates that require additional tests for correct identification (Persson and Olsen, 2005).

**Principles behind the biochemical tests**

Gram negative bacteria are decolourised by acetone alcohol and take the stain of the counter-stain (Carbol fuchsin).

The catalase-enzyme splits hydrogen peroxide to water and oxygen $\text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \Rightarrow \text{O}_2 + 2\text{H}_2\text{O}$. The peroxidase can only do this in presence of an organic substrate to donate a hydrogen atom to the reaction.

In oxidase test cytochrome C oxidises phenylene-diamine-derivatives leading to production of a bluish indophenol. Commercial kits are available.
### 2.11.2. Immunological based detection methods

There are several assays existing that rely on antigen antibody reaction to detect *Campylobacter* species.

Agglutination assays; these involve latex particles coated with anti-*Campylobacter* antibodies which react with antigenic outer membrane proteins on *Campylobacter* causing agglutination and can then be seen visually (Isaacson ., 2003). Agglutination assays have little sensitivity and specificity and are thus not intended for use on field samples but rather for confirmation of isolated colonies (Isaacson ., 2003).

Enzyme-linked immunosorbent assays are another group of immunological techniques that measure immunoglobulins G, M and A classes of antibodies to Campylobacter. Enzyme immunoassays are more sensitive than agglutination tests but less sensitive compared to culture techniques (Hindiye *et al.*, 2000).

Colony blotting: a colony lift immunoassay was developed that could enable faster identification and quantification of thermophilic *Campylobacter* from background microbes (Jensen, *et al.*, 2005). This test involves hybridization of nucleic acid from lysed colonies and is highly sensitive, however, it is associated with a high risk of cross reaction with background microbes (Rice *et al.*, 1996).

Immunomagnetic separation methods have also been employed in detection of *Campylobacter* resulting in increased sensitivity of subsequent cultures. Immunomagnetic separation technique uses antibody coated magnetic beads which bind antigens present on the surface thus *capturing* the cells. A magnet is used to facilitate the concentration of the cells as it attracts the beads toward itself (Isaacson ., 2003).

The last of the immunology based methods is the antibody based detection. These include ELISA and are very ideal for estimating *Campylobacter* prevalence in flock and herd levels.
(Isaacson., 2003). These are time and cost saving compared to traditional culture and isolation techniques.

**2.11.3. Nucleic acid based detection methods**

DNA based techniques are used to detect and identify *Campylobacter* genus and species. Generally these methods exist as either hybridization techniques or PCR assays (Isaacson., 2003).

DNA probes are usually used for hybridization assays and majority of the probes are developed from the *Campylobacter* 16S rRNA gene (Isaacson, 2003). Other gene probes also exist. The use of DNA probes has been tested on both food samples and pure culture isolates (Isaacson, 2003).

**2.11.3.1 PCR**

Polymerase Chain Reaction is an assay that uses the in vivo functioning of DNA replication (denaturation of double stranded DNA to single strand, and then copied) and this is done many times leading to exponential replication of the DNA strand. Polymerase chain reaction techniques can identify *Campylobacter* species both directly from stool samples (Inglis and Kalischuk, 2004), food samples (Schnider et al, 2010) and environmental samples (Rothrock et al, 2009) as well as from pure cultures and complex samples (Mily et al, 2011). PCR offers a more precise detection of *Campylobacter* species (Inglis and Kalischuk, 2004). Various PCR based methods have been designed to differentiate *C. jejuni* from *C. coli* using different primers (Jensen et al., 2005).

**2.12 Resistance testing methods**

Drug resistance can be tested by use of either phenotypic methods or molecular techniques. Phenotypic methods include; use of diffusion techniques (disk diffusion and E-test) and Minimum Inhibitory Concentration (MIC) techniques which include agar dilution, broth macro
and microdilution. These show whether the bacterial isolate is expressing the resistance. Molecular techniques on the other hand show the presence of resistance genes in the isolates. Identifying the presence of these resistance genes is important because they are responsible for transmission of resistance to other isolates e.g. human isolates to animal isolates. There are international standards that describe the methods in details.

“MIC is defined as the lowest concentration of antimicrobial agent required to inhibit growth of the bacteria” (WHO, 2003). The MIC (Minimal Inhibitory Concentration) of bacteria to a specific antimicrobial agent is the best measure of antimicrobial susceptibility as it tells you about the degree of resistance (WHO, 2003).

Here agar plates, tubes or microtitre plates with antimicrobial dilutions that the bacteria is inoculated into are used and the least concentration of antimicrobial at which visible growth of the bacteria can be seen is recorded as the MIC.

In agar dilution, a range of the antimicrobial agent concentration are serially diluted then mixed in an agar medium onto which bacteria are inoculated (OIE, 2012). Susceptibility testing using Agar dilution is considered to be the gold standard for all other antimicrobial susceptibility testing methods (WHO, 2003).

The agar dilution has several advantages over other techniques:

i) Testing of a number of bacteria at the same time on an agar plate with exeption of swarming colonies.

ii) It offers the freedom to extend the antimicrobial concentration range for testing thus in a way increases the identification of MIC end points.

iii) Semi-automated inoculation exists making work easier.
However its disadvantages include: being very laborious if not automated, short storage time for prepared plates (a week at most), and difficulty in reading the endpoints and verifying the purity of the inoculum (OIE, 2012).

Agar dilution is often recommended for fastidious organisms such as *Campylobacter* (CLSI, 2012).
CHAPTER 3.0 METHODOLOGY

3.1 Study area

The study was carried out in Korogocho and Viwandani locations in Kasarani and Makadara sub counties respectively (Fig.1). The two informal settlements; Korogocho and Viwandani are located about 5–10 km from the city center and occupy an area of 0.45 and 0.52 km$^2$ respectively. These areas were chosen because this research project forms part of a wider research (Epidemiology, Ecology and Socio-Economics of Disease Emergence in Nairobi-Urban Zoonosis research project) which aims to identify diversity of bacteria among livestock and humans with the aim of better understanding the mechanisms of pathogen emergence and mobility in densely populated areas and these two sites fulfilled this criterion.

Korogocho is located 12 km from the city centre; in Kasarani Sub County and has seven villages: Korogocho "A", Gitathuru "C", Highridge, Grogan "B" Nyayo/Kisumu Ndogo, Grogan "A", and Korogocho "B". Viwandani is 7 km from Nairobi city centre, and is located in Makadara Sub County. Viwandani has 5 villages: Donholm, Paradise, LungaLunga, Jamaica and Kingston. According to the 2009 population census, there was a total of 14,705 livestock across both informal settlements; 6,720 in Viwandani (45.7%) and 7,985 in Korogocho (54.3%).
Figure 1: A map of the study area showing the two sites within Nairobi. The legend shows the villages sampled within the study area.
3.2 Study design

This was a cross sectional study that involved the collection of faecal samples from livestock and rodents in the study areas as well as gathering questionnaire data on risk factors that predispose to the contamination and spread of pathogenic *Campylobacter* and development of antimicrobial resistance.

Information necessary to carry out this study was triangulated using two methods: Data from key informants (such as livestock production officers and village elders) and data from the APHRC Demographic Surveillance Survey 2013.

Ethical approval

Approval to conduct this study was given by the Institutional Research Ethics Committee at ILRI (International Livestock Research Institute) and the African Medical Research Foundation (AMREF) accredited International Ethical Review Committee (IERC). In addition, household heads signed consent form to allow sampling of their animals and to prove they willingly consented to giving the information collected in the questionnaires.

3.2.1 Sample and data collection

To attain the maximum sample size, a prevalence of 50\% was used. A design Effect of 2 was then employed to correct for any clustering that may have occurred within the households. The formula by Dohoo *et al.*, (2003) was used to come up with the working sample size.

\[
n = \frac{(Z\alpha/2)^2 \times p \times (1-p)}{L^2}
\]

Where 

\( n \) = sample size,

\( Z = Z \) value for a level of confidence,

\( p \) = expected prevalence or proportion (in proportion of one; 50\%, \( p = 0.5 \)), and
\[ L = \text{precision (in proportion of one; if 5\%, } L = 0.05) \].

\[ n = \frac{(1.96^2 \times 0.5(0.5))}{0.05^2} \]

\[ n = 384 \]

The calculated sample size without design effect was 384 animals. With study effect to correct for clustering within households, the sample size doubled to 768 animals.

3.3 Sampling
3.3.1. Random selection process

Proportional random sampling technique was used. Livestock keeping households were the primary sampling unit used. A list of households was obtained from the African Population and Health Research Center (APHRC) and used as the sampling frame. The households were classified into categories based on the type of livestock kept. These categories were further classified into number of livestock per species kept in the households. The households to be sampled in each category were then randomly selected using the computer generated random numbers in manner to get the required number of animals in each category. Number of livestock to be sampled from each household was proportional to the number of livestock kept as shown in the table 2 below.
Table 2: The sampling frame showing the total number of households in each category and their proportions in Korogocho and Viwandani.

<table>
<thead>
<tr>
<th>CATEGORIES</th>
<th>H.H VIWA</th>
<th>H.H KOCH</th>
<th>TOTAL H.H</th>
<th>NUMBER SAMPLED FROM CATEGORY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3 cattle</td>
<td>26</td>
<td>23</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>4-6 cattle</td>
<td>4</td>
<td>7</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>7-10 cattle</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>1-3 goats</td>
<td>28</td>
<td>8</td>
<td>36</td>
<td>1</td>
</tr>
<tr>
<td>4-10 goats</td>
<td>29</td>
<td>17</td>
<td>46</td>
<td>3</td>
</tr>
<tr>
<td>11-20 goats</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>21-39 goats</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>1-5 chickens</td>
<td>118</td>
<td>25</td>
<td>143</td>
<td>2</td>
</tr>
<tr>
<td>6-10 chickens</td>
<td>62</td>
<td>32</td>
<td>94</td>
<td>3</td>
</tr>
<tr>
<td>11-50 chickens</td>
<td>14</td>
<td>21</td>
<td>35</td>
<td>10</td>
</tr>
<tr>
<td>51-200 chickens</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>1-10 Other</td>
<td>20</td>
<td>8</td>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>11-20 Other</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>20-30 Other</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>1-5 pigs</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>6-15 pigs</td>
<td>13</td>
<td>60</td>
<td>73</td>
<td>5</td>
</tr>
<tr>
<td>15-35 pigs</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Total livestock keepers</td>
<td><strong>335</strong></td>
<td><strong>213</strong></td>
<td><strong>548</strong></td>
<td></td>
</tr>
</tbody>
</table>

3.3.2. Sample collection:

Faecal samples were collected from the livestock which included cattle, goats, pigs and sheep after manual restraint was used to hold the animals still. Faeces were collected manually directly from the rectum while wearing a clean well lubricated glove. The sample were then
placed in a faecal pot then assigned a unique barcode and stored in a cool box for transportation to the laboratory.

For poultry (chickens, ducks, quails etc.) and rabbits, cloacal swabs were obtained as samples and stored in Cary-Blair transport medium tube, labelled with a barcode and placed in a cool box for transportation to the laboratory.

Rodent traps were set in the selected households keeping livestock in Viwandani and Korogocho informal settlements falling within the homesteads under the Nairobi urban health demographic surveillance system (NUHDSS) which is operated by the Africa population health and research centre (APHRC). Between 2 to 5 traps were placed within the households to avoid interference by other animal or theft. A type of fish; *Rastrineobola argentea* also known as the silver cyprinid and locally known as “omena” was used to attract rats into the traps. The traps were checked every 24 hours and every 12 hours where possible. Trapped rodents were transported directly to PHPT laboratory (UoN) and post mortem examination on the same day of collection. Intestinal scrapping and faecal samples were taken for laboratory culture.

The questionnaire data collected included information on level of education, source of feed and water for the animals, animal treatment (who treats and what treatment is given to the different animals), location of animal house (whether they sleep in the house with the humans, just outside the house or outside the human’s compound) accessibility of feed and water by rodents; among other information.

### 3.4 Determination of the prevalence of zoonotic *Campylobacter*

To identify zoonotic Campylobacter, isolation was done by culture, gram staining and biochemical tests. Species confirmation then done by Multiplex PCR according to the WHO Global Salm- Surv protocol (2003).
3.4.1. Isolation by culture method

The cloacal swabs from poultry species and rectal swabs from rabbits transported in Preston broth were spread directly onto mCCDA plates while fecal samples from cattle, goats and pigs were emulsified in Preston broth, a sterile swab was then used to spread the emulsified sample onto a modified Charcoal Cefoperazone Deoxycholate agar plate. The plates were put in anaerobic jars loaded with Campygen® sachets to create a microaerophilic atmosphere required for optimal growth of Campylobacter. The jars were then incubated at 42ºc for 48 hours then examined for the growth characteristics.

3.4.2. Identification by gram staining (morphology)

One drop of saline was mixed with single colonies obtained from mCCDA plates and then used to make a smear on a glass slide. The smears were air dried; heat fixed using a Bunsen flame and then left to cool at room temperature. Thereafter, the smears stained with gram staining method using the following procedure: Crystal violet 60 seconds, Gram’s iodine 60 seconds, ethanol decolorizer, Carbol fuchsine 60 seconds. Before every step, the smear was rinsed under a stream of water running gently and excess water removed by tipping off. After the last staining and rinsing, the smear was air dried. The slides were then visualized under an oil emersion lens magnification x100 using a light microscope. Campylobacter is seen as gram negative curved or gull shaped rods.

3.4.3. Identification by Biochemical tests

To identify Campylobacter species by biochemical methods, catalase and oxidase tests were performed according to the protocol described by Global Salm-Surv (2003). For catalase test, a drop of hydrogen peroxide (H₂O₂) was mixed with a single colony of Campylobacter on a slide. Catalase positive result was confirmed on observation of formation of gas bubbles. Oxidase test was done by transferring a single colony onto an oxidase disc and a positive result was confirmed by the appearance of a blue color within 10 seconds.
3.4.4. Confirmation of *Campylobacter* species by Polymerase Chain Reaction (PCR)

Singleplex and multiplex PCR were used to confirm *Campylobacter* species using specific primers targeting 16S RNA, lpxA, and GlyA genes outlined in Table 5. *Campylobacter* species included: *C. jejuni*, *C. coli*, *C. fetus*, *C. lari* and *C. hyointestinalis*. DNA extraction and PCR were performed as outlined in the following sections 3.4.4.1 and 3.4.4.2, below.

3.4.4.1. Extraction of Campylobacter DNA

DNA was extracted from Campylobacter colonies initially stored in sterile skimmed milk at -40°C. The stored *Campylobacter* isolates were revived on mCCDA plates. Thereafter, the DNA was extracted by boiling for 5 minutes at 95°C in a heating block and then centrifuged at 12,000 revolutions per minute for 5 minutes. The supernatant containing the DNA was recovered and stored pending further analysis by PCR.

3.4.4.2. Identification by PCR

To confirm members of the genus *Campylobacter*, primers (C412F and C1228R) targeting the 16SRNA were used (Table 5). For species identification, the lpxA gene, Gly A gene and 16S RNA were used to identify the *C. jejuni*, *C. coli*, *C. lari*, *C. fetus* and *C. hyointestinalis*.

A PCR mixture containing a total volume of 25 μl was prepared. This mixture consisted of 1PCR bead, 19.8 μl of sterile distilled water, 0.1 μl each of forward and reverse primers and 5 μl of DNA template. The DNA was amplified using a thermocycler (make: Veriti, manufacturer: Applied Biosystems) by an initial denaturation at 95°C for 10 min, followed by another denaturation at 95°C for 30 sec, annealing at 59°C for 90 sec, and an extension at 72°C for 60 sec. The processes of second denaturation, annealing and extension were repeated for 35 cycles and a final extension done at 72°C for 10 min. DNAse free water was used as negative controls.

The thermocycling conditions for specific identification of *C. jejuni*, *C. coli*, *C. fetus*, *C. lari* and *C. hyointestinalis* as follows: initial denaturation at 94°C for 5 min, followed by another denaturation at 94°C for 60 sec, annealing at 50°C for 60 sec, and an extension at 72°C for 60 sec.
sec. The processes of second denaturation, annealing and extension were repeated for 30 cycles and a final extension done at 72°C for 10 min.

Ten microliters of the PCR amplicons were separated by gel electrophoresis in 2% agarose gel prepared by warming 2g of agarose in 100ml of Tris Acetate Edta (TAE Ph 8.0) buffer. Seven microlitres of Ethidium bromide was added to the TAE buffer for staining the DNA. The gel electrophoresis was run for 45 minutes at 200V. A molecular ladder of 100bp (Qiagen GelPilot® DNA molecular weight markers) was run together with the PCR amplicons. The amplified DNAs were then visualized under UV light using a gel documentation apparatus (gel max system interfaced with the UVP computer software.)
Table 3: Primers used for species identification

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Accession number</th>
<th>Product length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter spp</em></td>
<td>16s RNA</td>
<td>C412F, C1228R</td>
<td>GGATGACACTTTTTCGGAGC CATTGTAGCAGCTGTGTC</td>
<td>CP007769</td>
<td>816</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>LpxA gene</td>
<td>CjejlpxAF, CjejlpxAR</td>
<td>ACAACTTGTTGACGATGTGTA CAATCATGCDATATGASAAT AHGCCAT</td>
<td>PMC53526</td>
<td>331</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>lpxA gene</td>
<td>CcollpxAF, CjejlpxAR</td>
<td>AGA CAA ATA AGA GAG AAT CAG CAATCATGCDATATGASAAT AHGCCAT</td>
<td>PMC53526</td>
<td>391</td>
</tr>
<tr>
<td><em>C. lari</em></td>
<td>GlyA gene</td>
<td>CLF, CLR</td>
<td>TAGAGAGATAGCCAAAAGAGA TACACATAATAATCCCACCC</td>
<td>AF136495</td>
<td>251</td>
</tr>
<tr>
<td><em>C. fetus</em></td>
<td>16sRNA</td>
<td>CFCH57F, CF1054R</td>
<td>GCAAGTCGAACGGGTATTA GCAGCACCTGTCTCAACT</td>
<td>CP008810</td>
<td>978</td>
</tr>
<tr>
<td><em>C. hyointestinalis</em></td>
<td>16sRNA</td>
<td>CFCH57F, CH1344R</td>
<td>GCAAGTCGAACGGGTATTA GCAGCTCCGGCTTCATGTGTC</td>
<td></td>
<td>1267</td>
</tr>
</tbody>
</table>
3.5 Identification of risk factors associated with the prevalence of zoonotic Campylobacter

To identify the risk factors, questionnaires were administered to the household owner or person taking care of the livestock. The risk factors investigated during these interviews were on value chain used, farm hygiene and disease control and personal perceptions towards several themes such as food safety, antimicrobial use, functioning of the market and other value chain aspects.

3.6 Determination of antibiotic resistance by Campylobacter

Antibiotic resistance was identified in the zoonotic isolates by phenotypic and genotypic methods. Due to the lack of set breakpoints for MIC testing of Campylobacter for most antimicrobials, six drugs were tested phenotypically: gentamicin, erythromycin, tetracycline, chloramphenicol, ciprofloxacin and nalidixic acid. While for the genotypic drug resistance testing, genes for resistance to tetracyclines, aminoglycosides, quinolones, macrolides and betalactams were investigated.

3.6.1. Phenotypic Antibiotic sensitivity testing

Campylobacter colonies were used to make a 0.5 McFarland solution that was used for the agar dilution as per the WHO protocol (“Global Salm-Surv,” 2003, a) in appendix 2.

Preparation of antimicrobial solutions was done according to the international guidelines given by the NCCLS (CLSI, 2012).

The MIC was read at the minimal concentration of each antimicrobial without visible bacterial growth. These values were compared to the antimicrobial breakpoint set for Campylobacter by (Narms, 2011)as seen in appendix 3.
3.6.2. Detection of antibiotic resistance genes.

Presence of resistance genes was investigated using conventional PCR method using specific primers targeting the tet O gene for tetracycline, Aac lb-cr (aminoglycoside N (6')-acetyltransferase ) gene for aminoglycoside resistance, TEM and OXA genes for betalactam resistance, gyr A and gyrB for quinolone resistance and 23srna and l4 protein(rpld) genes for macrolide resistance as listed in Table 7. The plasmid carried aac (6) lb cr gene was investigated because it has not been identified in Campylobacter before and has been reported to mediate both aminoglycoside and quinolone resistance in other enteric bacteria. Presence of Integrons was also investigated. Other mobile genetic elements were not investigated.

A PCR mixture containing a total volume of 25 μl was prepared. This mixture consisted of 2.5 μl PCR buffer, 0.5 μl dNTPs, 0.125 μl Taq polymerase, 5μl DNA template and different volumes of forward and reverse primers; 1μl for TEM and OXA, 3 μl for aac (6) lb cr, 4μl for integrons and 5 μl for tetracyclines. Sterile RNase free water was then used to top up to 25 μl required volume per reaction.

Ten microliters of the PCR amplicons were electrophoresed in 2% agarose gel. Seven microlitres of Ethidium bromide was added to the TAE buffer for staining the DNA. The gel electrophoresis was run for 45 minutes at 200V. A molecular ladder of 100bp (Qiagen GelPilot® DNA molecular weight markers) was run together with the PCR amplicons. The amplified DNAs were then visualized under UV light using a gel documentation apparatus (gel max system- manufactured in USA) interfaced with the UVP computer software.

Amplicons for the gyrA, gyrB genes, 23s RNA and L4 protein from five representative samples were further sequenced to investigate present mutations related to Quinolone and Macrolide resistance respectively. Purification of the PCR product, sequence reaction, sequence
purification and analysis were done using the ABI 3130XL genetic analyzer. This was done according to the protocol by Applied Biosystems for the BIG DYE v3.1 sequencing kit.
Table 4: Primers used to identify of resistance genes present in the isolates

<table>
<thead>
<tr>
<th>Genetic element and antimicrobial agent.</th>
<th>Target gene</th>
<th>primer name</th>
<th>Primer sequence</th>
<th>Product length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTEGRON</td>
<td>Class 1 integrons</td>
<td>INT-1U</td>
<td>GTTCGGTCAAGGTTCCTG</td>
<td>923</td>
<td>Guney et al 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>INT-1D</td>
<td>GCCAACCTTTCACGACATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TETRACYCLINE</td>
<td>Tet O gene</td>
<td>Forward</td>
<td>AACTTAGCGATTCTGGCTCAG</td>
<td>515</td>
<td>Bahman et al (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCCCACGGTTCTCATCATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMINOGYCOSIDES</td>
<td>Aac(6) lcr</td>
<td>Forward</td>
<td>TTGCGATGCTCTATGAGTGGCTA</td>
<td>482</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTCGAAATGGCGTCTTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cjgyrA2</td>
<td>TCAGTATAACGCATCGCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTGATCCATCAACATCCGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CCCACCTATTCCTGCACATTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L4</td>
<td>Forward</td>
<td>AAGTTTAAGAGCAAATACAGCTCAT</td>
<td>270</td>
<td>“</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>ACTAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATAGCCAAGAATCAGGATGAAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1. Sample collection
2. Culture on mCCDA
3. Oxidase, catalase and gram staining tests
4. Storage in skimmed milk
5. Revive on mCCDA
6. Oxidase and catalase test
7. DNA extraction
8. PCR to identify Campy genus
9. Species identification

- C. jejuni and C. coli
- C. lari
- C. hyointestinalis
- C. fetus

Confirmed zoonotic isolates tested for presence of resistance genes

Phenotypic drug resistance testing by agar dilution

Figure 2: A flow chart of the laboratory processes described above from sample collection to species identification and drug resistance testing
3.8. Data handling and analysis

A database was created in Microsoft Excel 2010 where data from both laboratory and questionnaire data were entered in separate spreadsheets before merging them. Data was then imported to Rstudio software for further statistical analysis which included descriptive summary statistics carried out for the laboratory and questionnaire data to calculate prevalence and general household characteristics and secondly logistic regression analysis performed to identify risk factors associated with pathogenic *Campylobacter*. Univariate analysis of the individual exposure variables was carried out so as to obtain probabilities of individual variables and only those associations that were considered significant at a p value ≤0.1 were carried forward to the multiple regression model. Multivariate analysis: a backward fitting the logistic regression model was done using the variables identified from the univariate analysis. Factors in the multivariate logistic regression model that were giving a p≤0.05 were retained in the model. The odds ratios for each of the significant risk factors were obtained from the model.

The sequence data in ABI files was opened and edited using Bio edit software. Blast analysis was done using the National Center for Biotechnology Information (NCBI) blast and sequence alignment was done using Bio edit and seaview4 softwares. Clustal omega online software was also employed in the analysis in aligning the sequences. Reference sequences were found from the NCBI nucleotide database and the European Bioinformatics Institute (EBI) database.
4.0 RESULTS

4.1 Household characteristics

This information was collected by administering questionnaires to household owners in 203 livestock keeping households from which the samples were also collected.

Table 5: Household characteristics including gender roles in cleaning livestock premises and feeding the livestock; livestock species kept; sources of feed and water.

<table>
<thead>
<tr>
<th>ATTRIBUTE</th>
<th>SPECIFICS</th>
<th>NUMBER OF HOUSEHOLDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>HOUSEHOLDS</td>
<td></td>
<td>203</td>
</tr>
<tr>
<td>GENDER OF PERSON CLEANING HOUSING</td>
<td>Male</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Not cleaned</td>
<td>6</td>
</tr>
<tr>
<td>GENDER OF PERSON FEEDING LIVESTOCK</td>
<td>Male</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Not fed by either gender</td>
<td>3</td>
</tr>
<tr>
<td>LIVESTOCK</td>
<td>Poultry</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>Rabbits</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Dairy cows</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Goats</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Pigs</td>
<td>20</td>
</tr>
<tr>
<td>FEED SOURCE</td>
<td>Scavenging</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>HH leftovers</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>Purchase feed</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>Bring in forage</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Swill</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Graze by road side</td>
<td>10</td>
</tr>
<tr>
<td>LIVESTOCK WATER SOURCE</td>
<td>Tap</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>Road</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Sewer</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>HH waste water</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>River</td>
<td>4</td>
</tr>
</tbody>
</table>
There was no significant difference between the role of men and women in cleaning the livestock housing in the study area. However, a larger proportion women (47%; (66/141)) cleaned the livestock houses in Korogocho compared to Viwandani where cleaning of the livestock housing was done mostly by men (53%;(33/62) b).

Similarly, more women than men were involved in the livestock feeding in Korogocho (45%) compared to Viwandani where men were more involved (50%). The difference was however not statistically significant. The feed and water sources for the livestock were obtained from a variety of sources.

The bulk of the respondents in these households (mainly farm owners and animal care givers) had attained primary education as their highest level of education 107 (53%) respondents), 59 (29%) of them had attained secondary school education, only 3 (2%) had studied to certificate level and 4 (2%) respondents to diploma level while 28 (14%) of them did not have any education background. In a comparison between the two localities, the highest level of education of the respondents from Viwandani was secondary school education while the proportion of respondents with up to certificate and diploma education came from Korogocho informal settlement (Table 6).

Three percent of the respondents (6/203) of them had received some form of training on animal disease management and 10 (5%) had received some training on livestock management in general. Five (2%) of these respondents had been trained in both livestock management and livestock disease management, none of the respondents from any household had received any training on food safety (Table 6).

A good number (49.8%) of the respondents in the study area reported to have heard about zoonoses; antimicrobial resistance; antimicrobial residues and withdrawal periods; aflatoxins and presence of pathogens in livestock manure. The proportion of respondents aware about these issues was
considered to have some level of awareness on the issues and is presented in the awareness section of Table 6 below.

Table 6: A summary of household characteristics showing the level of education of the respondents; training attained; level of awareness/ knowledge of the respondents.

<table>
<thead>
<tr>
<th>ATTRIBUTE</th>
<th>SPECIFICS</th>
<th>NUMBER OF HOUSEHOLDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>HOUSEHOLDS</td>
<td></td>
<td>203</td>
</tr>
<tr>
<td>EDUCATION</td>
<td>None</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Primary school</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Secondary school</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Certificate</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Diploma</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>1</td>
</tr>
<tr>
<td>TRAINING</td>
<td>Animal disease</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>management</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Livestock management</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Food safety</td>
<td>0</td>
</tr>
<tr>
<td>AWARENESS</td>
<td>Antimicrobial resistance</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Antimicrobial residues</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Drug withdrawal periods</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Zoonosis</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Aflatoxins</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Pathogens in manure</td>
<td>58</td>
</tr>
</tbody>
</table>

Majority of the respondents were aware of more than one of these issues hence this number adds up to >203 (445).

Treatment was administered mostly by the owners themselves (67.5%) followed by others who weren’t veterinary professionals but knew how to administer medication and would therefore help the owners in doing so (19.7%). Veterinary personnel ranked third in 4.4% of the livestock keeping households. Some of the livestock owners (3.9%) did not know who administered the treatment since they were not directly involved with caring for the animals and in this cases the persons who were directly involved could not be reached at the time (Table 7).
Most respondents (47.8%) obtained veterinary medications from agro vet retail shops with those sourcing the drugs from qualified veterinary professionals being 3.9% of respondents (Table 7).

Table 7: The proportion of people who carry out livestock treatment and who the sources are for the veterinary drugs used in the different farms.

<table>
<thead>
<tr>
<th>Person treating livestock in the different households</th>
<th>PROPORTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Owner</td>
<td>67.5%</td>
</tr>
<tr>
<td>Owner and vet</td>
<td>2.5%</td>
</tr>
<tr>
<td>Agrovet</td>
<td>2.0%</td>
</tr>
<tr>
<td>Veterinarian</td>
<td>4.4%</td>
</tr>
<tr>
<td>Other</td>
<td>19.7%</td>
</tr>
<tr>
<td>Don’t know</td>
<td>3.9%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medicine sources</th>
<th>PROPORTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Owner</td>
<td>18.2%</td>
</tr>
<tr>
<td>Owner and vet</td>
<td>3.9%</td>
</tr>
<tr>
<td>Agro-Vet</td>
<td>47.8%</td>
</tr>
<tr>
<td>Veterinarian</td>
<td>3.9%</td>
</tr>
<tr>
<td>Other</td>
<td>21.2%</td>
</tr>
<tr>
<td>Don’t know</td>
<td>4.9%</td>
</tr>
</tbody>
</table>

4.2 summary of livestock and rodents sampled

The total number of households sampled in the study was 203 from which 802 livestock were sampled from in both informal settlements. A representation of the proportions of sampled livestock and rodents is shown by the pie chart in Figure 3 below.
Figure 2: Pie chart showing the distribution of livestock species sampled in the study in numbers.

The most common type of livestock kept in the study area was poultry. However among the poultry species, ducks are the second most common after the chicken and doves come third and geese fourth. Poultry are followed by goats in terms of numbers in the study area.

4.3 Prevalence of campylobacter in livestock and rodents.

Sixteen rats were trapped from the households and from these only two were identified as carrying Campylobacter species. However, none of the Campylobacter isolated from rats was identified as belonging to any of the five zoonotic species investigated in this study.

One hundred and seventy (21.2%) of the livestock isolates were confirmed to belong to the genus Campylobacter. Of these isolates, 16% were Campylobacter jejuni, only 1 sample was identified as Campylobacter coli (0.6%) and the remaining 81% were Campylobacter species which did not belong to the five zoonotic species identified (C. coli, C.fetus, C.lari, C.hyointestinalis and C.jejuni). Five isolates (3%) were identified as Campylobacter hyointestinalis, however, upon sequencing they were found not to be C.hyointestinalis, thus were not considered among the zoonotic species identified in this study. Sequence results of the three other isolates, isolates 30 (Campylobacter coli), isolates 884 and 889 (Campylobacter jejuni) confirmed that they were indeed Campylobacter coli (with 98% identity to sequences in Genbank) and Campylobacter jejuni (with 100% and 99% identities to sequences in genbank) respectively based on blast analysis results as shown in appendix….

The prevalence of Campylobacter in Korogocho was 23.8% while that in Viwandani was 21.1%. This difference in prevalence between the two informal settlements was not statistically significant (p value=1). The isolated C. coli came from Korogocho informal settlement while none was isolated from Viwandani. Of the C. jejuni isolates, 76.9% of the isolates came from Korogocho while only 23.1% were isolated from Viwandani.
Table 8: Zoonotic *Campylobacter* species isolated from the different livestock sampled.

<table>
<thead>
<tr>
<th></th>
<th><em>C. coli</em></th>
<th><em>C. jejuni</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy cow</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dairy goat</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other goat</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Other livestock</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pigs</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Chicken</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>Rabbits</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sheep</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>1</strong></td>
<td><strong>26</strong></td>
</tr>
</tbody>
</table>

24 (89%) of *Campylobacter jejuni* were identified in chicken, 7% in goats and 4% in pigs and the single *Campylobacter coli* isolate was isolated from a chicken.

4.4 Risk factors for prevalence of *campylobacter*.

The univariate analysis of the individual exposure variables identified 32 factors that were considered significant at a p value ≤0.1. These included presence of fecal contamination in the livestock drinking water, river as a source of livestock drinking water, surface road water as a source of livestock drinking water, tap water as a source of livestock drinking water, sewer water as a source of livestock drinking water, Concrete bedding, wood shavings as livestock bedding, a wareness on antibiotic resistance, awareness on zoonoses, forage bought as animal feed, house hold overs as animal feed, agro vet feed as animal feed, scavenging as animal feed source; just to mention a few. These identified factors were all carried forward to the multiple regression models. The results of the model of best fit from the backward fitted model included four factors that contribute to the risk of *Campylobacter* infection in livestock from Korogocho and Viwandani informal settlement areas at the 95% confidence interval.
Presence of animal faeces in the livestock drinking water, surface road water as a source of drinking water, awareness on antibiotic resistance and awareness on zoonoses were the associated factors (Table 9).

### Table 9: Results of the model selected from the backward fitted multivariate analysis.

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>Std. Error</th>
<th>z value</th>
<th>P value</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faeces in water</td>
<td>1.1425</td>
<td>0.3487</td>
<td>3.276</td>
<td>0.00105</td>
<td>3.790</td>
</tr>
<tr>
<td>Water source from Road</td>
<td>0.7234</td>
<td>0.3258</td>
<td>2.220</td>
<td>0.02640</td>
<td>0.039</td>
</tr>
<tr>
<td>Awareness on antibiotic resistance</td>
<td>0.7029</td>
<td>0.2905</td>
<td>2.420</td>
<td>0.01554</td>
<td>0.029</td>
</tr>
<tr>
<td>Awareness on zoonoses</td>
<td>0.5316</td>
<td>0.2391</td>
<td>2.224</td>
<td>0.02618</td>
<td>0.002</td>
</tr>
</tbody>
</table>

#### 4.5 Drug resistance patterns
Six antimicrobials were tested by agar dilution method: erythromycin, gentamicin, chloramphenicol, tetracycline, ciprofloxacin and nalidixic acid.

Only one isolate identified as *Campylobacter jejuni* from a pig sample was susceptible to all the six tested antibiotics although it showed intermediate susceptibility to the quinolones (Nalidixic acid and Ciprofloxacin).

All the other isolates were resistant to more than one drug and also all isolates were resistance to the macrolide Erythromycin.

For *Campylobacter jejuni* isolates, 9 were resistant to all 6 antimicrobials tested, 10 isolates were resistant to a combination of 5 antimicrobials (Table 9), 2 isolates were resistant to 4 antimicrobials, and 1 isolate was resistant to a combination of 3 antimicrobials and 2 isolates to 2 antimicrobials.

All isolates (except the one mentioned above from a pig sample) were resistant to Erythromycin which is a Macrolide while 19 *C.jejuni* isolates were resistant to both tested quinolones (Nalidixic
acid and Ciprofloxacin) and 2 other isolates were resistant to Nalidixic acid but not to Ciprofloxacin. These two antimicrobial groups are the first line of *Campylobacter* treatment in human beings and *C. jejuni* is the leading zoonotic *Campylobacter* species.

The single *Campylobacter coli* isolated in this study from a chicken sample was resistant to 3 antimicrobials (Table 10) and was completely susceptible to Gentamicin, Chloramphenicol and Ciprofloxacin.

The bulk of the resistant *Campylobacter* isolates belonged to other *Campylobacter* groups. Fifty three point three percent 53.3% (72/135) of these isolates showed resistance to all six antimicrobials tested. Thirty three point three percent [33.3% (45/135)], of this group were resistant to different combinations of 5 antimicrobial drugs 3% (4/135) were resistant to a combination of four antimicrobials, 5.2% (7/135) showed resistance patterns to different combinations of 3 of the antimicrobial drugs and 5.2% (7/135) of other *Campylobacter* group were resistant to both tetracycline and Erythromycin drugs only.

The isolates showed highest susceptibility to Chloramphenicol with 38.6% susceptibility (33% susceptible and 67% intermediate susceptibility). Second in terms of susceptibility was Gentamicin with 20.5% susceptibility (91% susceptible and 9% intermediate susceptibility). Gentamicin was followed by Ciprofloxacin with 13.9% of the isolates being susceptible (61% susceptible and 39% intermediate susceptibility).
Table 10: summary of antimicrobial resistance results and the resistance patterns

<table>
<thead>
<tr>
<th>Livestock</th>
<th>Zoonotic Campylobacter</th>
<th>ANTIBIOTICS TESTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gentamicin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>CHICKEN</td>
<td>C.jejuni *</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>C.coli **</td>
<td>0</td>
</tr>
<tr>
<td>GOATS</td>
<td>C.jejuni ***</td>
<td>1</td>
</tr>
<tr>
<td>PIGS</td>
<td>C.jejuni ****</td>
<td>0</td>
</tr>
</tbody>
</table>

Footnotes:

*9 C.jejuni isolates from chicken were resistant to all six drugs; 9 isolates were resistant to five antibiotics in the following patterns: 7 isolates were resistant to the combination of gentamicin, nalidixic acid, ciprofloxacin, tetracycline and erythromycin, while 2 isolates were resistant to the combination of Chloramphenicol, nalidixic acid, ciprofloxacin, tetracycline and erythromycin; 2 isolates were resistant to four antibiotics: Chloramphenicol, nalidixic acid, Tetracycline and erythromycin; a single isolate resistant to three: Chloramphenicol, tetracycline and erythromycin; and finally 2 isolates were resistant to only 2 drugs: Tetracycline and erythromycin.

** The single C.coli isolate from chicken was resistant to 3 antimicrobials: nalidixic acid, tetracycline and erythromycin.

*** One isolate from goats was zoonotic and was resistant to 5 antimicrobials: Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin.

**** The single Campylobacter jejuni isolate from a pig sample was susceptible to all antimicrobials.
4.6 antimicrobial resistance genes detected in zoonotic *Campylobacter* isolates.

4.6.1. Resistance to aminoglycosides and tetracycline

Tet O gene was found to be present in 50% of the tested samples while the aminoglycoside N 6 acetyltransferase (aac (6) lb cr) gene was present in 53% of the isolates tested for its presence by PCR (Table 11).

4.6.2. Resistance to betalactams

Genes coding for resistance to betalactams via the TEM and OXA enzymes were also identified in 14% and 57% of the zoonotic Campylobacter isolates tested for their presence respectively.

Table 11: A summary of the PCR results showing the percentage of positive isolates containing the resistance genes tested in the study.

<table>
<thead>
<tr>
<th>ANTIBIOTIC</th>
<th>GENE</th>
<th>Number tested (n)*</th>
<th>NUMBER POSITIVE (PROPORTION)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracyclines</td>
<td>Tet O</td>
<td>32</td>
<td>16 (50% )</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Aac (6) lb cr</td>
<td>30</td>
<td>16 (53.33%)</td>
</tr>
<tr>
<td>Betalactams</td>
<td>TEM</td>
<td>14</td>
<td>2 (14.28%)</td>
</tr>
<tr>
<td></td>
<td>OXA</td>
<td>14</td>
<td>8 (57.14%)</td>
</tr>
<tr>
<td>Quinolones</td>
<td>DNA gyrase A</td>
<td>7</td>
<td>7 (100%)</td>
</tr>
<tr>
<td></td>
<td>DNA gyrase B</td>
<td>7</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Macrolides</td>
<td>23SRNA</td>
<td>7</td>
<td>7 (100%)</td>
</tr>
<tr>
<td></td>
<td>L4 protein (rpld)</td>
<td>7</td>
<td>7 (100%)</td>
</tr>
</tbody>
</table>

*n varies in each of the genes amplified via PCR due volume of primers available.*

Twenty two isolates were tested for presence of class 1 integrons; extra-chromosomal genetic elements, that may be involved in horizontal genetic transfer of drug resistance, and was found to be present in 68.18 % of the tested isolates.

The results of the resistance genes and Class 1 integrons are shown in the graphical presentation in Figure 4.
Figure 3: A Heat map showing the isolates with multi-resistant genes and Class 1 integrons.

KEY:

- The numbers on the x axis are sample numbers and the labels on the y axis are the genes tested.
- Red colour represents absence of the resistance gene or integron while yellow colour represents samples which the resistance gene of interest was present and white colour represents samples which were not tested for the OXA and TEM genes. This is true with the exception of the TEM results where brown represents the tested samples that did not contain the TEM gene while the two white (s77 and s361) are the two samples which were positive for the TEM gene among those tested.

Example: sample 12(s12) carries integrin genes but does not have any of the tested resistance genes while sample 878(s878) has all the tested resistance genes including presence of integrons (except for the TEM gene for betalactam resistance which was not tested in this isolate.)
4.6.3. Resistance of the isolates to quinolones and macrolides

Five samples were sequenced and analyzed to check for presence of mutations responsible for quinolone and macrolide resistance.

**QUINOLONES**

No mutations were seen in the gyrase A gene of all the five sequenced samples. However, in the gyrase B gene amino acid glutamine (Q) at position 407 was deleted in sample 30 (Campylobacter coli; susceptible to ciprofloxacin and resistant to nalidixic acid). This deletion of Q407 was as a result of deletion of nucleotide A 1220. Other mutations seen in gyrase B were missense mutations: A1182G in sample 30, G1209A in samples 30, 507 and 883, C1308T in sample 30, 507 and 883, T1360C in sample 77 and T1407C in sample 77.

![Figure 4: DNA Gyrase B amino acid sequences showing deletion of the amino acid Q at position 407.](image)

**MACROLIDES**

The region amplified in the sequences done in this study did not cover the area from which mutations have been reported from in other studies. However, from the area covered by the
sequences, there was substitution of C2268 T in the 23SRNA gene of sample number 507. There were no mutations affecting amino acid expression in the 23S RNA gene in all five samples.

Figure 5: nucleic acid sequences of the 23 SRNA genes showing the C2268T substitution in sample 507 similar to that of sequence ENAU09611 from genbank.

The l4 (rplD) sequences showed a substitution of nucleotide T to C 362 in sample 507. No mutations in the amino acids expressed in the protein sequences.

Figure 6: protein l4 aligned sequences showing the T362C substitution in sample 507.
CHAPTER 5.0 DISCUSSION

The role played by the different genders in livestock keeping in the two areas differs in terms of proportions of men and women who take care of the livestock in the two areas. There are more women involved in livestock caring than men in Korogocho and the reverse is true for Viwandani. Despite these apparent differences the role of men and that of women in the study area (the two informal settlements) is not significantly different.

Chicken are the most common livestock species kept by these livestock owners as shown in table 8 above. This can be corroborated by the national demographic census data of livestock keeping in 2009 (“Census Vol II Q 11: Livestock population by type and district - 2009 | Open Data Portal,” n.d.) which shows chicken are the majority livestock species in Nairobi. Taking this into consideration, chicken can be described as the most important livestock species. The results show a much higher isolation rate of Campylobacter from chicken than from the other livestock species as shown in figure 3 above. This emphasises the importance of this pathogen in the urban setting and especially the potential risk posed by these zoonotic Campylobacter species. This result is similar to that of (Turkson et al., 1988) that found a higher isolation rate of the pathogen from chicken than from other livestock species from households and slaughter houses in Nairobi.

The prevalence of Campylobacter in the Nairobi informal settlements is 21.2%. Despite this apparent difference in number of isolates from the two sides of the study area, the difference was not significant owing to the difference in sample sizes/ livestock numbers from the two areas. Prevalence of the isolated Campylobacter species was highest for Campylobacter jejuni. This finding is echoed by other studies that isolated more C.jeuni than C.coli isolates (Newell and Fearnley, 2003; Padungton and Kaneene, 2003; WHO, 2013). Campylobacter jejuni is reported to be the most common culprit of human Campylobacteriosis (Wilson et al., 2008) and also the most common zoonotic Campylobacter species (Acheson and Allos, 2001).
There is a scarcity of information on the prevalence of *Campylobacter* from livestock faeces with which to compare the results of this study, however, one such study was done in 1988 which showed a 32% isolation rate of thermophilic *Campylobacter* from livestock, dog and diarrhoeic human faecal matter (Turkson *et al.*, 1988). In the 1988 study the prevalence of *Campylobacter jejuni* and *Campylobacter coli* was substantially higher than the rates found in this study; however, the 1988 study sampled livestock from abattoirs and these may have come from areas outside Nairobi.

There have been other studies on *Campylobacter* in Kenya. A recent study on *Campylobacter* in humans reported an isolation rate of 6.5% (Kabiru, 2014) and 77% from raw meat (Osano and Arimi, 1999). The results of this study and that of Osano and Arimi (1999) show the importance of livestock and livestock products as a potential source of zoonotic transmission of *Campylobacter* pathogens.

The full picture of the impact of livestock being the potential source of human infection by *Campylobacter* is not known in Kenya due to a lack of studies on the association of *Campylobacter* isolated from human and livestock in Kenya.

The most important species of *Campylobacter* in chicken is *Campylobacter jejuni* (Newell and Fearnley, 2003). *Campylobacter jejuni* is also considered the most important species affecting humans followed by *Campylobacter coli* (World Health Organization, 2013). The results of this study concur with this statement, as *C. jejuni* was the most isolated zoonotic species with 16% from all livestock and 89% of these from poultry. Only one *C. coli* (0.6%) isolate was identified, making it not one common zoonotic species from livestock based on this study’s results. Three percent (3%) of the isolated *Campylobacter*, from chicken and a goat, were seen to be *Campylobacter hyointestinalis* on PCR. Despite the sequencing results showing otherwise, this suggests that there could be other zoonotic species of this bacterium in the region that could be playing a role in disease causation and transmission between livestock and humans. There is a deficit of published studies
done to identify other zoonotic *Campylobacter* species (other than *C. jejuni* and *C. coli*) in Kenya. Such studies have been done in other countries identifying other zoonotic species in both livestock and humans, *Campylobacter hyointestinalis* from Finland (Laatu *et al*., 2005), *Campylobacter fetus* in livestock (Kienesberger *et al*., 2011) and humans (Wagenaar *et al*., 2014), *Campylobacter lari* also in livestock and human (Debruyne *et al*., 2009). Zoonotic transmission of *Campylobacter hyointestinalis* has been reported (Laatu *et al*., 2005). A case of transmission of *Campylobacter hyointestinalis* from pig to humans has also been reported in a woman experiencing persistent diarrhoea, abdominal pain, and intermittent vomiting for more than 1 month (Gorkiewicz *et al*., 2002). This case illustrates the importance of studying other zoonotic *Campylobacter* species.

The isolation pattern of the *Campylobacter* species shows that majority of identified *Campylobacter* species was *Campylobacter jejuni*. This greatly agrees with other similar studies done to identify zoonotic thermophillic *Campylobacter* from livestock ((WHO), 2003). However, 81% of the isolated thermophilic *Campylobacter* could not be identified as belonging to any of the five tested species (*C. jejuni*, *C. coli*, *C. hyointestinalis*, *C. fetus* and *C. lari*). This leaves a gap in identification of these species especially since they form the bulk of all *Campylobacter* isolates from the urban informal settlement areas. They could belong either to zoonotic or non-zoonotic species. Studies should be done to identify which species of *Campylobacter* these isolates belong to and what could be their impact on either livestock production or human health in the region.

The risk factors for *Campylobacter* infection that have been identified are independently associated with increased risk of infection. Presence of animal faces in the livestock drinking water was the leading factor for *Campylobacter* infection. The results show that an animal drinking from such a contaminated source is 3.8 odds of get infected by *Campylobacter* compared to an animal drinking from a water source that is not contaminated by animal faecal matter (odds ratio 3.790). This can be explained by the fact that slight faecal contamination of the drinking water by an infected animal’s excrement could lead to infection given that the infective dose of *Campylobacter* bacteria
is less than 500 organisms / cells (Acheson and Allos, 2001). This is further supported by studies which have shown the survival rate of *Campylobacter* in water to be over 120 days (Whiley *et al.*, 2013).

The results of the study show that surface water as source of livestock water is a protective factor in reducing *Campylobacter* infections in livestock (odds ratio 0.039). Kapperud *et al* (2003) found that drinking direct from surface water was a risk factor for *Campylobacter* infections in livestock with an odds ratio of 1.5. They suggested that surface road water may play a role as a pool for transmitting *Campylobacter* infections across different animal species in an area e.g. between, dogs, birds, poultry etc. (Kapperud *et al*., 2003). Further studies need to be conducted to investigate why drinking surface road water in this study area was a protective factor however, no such study has been published so far.

Awareness on zoonoses and awareness on anti-microbial resistance have also been identified as protective factors for *Campylobacter* infection with an odds ratio of 0.029 and 0.002 respectively. This means that farmers who are awarenessable on zoonoses have less chance of their livestock getting *Campylobacter* infections than farmers who do not have this awareness (odds ratio 0.029). Farmers who have awareness on anti-microbial resistance also have lesser chance of their livestock getting infected by *Campylobacter* compared to other farmers who lack this awareness (0.002 odds ratio).

This clearly shows that awareness has an impact on controlling the prevalence of *Campylobacter* and should therefore be exploited. The protective impact of awareness in prevention of infections is a factor that has been reported to contribute to lesser *Campylobacter* infections (Whiley *et al*., 2013) and thus awareness creation should be encouraged.

There is a scarcity of information on the resistance patterns of *Campylobacter* isolates from livestock in Kenya. Most of the studies done in Kenya, as few as they are, are on humans. The
resistance pattern seen in this study is relatively high. It is actually higher than that recorded in previous studies on human isolates in Kenya. A recent study done in Kenya showed resistance to Ciprofloxacin (13.8%), Ampicillin (7.1%), Nalidixic acid (10.3%) and Cotrimoxazole (79.3%) while no resistance was seen to Chloramphenicol, gentamicin, doxycycline and Azithromycin (Kabiru, 2014). This trend is different from the results of this study showing resistance in all drugs with the highest resistance being to macrolides (Erythromycin) with 99% resistance followed by tetracycline (98.2%), Quinolones (Nalidixic acid and Ciprofloxacin, respectively), Aminoglycosides (Gentamicin) at 79.5% and the least resistance to Chloramphenicol (61.4%).

The drug resistance test results of the isolate that contained both C. jejuni and C. hyointestinalis are not shown since the resistance cannot be attributed to the results to either species in the isolate.

The high level of resistance of these isolates to macrolides and quinolones, especially from the identified zoonotic species raises concern in the public health sector should these pathogens be transmitted to humans (Gibreel and Taylor, 2006). These two groups of medication are the first line of treatment if human Campylobacter infections (Coker et al., 2002). The high resistance trend among the quinolones seen in this study (Nalidixic acid and Ciprofloxacin) is also of interest since the use of quinolones in food animals in Kenya has been on the rise. (Mitema et al., 2001).

The results of the five isolates sequenced to study the presence of mutations to the macrolides only revealed two nucleotide substitutions, one in the 23S rRNA gene and the other in the L4 (rplD) protein. None of these mutations have been described before in any study, however, there was not enough information to draw conclusions as to whether the mutations were related to expression of resistance phenotypically owing to the small number of isolates that was sequenced.

Documented mutations that have been reported to cause resistance of Campylobacter to quinolones were not identified in any of the tested isolates. There is no direct relation between the mutations seen in this study and the expression of resistance to quinolones by the Campylobacter isolates.
(silent mutation). The mutations seen in the DNA gyraseB gene are missense mutations some of which have been reported in other studies (Chatur et al., 2014; Piddock et al., 2003).

However, a larger sample size than was sequenced in this study needs to be done so as to draw a more conclusive picture of mutations linked to either Quinolone or macrolide resistance.

Resistance of Campylobacter to quinolones has for long been documented to be caused by mutations in the QRDR region of the DNA gyrase gene only. Despite the results of the sequencing on the five isolates showing absence of mutations, the resistance to quinolones may have been caused by some other mechanism like via the aminoglycoside 6 N-acetyltransferase gene as has been reported to occur by other studies (Eun et al., 2009). This study tested for the presence of plasmid mediated gene aac6 lb cr which has been reported to mediate resistance to quinolones and aminoglycosides in various other bacteria (Eun et al., 2009). This aac (6) gene was present in the isolates. This however, is a novel finding in Campylobacter isolates explaining potential mechanisms of the high resistance pattern seen phenotypically in this study. Further studies should be done to study the role of this gene in Quinolone resistance.

Also some of the resistance genes encoding for resistance of the bacterium to these antimicrobial drugs are transferable to other pathogens posing an even greater risk of high resistance pattern to other human pathogens. For example tetracycline resistance genes which are located on plasmids and transposons can be exchanged between different bacteria in different ecosystems and also between human and animal isolates (Abdi-Hachesoo et al., 2014). The genes for tetracycline resistance were identified to be present in (98.2%) of the zoonotic isolates from this study. Tetracyclines had the second highest rate of resistance among the study isolates. This rate is very important especially since studies done have shown tetracycline to be the most commonly used antimicrobial in food animals in Kenya (Mitema et al., 2001).
Campylobacter are inherently resistant to betalactam drugs (Wieczorek and Osek, 2013b). There are no set breakpoints for betalactam antimicrobial resistance testing in Campylobacter hence why the chemotherapeutic drug group was not tested for phenotypic pattern using agar dilution. However, the presence of resistance genes to beta lactams seen in this study raises concerns since there is the potential capacity/ risk of transferring these resistance genes to other bacteria in the body of livestock or human beings that are usually treated with and respond to betalactams.

Integrons were also found to be present in (68.2%) of the identified zoonotic isolates from this study. The presence of these extrachromosomal genetic elements in the isolates shows the potential of the pathogens to carry and transmit various genes that may be responsible for resistance to many other drugs of livestock or human importance. Integrons have not been investigated or been shown to exist in Campylobacter isolates before. Having shown their presence in these zoonotic isolates, studies need to be done to investigate further and draw a picture on what genes may be contained in the integrons carried by the isolates from this study area.

The bulk of the Campylobacter isolated in this study still remains unidentified to the species level. This group however still carries a huge risk in transfer of drug resistance as seen in the resistance pattern described above. Some of the Campylobacter in this group may be zoonotic although even the non-zoonotic species are capable of transferring drug resistance to the zoonotic species and to other zoonotic pathogens putting human health at risk of drug resistance.

The high resistance pattern to antimicrobials seen in the results of this study may be explained by the trend of antimicrobial usage on livestock at farm level in the study area. Seventy percent (70%) of the livestock were treated by the owners while 3.9% of the livestock were treated by the owners in collaboration with agro vet personnel. This is a huge number compared to only 5.1% of livestock being treated by professional veterinarians. The trend seen in this study agrees with the report of the rampant use of antimicrobials in livestock in Kenya (Mitema, et al, 2001). This situation is
further emphasized by the 63.8% who buy the antibiotics from agro vets compared to only 2.7% of the households that get their livestock examined and the medication prescribed by a veterinarian.

Mitema et al (2001) also reported that most of the drug use in livestock is mainly for treatment purposes. The information collected in this study backs this statement up showing that more farmers use antimicrobials to treat their livestock (49 households) and even fewer farmers in this study used antimicrobials as growth promoters (8 farmers).
CHAPTER 6.0 CONCLUSION AND RECOMMENDATIONS

Campylobacter is prevalent in livestock in Korogocho and Viwandani informal settlements in Nairobi and the factors associated with this prevalence is the contamination of livestock drinking water with fecal matter. Awareness of the respondents on zoonoses as well as antimicrobial resistance are the two factors found by this study to contribute to reducing the prevalence of zoonotic Campylobacter in livestock. A high antimicrobial resistance profile was observed in this study showing a worrying trend. Resistance genes and integrons class 1 were also found to be present in the zoonotic isolates.

*Campylobacter* has been shown to be an important pathogen in livestock in Korogocho and Viwandani informal settlements in Nairobi. The presence of zoonotic species has also been demonstrated to be of importance. This finding, together with the high prevalence of resistance to antimicrobials especially to the first line of treatment of *Campylobacter* infections in humans (macrolides and quinolones) shows the importance / risks of antimicrobial resistant (AMR) *Campylobacter* transmission to humans. Taking to consideration the implied consequences of zoonotic transmission of these strains, especially in reference to transmission of resistance genes, the public health sector has a huge role to play in controlling the infection in livestock and in reducing the risks of transmission of the pathogens to humans especially given the study population is an informal settlement with all the expected challenges of sanitation, education and health care.

These challenges may pose difficulty reducing the infection and spread of the bacteria, however, combating the risk factor identified above may be a good start to controlling the situation. The results and discussion section also give insight as to how awareness on aspects such as zoonoses and antimicrobial resistance may play a role to reducing *Campylobacter* infections, thus emphasizing on the need for creating and enhancing awareness in the urban livestock keepers.
Despite the main zoonotic species in focus being \textit{C. jejuni} and \textit{C. coli}, the focus should expand to include the other zoonotic species that may be of importance but are otherwise neglected in human research. This study opens up the gap of studying the prevalence of other zoonotic \textit{Campylobacter} species (other than \textit{C. jejuni} and \textit{C. coli}) in livestock.

There is need for further studies on the drug resistance genes in Campylobacter from Kenya. The drug resistance pattern shown is high however, a larger number needs to be sequenced to draw better conclusion about the mutations reported in other studies to be responsible for resistance to quinolones and macrolides. The aac6 lb cr gene has also come up of interest as it has been identified in this study, although its role in mediating resistance of Campylobacter to quinolones has not been studied. It could be considered as alternative mechanism for the resistance.

The recommendations from this study can be summed up as follows:

1. Education of the public about \textit{Campylobacter}, its effects, risk factors, prevention and control.

2. Sensitization of the need for increased diagnosis of \textit{Campylobacter} as one of the differential diagnoses of diarrhoea in Kenyan hospitals.

3. The need for education of the public on zoonoses, antimicrobial resistance among other issues that may contribute to reduction of livestock infections and also reduce zoonoses.

4. Studies should be done to identify the unidentified strains of \textit{Campylobacter} species prevalent in the area and their impacts.

5. Studies should be done to find out the importance of other zoonotic species (other than \textit{C. jejuni} and \textit{C. coli}) in humans and livestock in Kenya.

6. Sequencing of a larger sample size to study resistance to quinolones and macrolides and also the role of aac (6) lb cr gene.
CHAPTER 8.0 REFERENCES


Livestock-population-by-type-an/qbvv-8bjk


Global Salm-Surv

A global *Salmonella* surveillance and laboratory support project of the World Health Organization

*Laboratory Protocols*

Level 2 Training Course

Identification of thermotolerant *Campylobacter*

5th Ed. March. 2003

EDITED BY: RENE S. HENDRIKSEN (DFVVF), JAAP WADENAAAR (ASG), MARCEL VAN BERGEN (ASG)
Contents:

1. Introduction to identification of thermotolerant *Campylobacter* from food, faeces or water 3
2. Identification of thermotolerant *Campylobacter* from food, faeces or water .................5
3. Composition and preparation of culture media and reagents.......................................8

Record sheet: Isolation and identification of *Campylobacter* from faeces, food or water ..... 11
Appendix 1. Result sheet for identification of *Campylobacter* ........................................14
Appendix 2. Photographs of pos. and neg. reactions of biochemical tests on *Campylobacter* 15
1. Identification of thermotolerant *Campylobacter* from food, faeces or water

**Introduction**

The following procedures will guide you through the steps that are necessary to carry out a biochemical identification of *Campylobacter*.

*Campylobacter* are generally identified by:

- Slender helical or curved gram-negative rods.
- Highly motile by means of a single polar flagellum.
- Optimal oxygen concentration for growth 5-10%.
- Do not ferment or oxidize sugars.
- Do not produce indole (mind the different with hydrolysis of indoxyl acetate!).

According to ISO 10272 (Microbiology of food and animal feeding stuffs – Horizontal method for detection of thermotolerant *Campylobacter*) *Campylobacter* is identified by the following characteristics:

- morphology and motility
- morphology in Gram staining
- oxidase
- glucose
- lactose
- sucrose
- gas

In this course identification and differentiation of strains is performed by:

- morphology and motility
- morphology in Gram staining
- katalase
- oxidase
- hippurate hydrolysis
- hydrolysis of indoxyl acetate

*Campylobacter* from faeces, food or water

*Campylobacter* food poisoning occurs in most cases sporadically affecting individuals. Outbreaks due to Campylobacter infections are rare. Outbreaks due to contaminated milk and drinking water are described more often than food borne outbreaks. *Campylobacter jejuni* is the most common cause of human bacterial enteritis but *Campylobacter coli* may also be responsible. *Campylobacter jejuni* is commonly isolated from chicken and cattle, and chicken is expected to be one of the major sources of infection for humans. Pigs commonly carry *Campylobacter coli* in some countries where large quantities of pork are consumed. *Campylobacter coli* infections frequently occur.
Campylobacter may also be present in faeces or food in low numbers and they may be injured. To diminish the risk of obtaining false negative results, selective enrichment of a large food sample can be performed:

- Enrichment in selective enrichment broth (e.g. Preston).
- Selective plating on CCD-agar plates.

References
   ASM Press, Washington, D.C.
2. Identification of thermotolerant *Campylobacter* from food, faeces or water

**Materials**

**Equipment**
- Disposable inoculation loops (1 μl and 10 μl)
- Incubators at 37°C/42°C
- Microscope
- Slides
- Cover glass
- Mineral oil
- Paper disc 6 mm
- Pipettes for 0.2 ml (e.g. 1 ml pipettes)
- 200 ml flask
- Forceps
- Eppendorf tubes, 1.5 ml
- Drop counters

**Media**
- Sterile water
- 3%-H₂O₂
- 1%-hippurate solution
- 3.5%-ninhydrin solution
- 10%-indoxyl acetat solution
- Oxidase sticks
- Gram staining reagents
- Crystal violet
- Gram’s iodine
- Ethanol (95%)
- Carbol fuchsine

**Bacterial strains:**

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>ATCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter lari</td>
<td>ATCC 35221</td>
</tr>
<tr>
<td>Campylobacter coli</td>
<td>ATCC 33559</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>ATCC 700819</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 27853</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>ATCC 29212</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 29213</td>
</tr>
</tbody>
</table>

**Safety**

Carry out all procedures in accordance with the local codes of safe practice.
**Procedure**

**Identification**

**Microscopy (morphology and motility)**
One drop of sterile saline is placed on a slide. Colonies from the CCD agar plates are mixed with the saline. Place cover glass above the colonies, and place the slide in the microscope.

**Gram staining (morphology)**
One small drop of saline is placed on a slide. Colonies from the CCD agar plates are mixed with saline and smeared over the surface of the slide. The smears are allowed to dry thoroughly. The smears are fixed by passing the slide, smear up, quickly through the Bunsen flame three times. After cooling the smears can be stained. Between each staining reagent the smear is washed under a gently running tap, excess of aerated off before the next reagent is added.
1. Crystal violet (60 sec)
2. Gram’s iodine (60 sec)
3. Ethanol (decolouriser) (60 sec)
4. Carbol fuchsine (60 sec)

**Test for catalase**
Put a colony at a small spot on a slide (do NOT not make a suspension; just dry). Put one drop of 3% H2O2 on the spot with the bacterial material. Examine immediately for evolution of gas, which indicates catalase activity.

**Test for oxidase**
Transfer one colony to a filter paper. Soak the filter in an oxidase solution. Appearance of a blue color within 10 sec indicates a positive result.

**Theory / comments**

A striking character of *Campylobacter* is their helical or curved shape. Long spiral forms can resemble spirochaetes superficially, but campylobacters have flagella, usually single, at one or both poles and are highly motile, spinning around their long axes and frequently reversing direction.

Gram negative bacteria (like Campylobacter) are decolourised and stained red by the counter-stain (Carbol fuchsine). Campylobacter are curved or gull shaped forms. Old cultures may contain coccolid bacteria.

The catalase-enzyme cleaves the hydrogen peroxide $H_2O_2 + O_2 \rightarrow O_2 + 2H_2O$ The peroxidase is only able to reduce $H_2O_2$ if an organic substrate is present at the same time and serves as a donor for hydrogen.

The method is based on the principle that certain phenyl-diamin-derivatives are oxidised by cytochrom C to produce a bluish indophenol. Commercial kits are available.
Hippurate hydrolysis
Suspend a loopful of a growth from an 18-24 hour Columbia agar plate containing 5% cattle blood culture in 400 μl of a 1%-hippurate solution (take care not to incorporate agar). Incubate at 37°C for 2 hours. Then slowly add 200 μl 3.5%-ninthydrol solution to the side of the tube to form an overlay. Reincubate at 37°C for 10 min, and read the reaction. Positive reaction: dark purple/blue. Negative reaction: clear or gray.

Hydrolysis of hippuric acid releases benzoic acid. Hippuric acid is soluble in excess of an acidic solution of ferrichloride while benzoic acid precipitates.

1%-hippurate solution: freshly prepared or stored at -20°C for about 6 months.
3.5%-ninthydrol solution: Stable for about one month. Stored at room temperature in a dark bottle.

Identification
Hydrolysis of indoxyl acetate
Add 50 μl of a 10% (w/v) solution of indoxyl acetate in acetone to an absorbent paper disc 6 mm in diameter and allow to dry in air. Apply growth from a *Campylobacter* colony directly to disc and then wet with a drop of sterile distilled water. Appearance of a blue-green color within 5-10 minutes indicates a positive result.

The bacterial enzyme esterase releases indoxyl from indoxyl acetate which spontaneously forms indigo in the presence of oxygen. Dried discs are stable for at least 12 months if stored at 4°C in a dark glass bottle with silica gel. Discs should not be used if the color has changed from white, or if the expiration date has passed.

Appendix 1. Result sheet
Appendix 2. Photographs of pos. and neg. reactions of biochemical tests on *Campylobacter*
3. Composition and preparation of culture media and reagents

The media and reagents are available from companies like Oxoid, Merck and Difco. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also, the media should be prepared according to the manufacturers description if it differs from the description given here.

**Saline solution**

Sodium chloride 8.5 g  
Water 1000 ml

**Preparation:**  
Dissolve the sodium chloride in the water, by heating if necessary. Adjust pH ~ 7.0 after sterilisation. Dispense the solution into tubes so 4 ml is obtained after autoclaving at 121°C for 20 min.

**3.5 % Ninhydrin solution**

Ninhydrin (C₈H₇O₃) 3.5 g  
Acetone (C₆H₁₂O) 50 ml  
Butanol (C₄H₉O) 50 ml

Dissolve the chemical in the solutions. Stored at + 5°C in dark bottles of 20 ml.

**1% Hippurate solution**

Natriumhippurat (C₉H₁₂NNaO₄) 1 g  
PBS 99 ml

Dissolve the chemical with the solutions. Stored at -20°C in tubes of 15 ml.

**Gram-staining**

- **Crystal violet**  
  - Crystal violet 2.0  
  - Ethanol 95% (vol/vol) 20.0 ml  
  - Ammonium oxalate 0.8 g  
  - Distilled water 80.0 ml

The crystal violet is first dissolved in the ethanol, then the ammonium oxalate is dissolved in the distilled water. The two solutions are added together. To aid the dissolving process, both mixtures are agitated in a bath of hot water.
Gram’s iodine
Iodine crystals 1.0 g
Potassium iodide 2.0 g
Distilled water 200 ml

The iodine crystals and the potassium iodide are ground together in a mortar and the distilled water is added slowly. If necessary the mixture can be agitated in a bath of hot water to aid dissolution.

Decolourizer
Ethanol 95% (vol/vol)

Carbol fuchsin (counterstain)
Concentrated carbol fuchsin 10.0 ml
Distilled water 90.0 ml

10% (wt/vol) Indoxylacetate solution
Indoxylacetate \((C_{10}H_8NO_2)\) 10 g
Acetone \((C_3H_6O)\) 90 ml

Dissolve the chemical in acetone. Stored at +4°C in a dark bottle.

Oxidase solution

L(+)-Ascorbic acid 0.03 g
N,N,N’,N’- Tetramethyl-p-Phenylenediamine
Dihydrochloride \((C_{16}H_{12}N_2\cdot 2HCl)\) 0.03 g
Sterile water 30 ml

Dissolve the chemicals in water, and store the solution in a dark bottle at +5°C for 3 weeks.

References
1. BARROW & FELTHAM (eds.): Cowan and Steel’s Manual for the Identification of Medical Bacteria, 3 rd edn.
2. NMKL method no. 119, 2nd ed, Campylobacter Jejuni Coli detection in foods. Nordic committee on food analysis.
<table>
<thead>
<tr>
<th>QC-Strain</th>
<th>C. jejuni</th>
<th>C. coli</th>
<th>C. lari</th>
<th>E. faecalis</th>
<th>S. aureus</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCC 700819</td>
<td>ATCC 33559</td>
<td>ATCC 35221</td>
<td>ATCC 29212</td>
<td>ATCC 29213</td>
<td>ATCC 27853</td>
</tr>
</tbody>
</table>

Gram staining

Test for catalase

Test for oxidase

Hippurate hydrolysis

Hydrolysis of indoxyl acetate
Record sheet: Identification of Campylobacter.

Date: ____________________ Init.: ____________________

Biochemical tests

<table>
<thead>
<tr>
<th></th>
<th>Strain #</th>
<th>Strain #</th>
<th>Strain #</th>
<th>Strain #</th>
<th>Strain #</th>
<th>Strain #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology of the cell (microscopy)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility (microscopy)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for catalase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for oxidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippurate hydrolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolysis of indoxyl acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Species: __________ __________ __________ __________ __________ __________
Record sheet: Isolation and identification of Campylobacter from faeces, food or water.

Date: ___________________ Init.: ___________________

Biochemical tests

<table>
<thead>
<tr>
<th></th>
<th>Faeces-sample 1</th>
<th>Faeces-sample 2</th>
<th>Food-sample 1</th>
<th>Food-sample 2</th>
<th>Water-sample 1</th>
<th>Water-sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology of the cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(microscopy)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility (microscopy)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for catalase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for oxidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippurate hydrolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolysis of indoxyl acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Global Salm-Surv

A global *Salmonella* surveillance and laboratory support project of the World Health Organization

Laboratory Protocols

Level 2 Training Course

**MIC susceptibility testing of *Salmonella* and *Campylobacter***

**4th Ed. January. 2003**

Edited by: Rene S. Hendriksen (DFVF)
Contents:

1. Susceptibility testing: Determination of phenotypic resistance ........................................ 3
2. MIC determination by agar dilution on Salmonella and Campylobacter .......................... 4
3. Composition and preparation of culture media and reagents ........................................... 11
   Laboratory record sheets .................................................................................................. 13

Appendix 1. Example of preparing the dilutions of antimicrobial agents used in agar dilution.
Appendix 2. Scheme for preparing dilutions of antimicrobial agents used in agar dilution.
Appendix 3. Record form for MIC determination by agar dilution.
Appendix 4. Record form for MIC determination by microdilution broth testing.
Appendix 5. Ranges for MIC determination on Campylobacter.
Appendix 6. Quality control ranges for MIC determination on Enterobacteriaceae.
Appendix 7. Quality control ranges for MIC determination on Campylobacter.
1. Susceptibility testing: Determination of phenotypic resistance

1) Agar diffusion with disk
2) Agar diffusion with E-test
3) MIC-determination using Agar dilution method.

Introduction

The MIC (Minimal Inhibitory Concentration) of a bacterium to a certain antimicrobial agent can be determined and today gives the best quantitative estimate for susceptibility.

MIC is defined as the lowest concentration of antimicrobial agent required to inhibit growth of the bacteria. The principle is simple: Agar plates, tubes or microtitre trays with two-fold dilutions of antibiotics are inoculated with the bacteria and incubated. The next day the MIC is recorded as the lowest concentration of antimicrobial agent with no visible growth.

The MIC tells you about the degree of resistance and might give you important information about the resistance mechanism and the resistance genes involved. MIC-determination performed as agar dilution is regarded as the golden standard for susceptibility testing.

In contrast, diffusion tests are primarily qualitative methods that normally should only be used to report whether a bacterium is resistant or not. Principle: After an agar plate is inoculated with the bacteria, a tablet, disk or paperstrip with antimicrobial agent is placed on the surface. During incubation the antimicrobial agent diffuses into the agar and inhibits growth of the bacteria if sensitive. Diffusion tests are cheap compared to most MIC-determination methods. E-test is a diffusion test, but has been developed to give an approximate MIC-value.

Well standardised methods are essential for all kinds of susceptibility testing, since the methods are highly sensitive to variations in several factors, for example, size of inoculum, contents and acidity of the growth medium, time and temperature of incubation. The agar diffusion methods are also strongly influenced by factors such as agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria.

The MIC-determination and disk diffusion methods described in this protocol are in accordance with the international recommendations given by the National Committee for Clinical Laboratory Standards (NCCLS). The NCCLS describes how to perform the testing and sets international guidelines for interpretation of the results.

Quality control is regularly performed by running specific control strains as recommended by NCCLS.
2. Antimicrobial susceptibility testing by agar dilution (MIC)

Introduction

Agar dilution susceptibility testing is regarded as the golden standard for all other susceptibility testing methods. It is of course extremely important to be able to prepare the agar plates in such a way that the obtained antimicrobial concentration in the plates are exactly or very close to the desired concentrations.

When preparing antimicrobial solutions and agar plates for agar dilution susceptibility testing, we therefore strongly recommend following the international guidelines given by the NCCLS (NCCLS document M7-A5 "Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically"). Instructions on how to prepare the antimicrobial solution are outlined in table 5 (NCCLS document M100-S12) and further explained in appendices 1 and 2. The dilution procedure might at first seem a little complicated, but this method ensures that there is minimal risk of making out-of-scale-dilutions for the smallest concentrations in the test range.

Materials

Equipment
- McFarland standard 0.5
- Nephelometer or white paper with black lines
- Multi-point inoculator (applies up to 30 inocula to the same agar plate)
  At this course only parts from a multi-inoculator will be used. A stand with inoculation pins, and a well inoculation pot.
- Graduated pipettes (20 μl - 1000 μl)
- Disposable loops (1 μl and 10 μl)

Media
- Sterile normal saline, 4 ml volumes in tubes for nephelometer
- Eppendorf-tubes with 900 μl sterile normal saline
- Mueller-Hinton II agar plates (9 mm) for Salmonella with two-fold dilutions of antibiotic:
  - Chloramphenicol (1-64 μg/ml)
  - Ampicillin (0.5-32 μg/ml)
  - Tetracycline (1-32 μg/ml)
- Mueller-Hinton II agar plates (9 mm) containing 5% cattleblood for campylobacter with two-fold dilutions of antibiotic:
  - Ciprofloxacin (0.125-16 μg/ml)
  - Nalidixan (1-128 μg/ml)
  - Tetracycline (0.5-32 μg/ml)
  - Erythromycin (0.25-32 μg/ml)

An example of the dilution procedure for preparing agar plates is shown in Appendix 1.
- Mueller-Hinton II agar plates (9 mm) for Salmonella without antibiotic for growth control (2 per test-antibiotic)
• Mueller-Hinton II agar plates (9 mm) containing 5% cattle blood for campylobacter without antibiotic for growth control (2 per test-antibiotic)
• Nutrient agar plates for purity control of inoculum suspension

**Bacterial strains**
• *Salmonella* strains on non-selective agar.
• *Campylobacter* strains on non-selective agar.
• 4 strains for quality control: *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 and Campylobacter jejuni ATCC 33560

**Safety**
Carry out all procedures in accordance with the local codes of safe practice.
Preparing antimicrobial solutions and agar-plates for agar dilution MIC testing.

Procedure

Day 1
1. Find the highest concentration in your test-range in the column: “Final Concentration at 1:10 Dilution in Agar”. The row where you read the highest concentration will be your starting point in the dilution of the antimicrobials.

2. Find the stock solution for your test-range in the column: “Concentration”.

3. From the number of agar plates you want to prepare for each concentration, calculate the needed volume of agar per concentration.

4. 10% of this volume will be antimicrobial solution. Bear this in mind when you calculate the needed volume of antimicrobial solution.

5. In the columns “Volume + Distilled Water” you will find the scale of dilution between the stock solution and the solvent.

6. You have to multiply the sum of the two columns by a digit large enough so that you are sure to have enough solution for preparing the agar plates and for further dilution of the antimicrobial solutions for rows 3, 6, 9 and 12.

7. When you incorporate the further dilution in the calculation of the antimicrobial solution for rows 3, 6, 9 and 12 bear in mind that at this step you have to add the needed volumes of antimicrobial solution for the next three rows.

Theory / comments

NCCLS manual M100-S12 page 114
Procedure

8. You have now finished the first line of plates. Continue with the next concentration using the same procedure. Be aware of the change in the column: “Source”. This step number refers to the solution from which the next line of solutions is made. Remember to multiply by a digit large enough so that you have enough of your solution for the agar plates and for preparing the next solutions.

9. When you prepare the stock solution remember to multiply so that the amount of antimicrobial to be weighed exceeds more than 100mg (for better accuracy).

10. When you plan your preparation of the antimicrobial solution, it may be an advantage to use the scheme in appendix 2 for the calculation of the solutions. Appendix 1 is an example of a calculation.

Theory / comments

How to prepare the agar for producing plates to the agar dilution method

Day 1
1. The Müller Hinton II agar are melted and warmed in a water bath to approximately 50°C.

2. The different solutions (concentrations) of antimicrobials are poured into measuring glasses and labelled.

3. Add the agar to the measuring glasses with the antimicrobials and mixed gently. (If necessary, add blood to the agar before you pour it into the measuring glasses).

4. Pour the agar into empty petri dishes, which have been labelled. (The agar depth is crucial using this method).
Procedure

5. Wait until they are set then reverse them and incubate them overnight for control of the purity.

6. Allow the surface of the agar-plates to dry before use. (Use plates within 14 days).

MIC determination by agar dilution

Day 1

Standardisation of inoculum
From a pure o/n culture, pick material from at least 3-4 colonies. Resolve totally in 4 ml NaCl in tubes. Mix.

Adjust to McFarland 0.5 (nephelometer). Calibrate the nephelometer before use and gently turn all suspensions upside-down before measuring. Adjust turbidity of inoculum to match that of the standard.

If a nephelometer is not available: Compare visually with the McFarland 0.5 standard using white paper with black lines as background.

The McFarland 0.5 suspension is diluted 10-fold to yield the final inoculum suspension. Transfer 100 µl to 900 µl saline in Eppendorf tubes. Turn the tube up-side-down two times.

The inoculum suspension should be used for inoculation within 15 minutes.

Inoculation and incubation
Transfer 400 µl of the inoculum suspension to the multi-point inoculator wells.

Theory / comments

This is done to minimize the risk of picking bacteria which have lost their resistance.

McFarland 0.5 ~ approximately $10^8$ CFU/ml

The inoculum suspension ~ approximately $10^7$ CFU/ml.

To avoid further growth of inoculum.

This procedure must be carried out in a flow bench to avoid contamination.
**Procedure**

Place the control strains as shown on the result sheet (Appendix 3) and write down the orientation of the other isolates too.

Inoculate plates starting with the lowest concentration. Remember to inoculate one of the growth control plates before and after. It is important that all plates are dry before inoculation.

Allow the inoculum-spots to dry upside down before incubation. (37°C for 16-20 h for Salmonella and 42°C for 48 h for Campylobacter).

Purity control: Spread 10 µl of the inoculation-suspension on a non selective agar plate. Incubate at 37°C /42°C overnight.

**Day 2**

**Reading plates/interpretation of results**
Check purity of the inoculum suspension. If not OK, results should not be reported.

Read plates as follows on a dark background:
- Use the result sheet (Appendix 3) for orientation of the isolates on the plates.
- Check growth on the two control plates. If growth is weak (faint haze, pinpoint colonies or <10 colonies), results cannot be reported.
- The MIC is read as the lowest concentration without visible growth. A faint haze, pinpoint colonies or growth of a single colony should be ignored.

Be aware of special reading for trimethoprim and sulphonamides. In these cases the MIC is recorded as the lowest concentration where a growth reduction of 80-90 % can be seen.

**Theory / comments**

Most multi-point inoculators apply 1-2 µl of the suspension to the agar surface. The final inoculum on the agar will then be approximately $10^4$ CFU per spot.

The MIC is determined from two-fold dilutions of the antimicrobial agent. Be aware that "the true" MIC can be anywhere between the observed MIC and the dilution step below.

The antibiotic trimethoprim and the sulphonamides allow growth of the bacteria for some generations before inhibition occurs.
**Procedure**

Further interpretation of the MIC is done according to the NCCLS recommendations (breakpoints for Enterobacteriaceae are visualised in the result sheet for microdilution broth testing. Appendix 4 and in Appendix 5 regarding Campylobacter).

The acceptable MIC-ranges for the quality control strains as recommended by the NCCLS for Enterobacteriaceae are shown in Appendix 6. For Campylobacter the MIC-ranges of the quality control strains are based on population-distribution in Appendix 7.

**Theory / comments**

The NCCLS standard do not include breakpoint-recommendations for all of the compounds and organisms tested. In these cases breakpoints are assigned in accordance to the population-distribution after testing a large number of isolates. (Appendix 5 and Appendix 7).
3. Composition and preparation of culture media and reagents

The media and reagents are available from companies like Oxoid, Merck and Difco. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also the media should be prepared according to the manufacturers description if it differs from the description given here.

Mueller Hinton II agar (e.g. from BBL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Acid hydrolysate of casein</td>
<td>17.5 g</td>
</tr>
<tr>
<td>Starch</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>17.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Preparation:
Dissolve the dehydrated medium in water by heating if necessary. Adjust pH to 7.2 - 7.4, transfer into bottles and autoclave at 110°C for 20 min.

Saline solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>8.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Preparation:
Dissolve the sodium chloride in the water, by heating if necessary. Adjust pH ~ 7.0 after sterilisation. Dispense the solution into tubes so 4 ml is obtained after autoclaving at 121°C for 20 min.

Columbia-agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia agar base (Oxoid CM331)</td>
<td>1125 g</td>
</tr>
<tr>
<td>Water</td>
<td>25,000 ml</td>
</tr>
<tr>
<td>Natriumhydroxid 5N</td>
<td></td>
</tr>
<tr>
<td>Saltsyre 4N</td>
<td></td>
</tr>
</tbody>
</table>

Preparation:
Dissolve the Agar Base in water, and let it stand for 15 min. Boil the solution for 15 min., and adjust pH~7.1-7.5. The medium is poured into 1000 ml flasks and autoclaved at 121°C for 15 min.
Columbia-agar with cattle blood

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia agar</td>
<td>950 ml</td>
</tr>
<tr>
<td>Cattle blood</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Preparation:
Melt the agar and add cattle blood. Pour plates with about 15 ml melted medium in each. Incubate overnight at 37°C.

References
Before inoculation write the ID of the strains 1-26 above each circle.
Write the lowest concentration of antibiotic without growth (the MIC) in each circle.
Fill out the following table.
Record sheet: Salmonella / Tetracycline
MIC determination by agar dilution

Before inoculation write the ID of the strains 1-26 above each circle.
Write the lowest concentration of antibiotic without growth (the MIC) in each circle.
Fill out the following table.
Before inoculation write the ID of the strains 1-26 above each circle. Write the lowest concentration of antibiotic without growth (the MIC) in each circle. Fill out the following table.
### Record sheet: Salmonella

MIC determination by agar dilution

<table>
<thead>
<tr>
<th>No</th>
<th>Strain</th>
<th>Chloramphenicol</th>
<th>Ampicillin</th>
<th>Tetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC (µg/ml)</td>
<td>Interpretation (R-I-S)</td>
<td>MIC (µg/ml)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Date:______________ Record sheet: Camphylobacter / Ciprofloxacin
Initials:____________ MIC determination by agar dilution

Before inoculation write the ID of the strains 1-26 above each circle.
Write the lowest concentration of antibiotic without growth (the MIC) in each circle.
Fill out the following table.
Before inoculation write the ID of the strains 1-26 above each circle.
Write the lowest concentration of antibiotic without growth (the MIC) in each circle.
Fill out the following table.
Date: ____________ Record sheet: Campylobacter / Tetracycline 
Initials: ____________ MIC determination by agar dilution

Before inoculation write the ID of the strains 1-26 above each circle.
Write the lowest concentration of antibiotic without growth (the MIC) in each circle.
Fill out the following table.
Date: __________ Record sheet: Campylobacter / Erythromycin
Initials: __________ MIC determination by agar dilution

Before inoculation write the ID of the strains 1-26 above each circle.
Write the lowest concentration of antibiotic without growth (the MIC) in each circle.
Fill out the following table.

E. coli
ATCC 25922

S. marcescens
ATCC 25921

C. jejuni
ATCC 33560
<table>
<thead>
<tr>
<th>No</th>
<th>Strain</th>
<th>Ciprofloxacin</th>
<th>Nalidixic Acid</th>
<th>Tetracycline</th>
<th>Erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC (µg/ml)</td>
<td>Interpretation (R-I-S)</td>
<td>MIC (µg/ml)</td>
<td>Interpretation (R-I-S)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### APPENDIX 1

Example of preparing the dilutions of antimicrobial agents used in agar dilution. (NCCLS M100-S12 table 5)

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration</th>
<th>Source</th>
<th>Volume + Solvent</th>
<th>Upscale to use vol.</th>
<th>Final vol. of solvent</th>
<th>Final concentration at 1/10 dilution agar</th>
<th>Vol. media</th>
<th>Vol. solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5120</td>
<td>Stock</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>512</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>2</td>
<td>5120</td>
<td>Step 1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>586</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>3</td>
<td>5120</td>
<td>Step 1</td>
<td>1</td>
<td>3</td>
<td></td>
<td>128</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>4</td>
<td>1280</td>
<td>Step 3</td>
<td>1</td>
<td>1</td>
<td></td>
<td>64</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>5</td>
<td>1280</td>
<td>Step 3</td>
<td>1</td>
<td>3</td>
<td></td>
<td>32</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>1280</td>
<td>Step 3</td>
<td>1</td>
<td>7</td>
<td>3 + 21</td>
<td>16</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>160</td>
<td>Step 6</td>
<td>1</td>
<td>1</td>
<td>6 + 6</td>
<td>8</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>160</td>
<td>Step 6</td>
<td>1</td>
<td>3</td>
<td></td>
<td>4</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>160</td>
<td>Step 6</td>
<td>1</td>
<td>7</td>
<td>3 + 21</td>
<td>2</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>Step 9</td>
<td>1</td>
<td>1</td>
<td>6 + 6</td>
<td>1</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>20</td>
<td>Step 9</td>
<td>1</td>
<td>3</td>
<td>3 + 9</td>
<td>0.5</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>Step 9</td>
<td>1</td>
<td>7</td>
<td>3 + 21</td>
<td>0.25</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>13</td>
<td>2.5</td>
<td>Step 12</td>
<td>1</td>
<td>1</td>
<td></td>
<td>0.125</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Antimicrobial: *E. coli*

Antimicrobial gradient: 0.25 - 32 mg/mL.

Concentration of the stock solution: 1280 mg/mL.

Volume of antimicrobial to be weighed (1280 mg/mL * 6 mL) 1280 mg/mL * 90 = 108.24 mg.

Volume of agar: 90 mL.

Volume of antimicrobial solution (10% of agar vol): 10 mL.
APPENDIX 3: NARMS ANTIMICROBIAL SUSCEPTIBILITY BREAKPOINTS


On page 21, Table V, "Antimicrobial agents used for susceptibility testing of *Campylobacter* isolates, NARMS, 1997-2005." should read as follows:

**Table V: Antimicrobial agents used for susceptibility testing of *Campylobacter* isolates, NARMS, 1997-2005**

<table>
<thead>
<tr>
<th>CLSI Subclass</th>
<th>Antimicrobial Agent</th>
<th>Antimicrobial Agent Concentration Range (µg/mL)</th>
<th>Breakpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>Gentamicin</td>
<td>0.12-32 0.016-256*</td>
<td>≤2 4 ≥8</td>
</tr>
<tr>
<td>Ketolides</td>
<td>Telithromycin†</td>
<td>0.015-8</td>
<td>≤4 8 ≥16</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>Clindamycin</td>
<td>0.03-16 0.016-256*</td>
<td>≤2 4 ≥8</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Azithromycin</td>
<td>0.015-64 0.016-256*</td>
<td>≤2 4 ≥8</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>0.03-64 0.016-256*</td>
<td>≤8 16 ≥32</td>
</tr>
<tr>
<td>Phenics</td>
<td>Chloramphenicol‡</td>
<td>0.016-256*</td>
<td>≤8 16 ≥32</td>
</tr>
<tr>
<td></td>
<td>Florfenicol§</td>
<td>0.03-64</td>
<td>≤4 N/A N/A</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Ciprofloxacin</td>
<td>0.015-64 0.002-32*</td>
<td>≤1 2 ≥4</td>
</tr>
<tr>
<td></td>
<td>Nalidixic acid</td>
<td>4-64 0.016-256*</td>
<td>≤16 32 ≥64</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Tetracycline</td>
<td>0.06-64 0.016-256*</td>
<td>≤4 8 ≥16</td>
</tr>
</tbody>
</table>

†E-test dilution range used from 1997-2004.
‡Telithromycin added to NARMS panel in 2005.
§Chloramphenicol, tested from 1997-2004, was replaced by florfenicol in 2005.
§Currently only a susceptible breakpoint has been established. In this report isolates with a MIC ≥8 µg/mL are categorized as resistant.

On page 59 Table 5.01, the percentage of *Campylobacter jejuni* species isolated in NARMS, 2005 should read 88.9% and 11.0% for *Campylobacter coli*. The total percentage of *Campylobacter* species isolated in NARMS, 2005 should read 100%.

On page 60 Table 5.03, page 62 Table 5.04 and page 64 Table 5.08, the susceptible breakpoint for florfenicol should read (MIC ≤4).

APPENDIX 4: SEQUENCE BLAST RESULTS

NCBI NUCLEOTIDE BLAST RESULTS

1. Sample 30 (CCOL)

Blast result of the sequence from Campylobacter coli isolate 30 from this study (Query; CCOL) showing 99% identity to Campylobacter coli strain KLC5104 from genbank.

---

Key:
The Query refers to the sequence of the isolate from my study while the subject refers to the sequence from the genbank database. In this case the subject sequence is that if Campylobacter coli strain KLC5104.
2. **Sample 884 (36cjejlpxA)**

Blast results of the sequence for *Campylobacter jejuni* isolate number 884 from this study (Query: 36cjejlpxA) showing 100% identity to the complete genome of *Campylobacter jejuni* subsp. Jejuni strain F38011 from gen bank.
3. **Sample 889 (37cjejlpxA)**

Blast results of sequence for Campylobacter jejuni isolate 889 from this study (Query; 37cjejlpxA) showing 99% identity to the complete genome of Campylobacter jejuni subsp/ jejuni strain CG8421 from gen bank data base.
APPENDIX 5: DETAILS OF PHENOTYPIC DRUG RESISTANCE TEST RESULTS.

<table>
<thead>
<tr>
<th>LIVESTOCK SPECIES</th>
<th>CAMPYLOBACTER SPECIES</th>
<th>ANTIMICROBIALS</th>
<th>Resist-ant</th>
<th>Interme-di-ate</th>
<th>Suscep-ti-ble</th>
<th>RESISTANCE PATTERNS SUMMARY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chickens</td>
<td>Campylobacter jejuni</td>
<td>Gentamicin</td>
<td>16</td>
<td>1</td>
<td>6</td>
<td>Resistant to all 6 drugs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloramphenicol</td>
<td>13</td>
<td>7</td>
<td>3</td>
<td>Resistant to 5 antimicrobials</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>19</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciprofloxacin</td>
<td>18</td>
<td>1</td>
<td>4</td>
<td>Resistant to 4 antimicrobials</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracycline</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>Resistant to 3 antimicrobials</td>
</tr>
</tbody>
</table>

9 isolates

7 isolates

2 isolates

2 isolates

1 isolate
<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Campylobacter coli</th>
<th>Other Campylobacter species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>23 0 0</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0 0 1</td>
<td>87 0 0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0 0 1</td>
<td>69 0 0</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>1 0 0</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0 0 1</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1 0 0</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1 0 0</td>
<td></td>
</tr>
</tbody>
</table>

### Resistant to 2 antimicrobials:
- Tetracycline and erythromycin

This was only 1 isolate which was resistant to 3 antimicrobials: nalidixic acid, tetracycline and erythromycin.

### Resistant to all 6 antimicrobials:
- Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin

59 isolates

### Resistant to 5 antimicrobials:
- Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin

28 isolates

### Resistant to 4 antimicrobials:
- Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline

7 isolates
<table>
<thead>
<tr>
<th>LIVESTOCK SPECIES</th>
<th>CAMPYLOBACTER SPECIES</th>
<th>ANTIMICROBIALS</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni</td>
<td>I. Gentamicin</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II. Chloramphenicol</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Resistant to 4 antimicrobials</th>
<th>Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 isolates</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Resistant to 3 antimicrobials</th>
<th>Ciprofloxacin, Tetracycline and Erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 isolate</td>
<td>Nalidixic acid, Tetracycline and Erythromycin</td>
</tr>
<tr>
<td>2 isolates</td>
<td>Chloramphenicol, Tetracycline and Erythromycin</td>
</tr>
<tr>
<td>3 isolates</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Resistant to 2 antimicrobials</th>
<th>Tetracycline and Erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 isolates</td>
<td>One isolate grew upon reviving and was resistant to 5 antimicrobials: Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin</td>
</tr>
<tr>
<td></td>
<td>I.</td>
</tr>
<tr>
<td>----------------</td>
<td>-----</td>
</tr>
<tr>
<td>I. Gentamicin</td>
<td>11</td>
</tr>
<tr>
<td>II. Chloramphenicol</td>
<td>7</td>
</tr>
<tr>
<td>III. Nalidixic acid</td>
<td>11</td>
</tr>
<tr>
<td>IV. Ciprofloxacin</td>
<td>10</td>
</tr>
<tr>
<td>V. Tetracycline</td>
<td>12</td>
</tr>
<tr>
<td>VI. Erythromycin</td>
<td>12</td>
</tr>
</tbody>
</table>

**Other Campylobacter species**

- **Resistant to all 6 antimicrobials**
  - Gentamicin: 6 isolates
- **Resistant to 5 antimicrobials**
  - Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin: 4 isolates
- **Resistant to 2 antimicrobials**
  - Tetracycline and Erythromycin: 1 isolate
<table>
<thead>
<tr>
<th>LIVESTOCK SPECIES</th>
<th>CAMPYLOBACTER SPECIES</th>
<th>ANTIMICROBIALS</th>
<th>Resist</th>
<th>Intermediate</th>
<th>Susceptible</th>
<th>RESISTANCE PATTERNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIGS</td>
<td><em>Campylobacter jejuni</em></td>
<td>VII. Gentamicin</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>The single <em>Campylobacter jejuni</em> isolate from a pig sample was <strong>susceptible to all antimicrobials</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VIII. Chloramphenicol</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IX. Nalidixic acid</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>X. Ciprofloxacin</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>XI. Tetracycline</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>XII. Erythromycin</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other <em>Campylobacter</em></td>
<td>Gentamicin</td>
<td>10</td>
<td></td>
<td></td>
<td><strong>Resistant to all 6 antimicrobials</strong></td>
</tr>
<tr>
<td></td>
<td>species</td>
<td>Chloramphenicol</td>
<td>8</td>
<td></td>
<td></td>
<td><strong>6 isolates</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>9</td>
<td></td>
<td></td>
<td><strong>Resistant to 5 antimicrobials</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>4 isolates</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

111
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/mL)</th>
<th>Number</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>8</td>
<td>1 isolate</td>
<td>Gentamicin, Chloramphenicol, Nalidixic acid, Tetracycline, and Erythromycin</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10</td>
<td>1 isolate</td>
<td>Gentamicin, Chloramphenicol, Tetracycline, and Erythromycin</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>10</td>
<td>1 isolate</td>
<td>Gentamicin, Chloramphenicol, Nalidixic acid, Tetracycline, and Erythromycin</td>
</tr>
<tr>
<td><strong>LIVESTOCK SPECIES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1</td>
<td>1 isolate</td>
<td>Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline, and Erythromycin</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1</td>
<td>1 isolate</td>
<td>Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline, and Erythromycin</td>
</tr>
</tbody>
</table>

**Resistant to 4 antimicrobials**:
- Gentamicin
- Chloramphenicol
- Nalidixic acid
- Tetracycline
- Erythromycin

**Resistant to 5 antimicrobials**:
- Gentamicin
- Nalidixic acid
- Ciprofloxacin
- Tetracycline
- Erythromycin

**Intermediate Sensitivity**
- Campylobacter species

**Susceptible**
- Campylobacter species

**Other Campylobacter species**
- Gentamicin: 1
- Chloramphenicol: 1
<table>
<thead>
<tr>
<th>LIVESTOCK SPECIES</th>
<th>CAMPYLOBACTER SPECIES</th>
<th>ANTIMICROBIALS</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>RABBITS</td>
<td></td>
<td>Nalidixic acid</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciprofloxacin</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracycline</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erythromycin</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CATTLE</td>
<td>Other <em>Campylobacter</em> species</td>
<td>Gentamicin</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloramphenicol</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciprofloxacin</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracycline</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erythromycin</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Resistant to 3 antimicrobials**
Chloramphenicol, Nalidixic acid and Erythromycin

**Resistant to 2 antimicrobials**
Tetracycline and Erythromycin

**RESISTANCE PATTERNS**
This was only 1 isolate resistant to 5 antimicrobials: Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin.
<table>
<thead>
<tr>
<th>LIVESTOCK SPECIES</th>
<th>CAMPYLOBACTER SPECIES</th>
<th>ANTIMICROBIALS</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
<th>RESISTANCE PATTERN</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUCKS</td>
<td>Other Campylobacter species</td>
<td>Gentamicin</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>Resistant to all 6 antimicrobials</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloramphenicol</td>
<td>2</td>
<td></td>
<td></td>
<td>2 isolates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciprofloxacin</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>Resistant to 5 antimicrobials</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetracycline</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1 isolate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erythromycin</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline, Erythromycin</td>
</tr>
</tbody>
</table>