PREVALENCE AND ANTIMICROBIAL RESISTANCE OF ZOONOTIC *CAMPYLOBACTER* ISOLATED FROM LIVESTOCK AND RODENTS IN URBAN INFORMAL SETTLEMENTS IN NAIROBI.

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DECLARATION

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

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DEDICATION

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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.

*Campylobacter*s 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 poutry

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 *Campylobacter*s 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*.

1.3 Hypothesis

Antimicrobial resistant zoonotic Campylobacter phenotypes are prevalent in 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 charactestic 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 *Campylobacter*iosis 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 *Campylobacter*s: 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 iolates 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 habor 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 diarrhoes 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 *tetO*. (Wieczorek and Osek, 2013a). Examples of these could be integrons and transposons. Studies show the likelihood of *Campylobacter tetO* having been obtained from *Streptomyces, Streptococcus*, or *Enterococcus species* through horizontal genetic transmission (Batchelor, *et al* 2004).

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 *et al*., 2004). Replacement of nucleotides at positions 2074 and 2075 of the adenine residues in the 23S rRNA gene in *Campylobacter* frequently occur in erythromycin resistance (Luangtongkum *et al*., 2009). The A2074C, A2074G, and A2075G mutations result in increased macrolide resistance in *C. jejuni* and *C. coli*, with erythromycin resistance corresponding with resistance to all other macrolides, lincosamides and streptogramin antimicrobials (Avrain, *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, *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 *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 *et al*, 2003). These work together to remove antimicrobials among other substances from a *Campylobacter* cell (Lin *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 *Campylobacter*iosis (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, vancomicin, 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 atypicall; round, convex, shiny morphology if plates are not moist (Isaacson ., 2003). Futher 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*

	c. jejuni	***C. coli***	***C. lari***	***C. hyointestinalis***	***C. fetus***
Gram stain morphology	Gram negative curved rods	Gram negative curved rods	Gram negative curved rods	Gram negative curved rods	Gram negative curved rods
Test for Catalase	+	+	+	+	+
Test for Oxidase	+	+	+	+	+
Hippurate hydrolysis	+	-	-	-	-
Indoxyl acetate hydrolysis	+	+	-	-	-

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 $H_2O_2 + H_2O_2 => O_2 + 2H_2O$. 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 thermophillic *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 susceptibilily 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%).

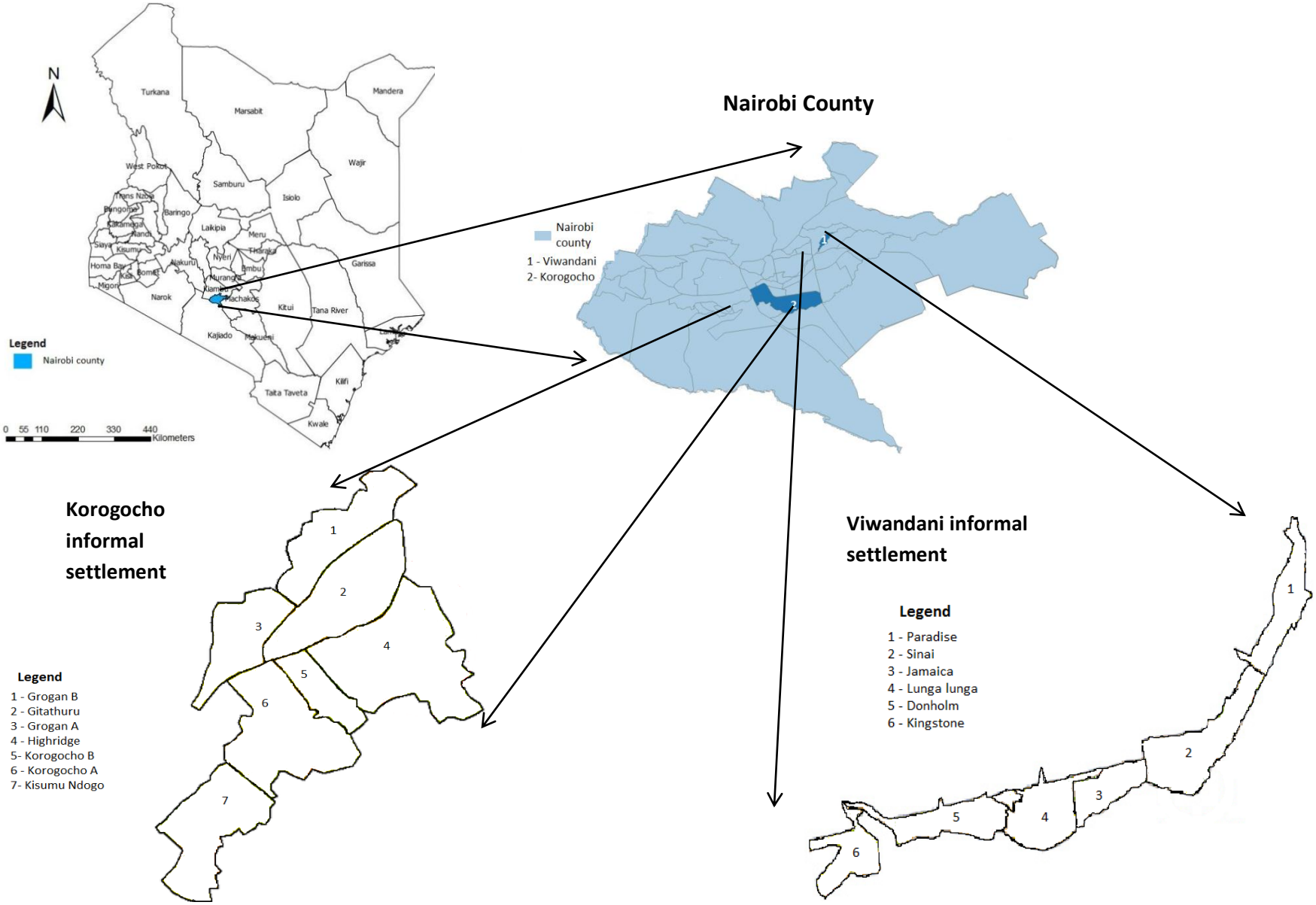

Figure 1: A map of the study area showing the two sites within Nairobi. The legend shows the villages sampled within the study area.

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.

$$n= ((Z\alpha/_2)^2 * p (1-p)) / L^2$$

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 = precision (in proportion of one; if 5%, L = 0.05).

$n= (1.96^2*0.5(0.5)) / 0.05^2$

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.

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

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 (H_2O_2) 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 ectracted from Campylobacter colonies initially stored in sterile skimmed milk at -40℃. The stored *Campylobacter* isolates were revived on mCCDA plates. Thereafter, the DNA was extracted by boiling for 5 minutes at 95℃ in a heating block and then centrifuged at 12,000 revolutions per minute for 5minutes. 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 ditilled water, 0.1 µl each of forward and reverse primers and 5µl of DNA template. The DNA was amplified using a thermocycler (make: Veriti, manufacturer: Applied Biosystems) by an initial denaturation at 95^0C for 10 min, followed by another denaturation at 95^0C for 30 sec, annealing at 59^0C for 90 sec, and an extension at 72^0C for 60 sec. The processes of second denaturation, annealing and extension were repeated for 35 cycles and a final extension done at 72^0C 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 94^0C for 5 min, followed by another denaturation at 94^0C for 60 sec, annealing at 50^0C for 60 sec, and an extension at 72^0C for 60

sec. The processes of second denaturation, annealing and extension were repeated for 30 cycles and a final extension done at 72^0C 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

	Target gene	primer name	Primer sequence	Accession number	Product length	Reference
Campylobacter spp	16s RNA	C412F C1228R	GGATGACACTTTTCGGAGC CATTGTAGCACGTGTGTC	CP007769	816	(Yamazaki *et al*., 2007).
C. jejuni	LpxA gene	CjejlpxAF CjejlpxAR	ACAACTTGGTGACGATGTTGTA CAATCATGDGCDATATGASAAT AHGCCAT	PMC535264	331	(Klena *et al*., 2004)
C. coli	lpxA gene	CcollpxAF CjejlpxAR	AGA CAA ATA AGA GAG AAT CAG CAATCATGDGCDATATGASAAT AHGCCAT	PMC535264	391	(Klena *et al*., 2004)
C.lari	GlyA gene	CLF CLR	TAGAGAGATAGCCAAAAGAGA TACACATAATAATCCCACCC	AF136495	251	(Yamazaki *et al*., 2007)
C, fetus	16sRNA	CFCH57F CF1054R	GCAAGTCGAACGGAGTATTA GCAGCACCTGTCTCAACT	CP008810	978	(Wangroongsarb *et al*; 2011)
C. hyointestinalis	16s RNA	CFCH57F CH1344R	GCAAGTCGAACGGAGTATTA GCGATTCCGGCTTCATGCTC		1267	"

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

Genetic element and antimicrobial agent.	Target gene	primer name	Primer sequence	Product length	Reference
INTEGRON	Class 1 integrons	INT-1U INT-1D	GTTCGGTCAAGGTTCTG GCCAACTTTCAGCACATG	923	Guney *et al* 2014
TETRACYCLINE	Tet O gene	Forward Reverse	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	515	Bahman *et al* (2014)
AMINOGLYCOSIDES	Aac(6) lb-cr	Forward Reverse	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	
QUINOLONES	DNA gyrase A gene	cjgyrA1 cjgyrA2	GCCTGACGCAAGAGATGGTT TCAGTATAACGCATCGCAGC	270	Piddock *et al* (2003)
	DNA gyrase B gene	Forward Reverse	ATGGCAGCTAGAGGAAGAGA GTGATCCATCAACATCCGCA	382	Piddock *et al* (2003)
MACROLIDES	23S Rna	Forward Reverse	CGAGATGGGAGCTGTCTCAAAG CCCACCTATCCTGCACATTCTT	147	Hao *et al* (2015)
	L4	Forward Reverse	AAGTTTAAGAGCAAATACAGCTCATACTAAAG ATAGCCAAAGAATCAGCAGTAAATAAC	270	“

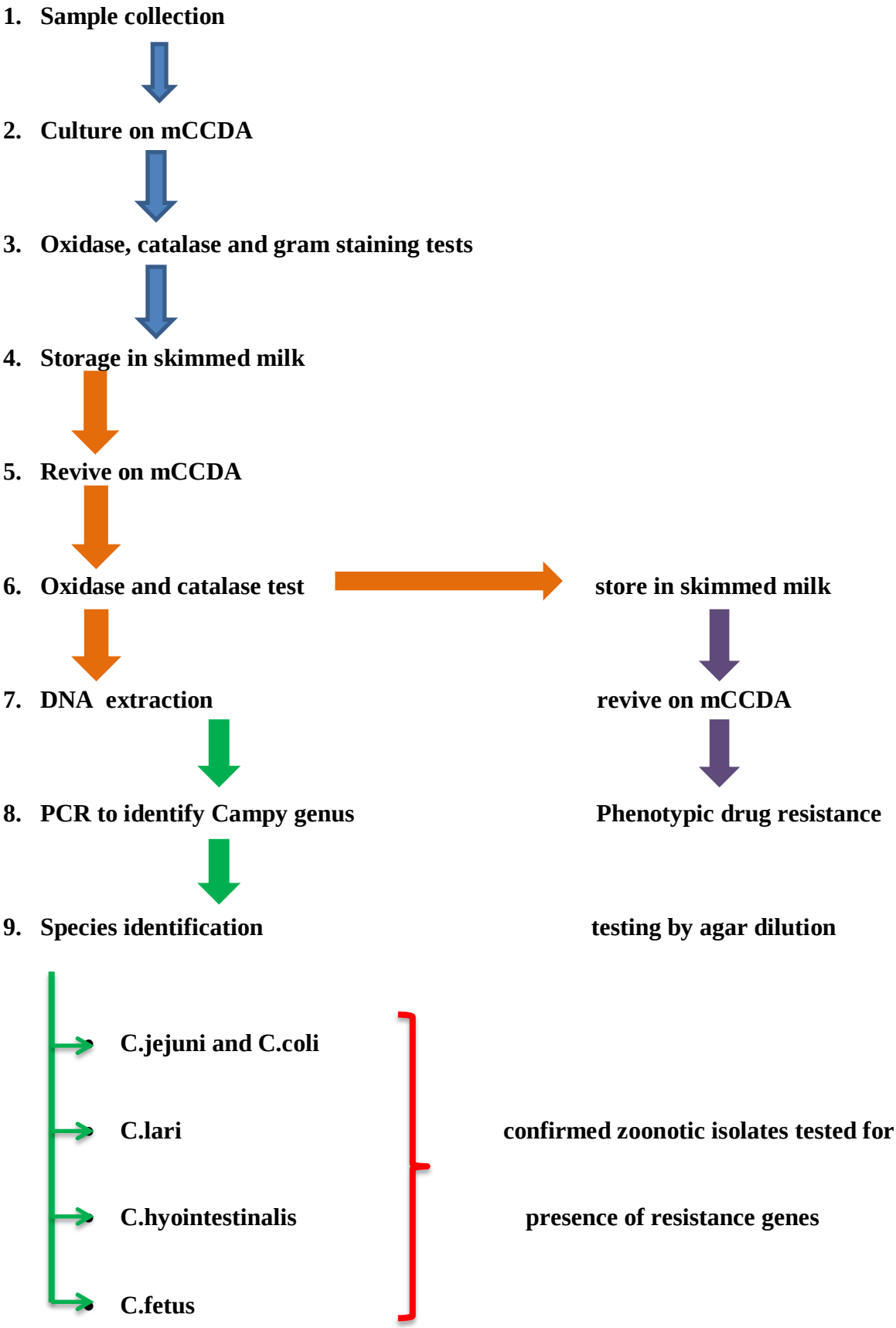

Figure 2: A flow chart of the laboratory processes described above from sample collection to species identification and drug resistance testing

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 $p \leq 0.05$ 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.

CHAPTER 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.

ATTRIBUTE	SPECIFICS	NUMBER OF HOUSEHOLDS		
		Total	**Korogocho (%)**	**Viwandani (%)**
HOUSEHOLDS		**203**	**141**	**62**
GENDER OF PERSON CLEANING HOUSING	Male	**90**	57 (63)	33 (37)
	Female	**86**	66 (77)	20 (23)
	Both	**21**	15 (71)	6 (29)
	Not cleaned	**6**		
GENDER OF PERSON FEEDING LIVESTOCK	Male	**82**	51(62)	31 (38)
	Female	**83**	64 (77)	19 (23)
	Both	**35**	25 (71)	10 (29)
	Not fed by either gender	**3**		
LIVESTOCK	Poultry	**167**	117(70)	50(30)
	Rabbits	**22**	99(28)	13(72)
	Dairy cows	**21**	12(57)	9(43)
	Goats	**48**	28(58)	20(42)
	Pigs	**20**	11(55)	90(45)
Some households kept more than one livestock species hence this section has >203 households				
FEED SOURCE	Scavenging	**71**	38(54)	33(46)
	HH leftovers	**117**	83(71)	34(29)
	Purchase feed	**157**	109(69)	48(31)
	Bring in forage	**31**	19(61)	12(39)
	Swill	**27**	20(74)	7(26)
	Graze by road side	**10**	8(80)	2(20)
LIVESTOCK WATER SOURCE	Tap	**184**	130(71)	54(29)
	Road	**21**	5(24)	16(76)
	Sewer	**15**	7(47)	8(53)
	HH waste water	**17**	15(88)	2(12)
	River	**4**	2(50)	2(50)

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 we 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 back ground. 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.

ATTRIBUTE	SPECIFICS	NUMBER OF HOUSEHOLDS		
		Total	**Korogocho**	**Viwandani**
HOUSEHOLDS		**203**	**141**	**62**
EDUCATION	None	**28**	21	7
	Primary school	**107**	69	38
	Secondary school	**60**	43	17
	Certificate	**3**	3	0
	Diploma	**4**	4	0
	Other	**1**	1	0
TRAINING	Animal disease management	**6**	3	3
	Livestock management	**10**	5	5
	Food safety	**0**	0	0
AWARENESS	Antimicrobial resistance	**76**	55	21
	Antimicrobial residues	**72**	16	56
	Drug withdrawal periods	**89**	67	22
	Zoonosis	**101**	72	29
	Aflatoxins	**49**	36	13
	Pathogens in manure	**58**	39	19
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 keepin g 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 s ourcing 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.

		PROPORTIONS
Person treating livestock in the different households	Owner	**67.5%**
	Owner and vet	**2.5%**
	Agrovet	**2.0%**
	Veterinarian	**4.4%**
	Other	**19.7%**
	Don't know	**3.9%**
Medicine sources	Owner	**18.2%**
	Owner and vet	**3.9%**
	Agro-Vet	**47.8%**
	Veterinarian	**3.9%**
	Other	**21.2%**
	Don't know	**4.9%**

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.

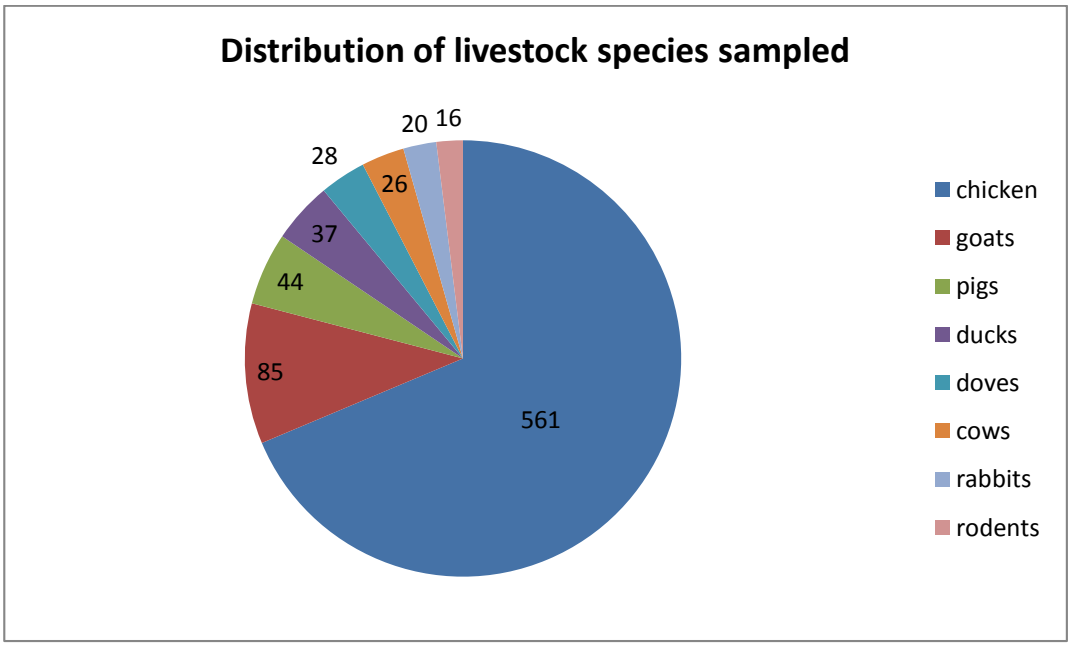

Figure 2 : Pie chart showing the distribution of livestock species sampled in the study in numbers.

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.

	C. coli	*C. jejuni*
Dairy cow	**0**	**0**
Dairy goat	**0**	**0**
Other goat	**0**	**2**
Other livestock	**0**	**0**
Pigs	**0**	**1**
Chicken	**1**	**23**
Rabbits	**0**	**0**
Sheep	**0**	**0**
Totals	**1**	**26**

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 hol d left 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.

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

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

Livestock	Zoonotic *Campylobacter*	ANTIBIOTICS TESTED											
		Gentamicin		Chloramphenicol		Tetracycline		Erythromycin		Ciprofloxacin		Nalidixic acid	
		R	S	R	S	R	S	R	S	R	S	R	S
CHICKEN	***C.jejuni*** *	16	7	13	10	23	0	23	0	18	5	19	4
	C.coli **	0	1	0	1	1	0	1	0	0	1	1	0
GOATS	***C.jejuni*** ***	1	0	0	1	1	0	1	0	1	0	1	0
PIGS	***C.jejuni*** ****	0	1	0	1	0	1	0	1	0	1	0	1

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.

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

***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.

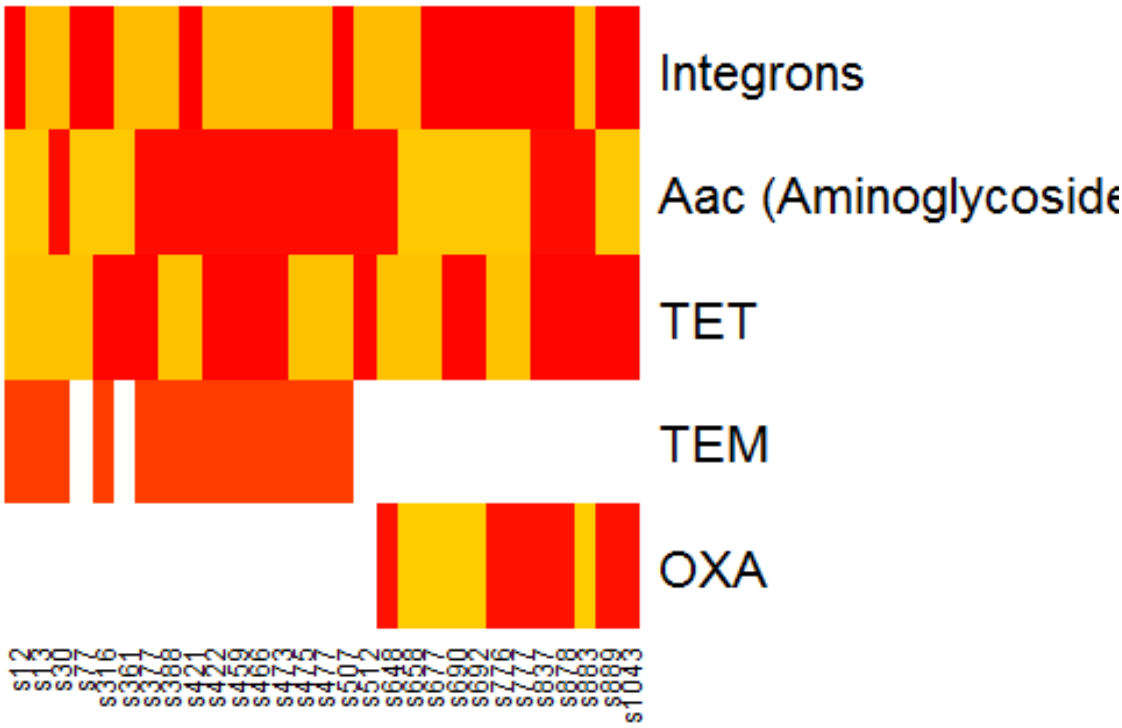

Figure 3: A Heat map showing the isolates with multi-resistant genes and Class 1 integrons.

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 foe 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 and883, C1308T in sample 30, 507 and 883, T1360C in sample 77 and T1407C in sample 77.

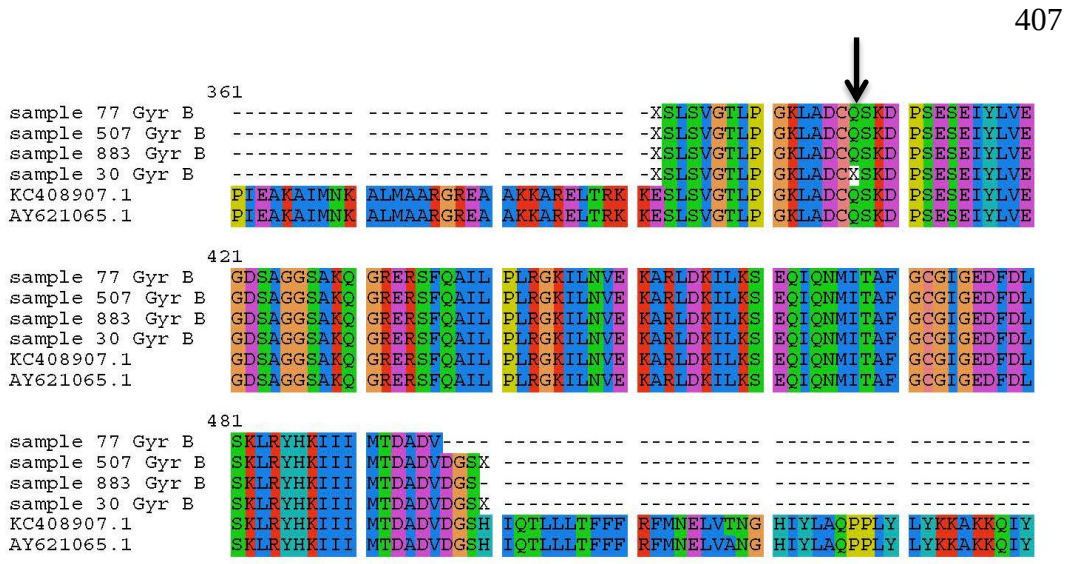

Figure 4: DNA Gyrase B amino acid sequences showing deletion of the amino acid Q at position 407.

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.

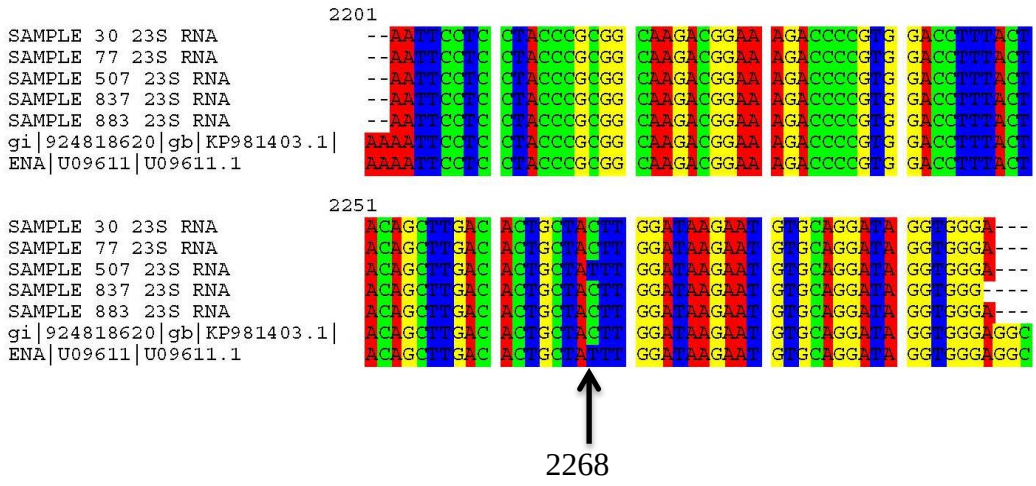

Figure 5: nucleic acid sequences of the 23 SRNA genes showing the C2268T substitution in sample 507 similar to that of sequence ENAU09611 from genbank.

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.

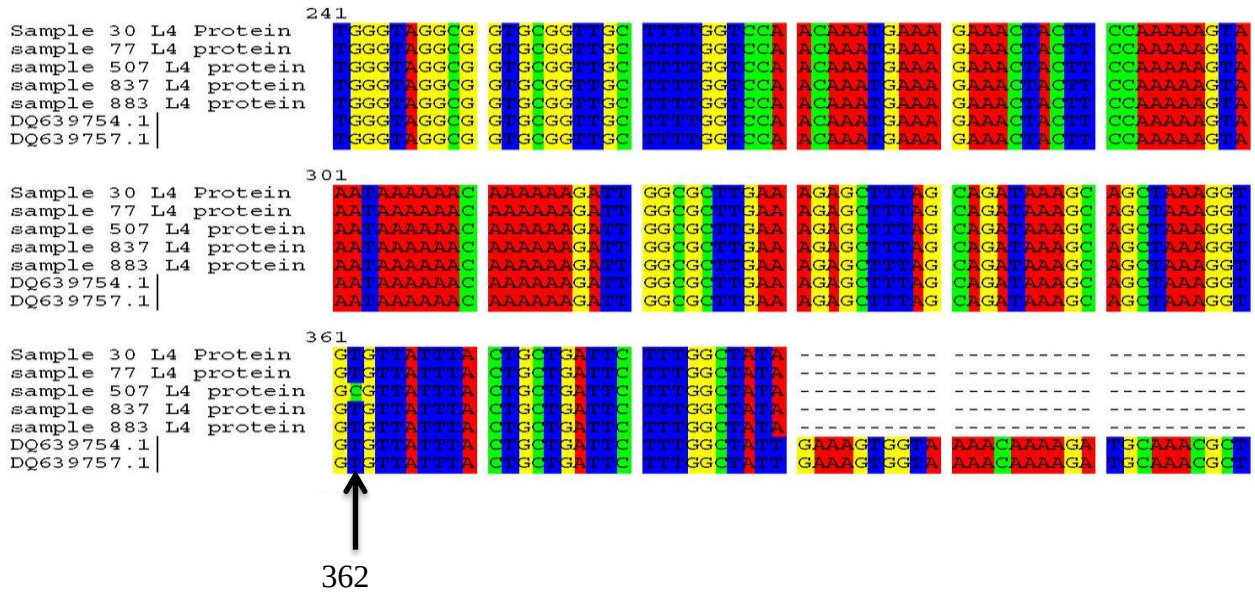

Figure 6: protein l4 aligned sequences showing the T362C substitution in sample 507

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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 *Campylobacte*r species was highest for *Campylobacter jejuni*. This finding is echoed by other studies that isolated more *C.jejuni* 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 thermophillic *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 Azithromicin (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. hyointestionalis 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 23Srna 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 batelactam 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 *C.jejuni* and *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 *Campylobacter* species (other than *C.jejuni* and *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 *Campylobacter*, its effects, risk factors, prevention and control.

2. Sensitization of the need for increased diagnosis of *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 *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 *C.jejuni* and *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.

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CHAPTER 9.0 APPENDICES

APPENDIX 1: PROTOCOL FOR CAMPYLOBACTER IDENTIFICATION

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 (DFVF), JAAP WAGENAAR (ASG), MARCEL VAN BERGEN (ASG)

Contents:

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

1. Nachamkin I. and M. J. Blaser (eds) (2000). *Campylobacter 2nd ed.* 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%-H_2O_2
- 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:

Campylobacter.lari	ATCC 35221
Campylobacter.coli	ATCC 33559
Campylobacter.jejuni	ATCC 700819
Pseudomonas aeruginasa	ATCC 27853
Enterococcus faecalis	ATCC 29212
Staphylococcus aureus	ATCC 29213

Safety

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

Procedure	Theory / comments
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.	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 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 ater tipped 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)	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 coccoid bacteria.
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%-H_2O_2 on the spot with the bacterial material. Examine immediately for evolution of gas, which indicates catalase activity.	The catalase-enzyme cleaves the hydrogen peroxide $H_2O_2 + H_2O_2 \Rightarrow O_2 + 2H_2O$ 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.
Test for oxidase Transfer one colony to a filter paper. Soak the filter in a oxidase solution. Appearance of a blue color within 10 sec indicates a positive result.	The method is based on the principle that certain phenylen-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%-ninhydrin 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 hippuracid releases benzoeacid. Hippuracid is soluble in excess of an acidic solution of ferrichloride while benzoeacid precipitates.

1%-hippurate solution: freshly prepared or stored at -20 °C for about 6 month.
3.5%-ninhydrin 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 ($C_9H_6O_4$) 3,5 g
Acetone (C_3H_6O) 50 ml
Butanol ($C_4H_{10}O$) 50 ml

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

1% Hippurate solution

Natriumhippurat ($C_9H_8NNaO_3$) 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 iodine 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 fuchsine (counterstain)

Concentrated carbol fuchsine	10.0 ml
Distilled water	90.0 ml

10% (wt/vol) Indoxylacetate solution

Indoxylacetat ($C_{10}H_9NO_2$)	10 g
Acetone (C_3H_6O)	90 ml

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

Oxidase solution

L(+)-Ascorbicacid	0,03 g
N,N,N′,N′- Tetramethyl-p-Phenylendiamine Dihydrochloride ($C_{10}H_{16}N_2 \cdot 2HCl$)	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.

Record sheet: Quality Control / Batch Control

Date:_____ Init.:_____

Biochemical tests

QC-Strain	*C.jejuni* ATCC 700819	*C.coli* ATCC 33559	*C.lari* ATCC 35221	*E. faecalis* ATCC 29212	*S. aureus* ATCC 29213	*P. aeruginosa* ATCC 27853
Gram staining						
Test for catalase						
Test for oxidase						
Hippurate hydrolysis						
Hydrolysis of indoxyl acetate						

Record sheet: Identification of Campylobacter.

Date:_____ Init.:_____

Biochemical tests

	Strain #	**Strain** #	**Strain** #	**Strain** #	**Strain** #	**Strain** #
Morphology of the cell (microscopy)						
Motility (microscopy)						
Gram staining						
Test for catalase						
Test for oxidase						
Hippurate hydrolysis						
Hydrolysis of indoxyl acetate						
Species:	_____	_____	_____	_____	_____	_____

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

Date:_____ Init.:_____

Biochemical tests

	Faeces-sample 1	Faeces-sample 2	Food-sample 1	Food-sample 2	Water-sample 1	Water-sample 2
Morphology of the cell (microscopy)						
Motility (microscopy)						
Gram staining						
Test for catalase						
Test for oxidase						
Hippurate hydrolysis						
Hydrolysis of indoxyl acetate						
Species:	_____	_____	_____	_____	_____	_____

APPENDIX2: GLOBAL SALM SURV ANTIMICROBIAL SUSCEPTIBILITY TESTING BY AGAR DILUTION PROTOCOL USED IN THIS STUDY.

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

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 susceptibililty testing is regarded as the golden standard for all other susceptibilily 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 camphylobacter with two-fold dilutions of antibiotic:
  Ciprofloaxin (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% cattleblood 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	Theory / comments
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.	

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	Theory / comments
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.	This is done to minimize the risk of picking bacteria which have lost their resistance.
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.	McFarland 0.5 ~ approximately 10^8 CFU/ml
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 ~ approximately 10^7 CFU/ml.
The inoculum suspension should be used for inoculation within 15 minutes.	To avoid further growth of inoculum.
Inoculation and incubation Transfer 400 µl of the inoculum suspension to the multi-point inoculator wells.	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 can not 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)

Beef extract	2.0 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled water	1000 ml

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

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.

Columbia-agar

	25 L
Columbia agar base (Oxoid CM331)	1125 g
Water	25,000 ml
Natriumhydroxid 5N	
Saltsyre 4N	

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

Columbia agar	950 ml
Cattle blood	50 ml

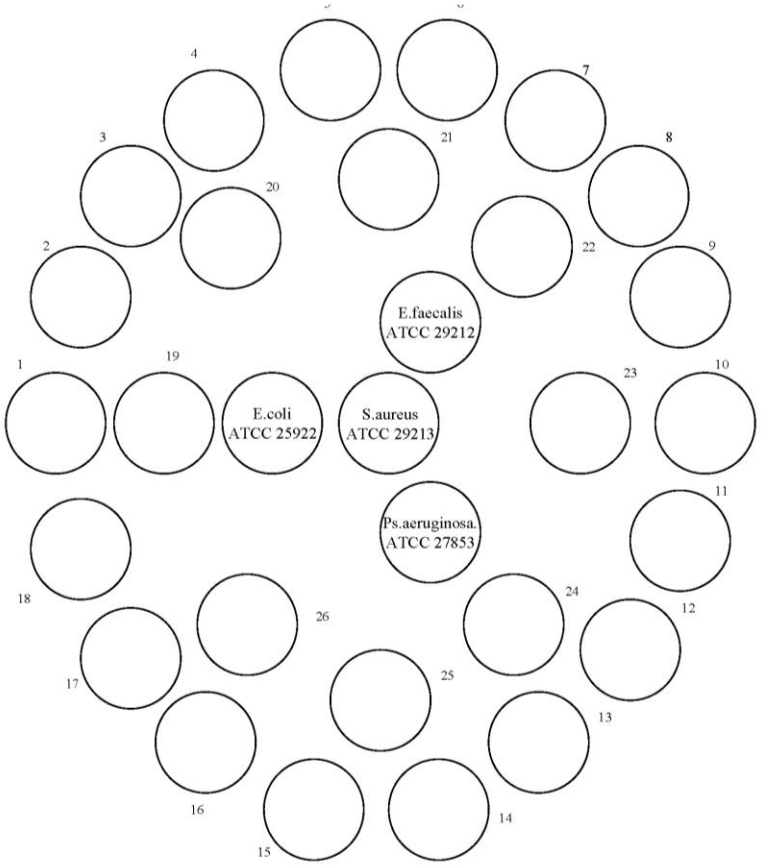
Preparation:
Melt the agar and add cattle blood. Pour plates with about 15 ml melted medium in each.
Incubate overnight at 37°C.

References

1. BARROW & FELTHAM (eds.): *Cowan and Steel's Manual for the Identification of Medical Bacteria,* 3 rd edn.

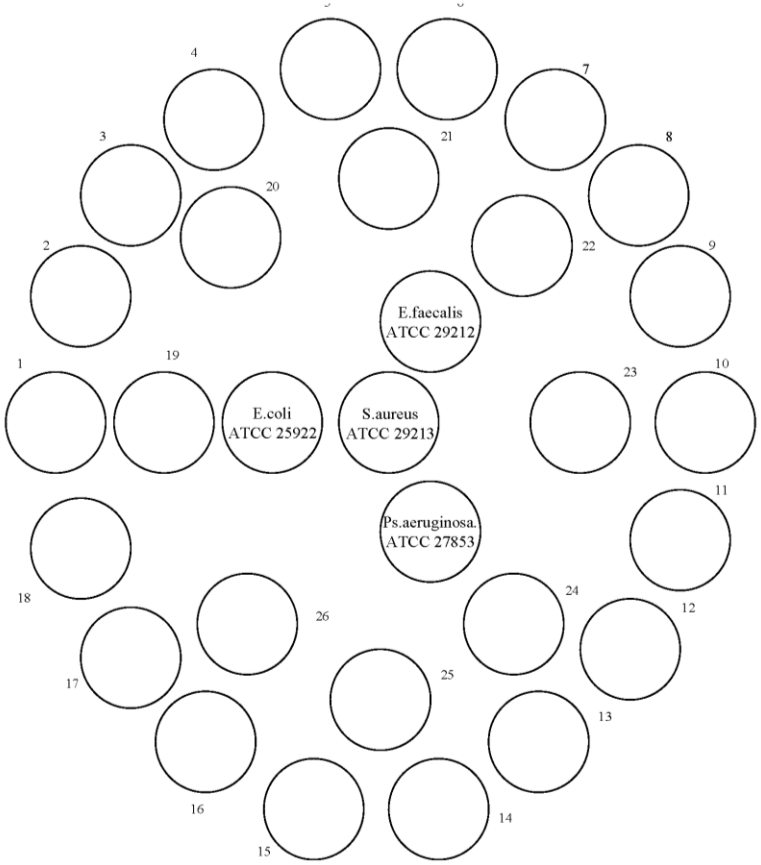
Date:_____**Record sheet: Salmonella / Chloramphenicol**
Initials:_____**MIC determination by agar dilution**

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.



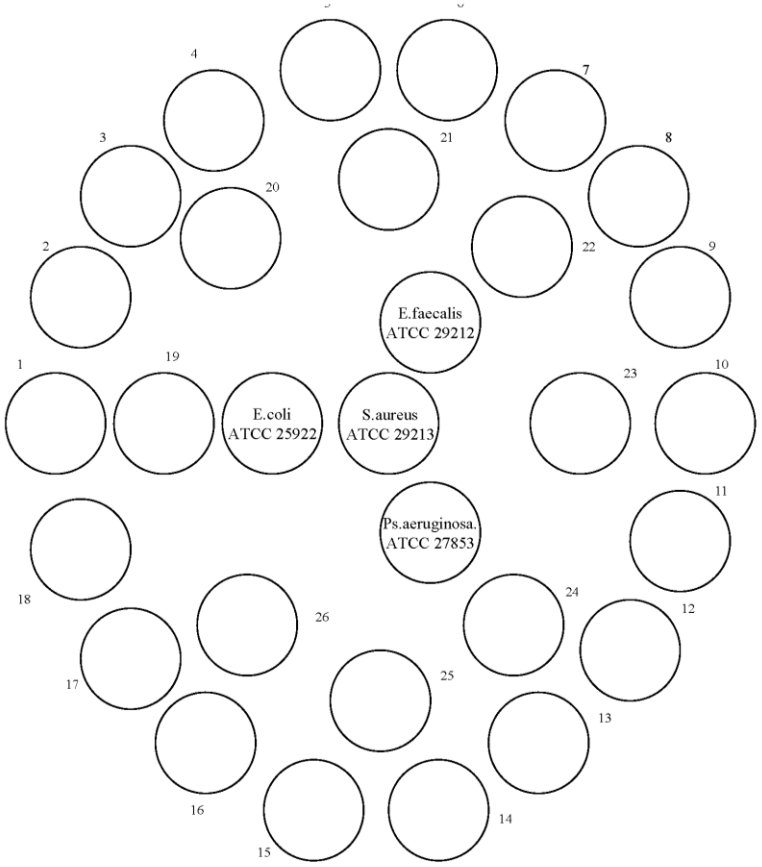
Date:_____**Record sheet: Salmonella / Tetrecycline**
Initials:_____**MIC determination by agar dilution**

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: Salmonella / Ampicillin**
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: Salmonella**

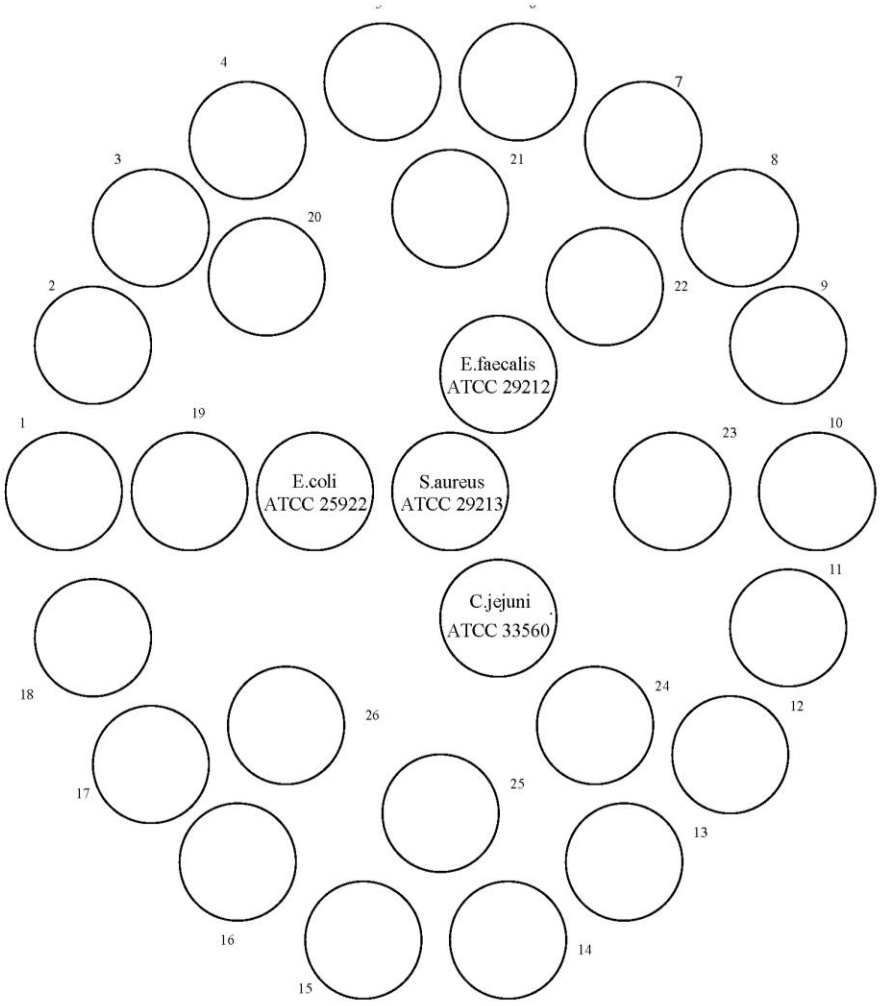
Initials:_____**MIC determination by agar dilution**

No	Strain	Chloramphenicol		Ampicillin		Tetracycline	
		MIC (μg/ml)	Interpretation (R-I-S)	MIC (μg/ml)	Interpretation (R-I-S)	MIC (μg/ml)	Interpretation (R-I-S)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							
21							
22							
23							
24							
25							
26							

Date:_____**Record sheet: Camphylobacter / Ciprofloxacin**

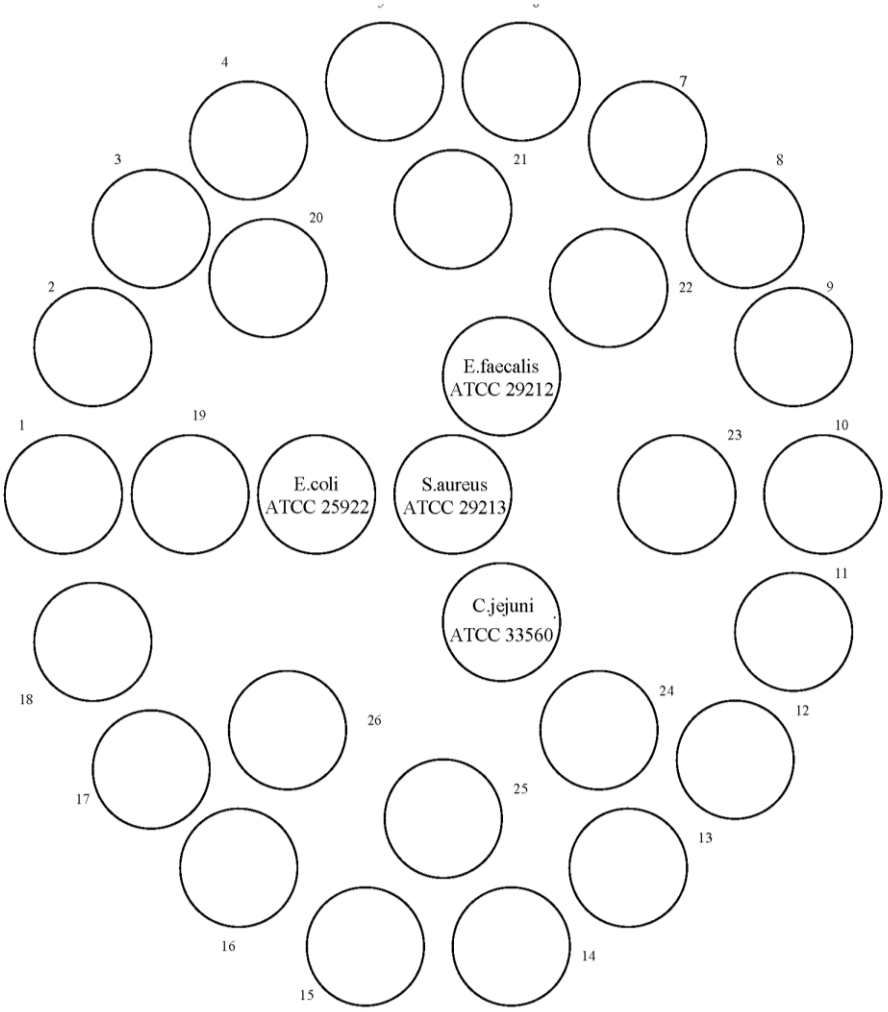
Initials:_____**MIC determination by agar dilution**

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.



Date:_____**Record sheet: Camphylobacter / Nalidixan acid**
Initials:_____**MIC determination by agar dilution**

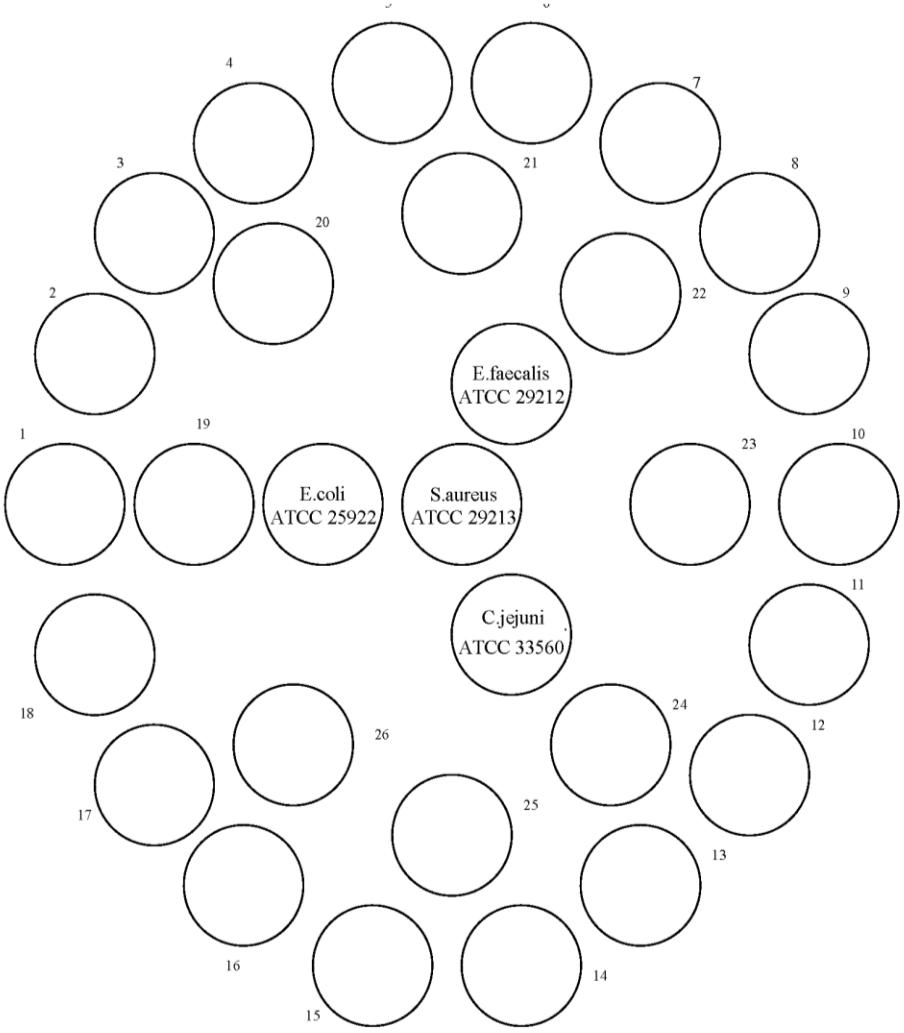
Before inoculation write the ID of the strains 1-26 above each circle.
Write the lowest concentration of antibiotic without growth (the MIC) in each circle.
Fill out the following table.



Date:_____**Record sheet: Camphylobacter / Tetracycline**

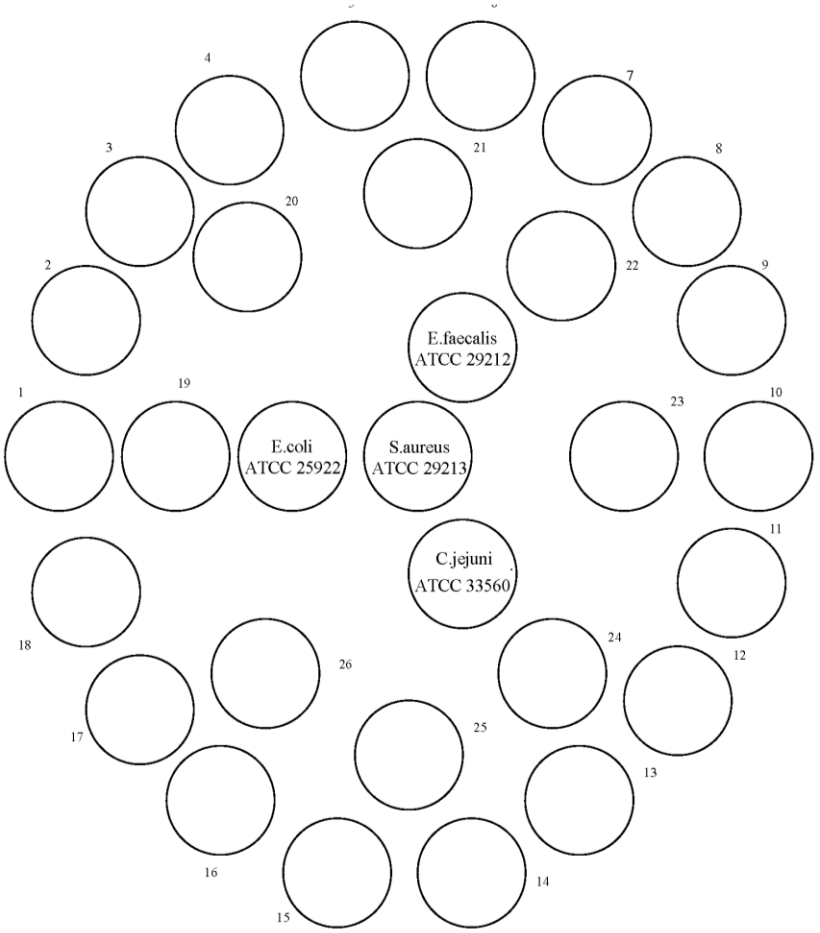
Initials:_____**MIC determination by agar dilution**

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: Camphylobacter / 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.



Date:_____**Record sheet: Camphylobacter**

Initials:_____**MIC determination by agar dilution**

No	Strain	Ciprofloxacin		Nalidixan acid		Tetracycline		Erythromycin	
		MIC (μg/ml)	Interpretation (R-I-S)	MIC (μg/ml)	Interpretation (R-I-S)	MIC (μg/ml)	Interpretation (R-I-S)	MIC (μg/ml)	Interpretation (R-I-S)
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
25									
26									

APPENDIX 1

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

Step	Concentration ug/ml.	Source	Volume + Solvent ml. ml.	Upscale to user vol. ml. ml.	Final vol. of solvent ml.	Final concentration At 1:10 dilution i agar	Vol. media ml.	Vol. solution ml.
1	5120	Stock	- -			512		
2	5120	Step 1	1 1			256		
3	5120	Step 1	1 3			128		
4	1280	Step 3	1 1			64		
5	1280	Step 3	1 3	***3 + 9***	***12***	***32***	***90***	***10***
6	1280	Step 3	1 7	***3 + 21***	***24***	***16***	***90***	***10***
7	160	Step 6	1 1	***6 + 6***	***12***	***8***	***90***	***10***
8	160	Step 6	1 3	***3 + 9***	***12***	***4***	***90***	***10***
9	160	Step 6	1 7	***3 + 21***	***24***	***2***	***90***	***10***
10	20	Step 9	1 1	***6 + 6***	***12***	***1***	***90***	***10***
11	20	Step 9	1 3	***3 + 9***	***12***	***0.5***	***90***	***10***
12	20	Step 9	1 7	***3 + 21***	***24***	***0.25***	***90***	***10***
13	2.5	Step 12	1 1			0.125		

Antimicrobial: ***Erythromycin.***
Antimicrobial gradient: ***0.25 – 32 ug/ml.***
Concentration of the stock solution: ***1280ug/ml.***
Volume of antimicrobial to be weight: (*1280ug/ml * 6ml) 1280ug/ml * 80 = 102.4mg*
Volume of Agar: ***90ml***
Volume of antimicrobial solutions (10% of agar vol):***10ml.***

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APPENDIX 3: NARMS ANTIMICROBIAL SUSCEPTIBILITY BREAKPOINTS

Posted online at http://www.cdc.gov/NARMS/ on December 12th, 2008, the "National Antimicrobial Resistance Monitoring System 2005 Human Isolates Final Report" contained errors in five tables.

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

CLSI Subclass	Antimicrobial Agent	Antimicrobial Agent Concentration Range (µg/mL)	Breakpoints		
			Susceptible	Intermediate	Resistant
Aminoglycosides	Gentamicin	0.12-32 0.016–256*	≤2	4	≥8
Ketolides	Telithromycin†	0.015-8	≤4	8	≥16
Lincosamides	Clindamycin	0.03-16 0.016–256*	≤2	4	≥8
Macrolides	Azithromycin	0.015-64 0.016–256*	≤2	4	≥8
	Erythromycin	0.03-64 0.016–256*	≤8	16	≥32
Phenicols	Chloramphenicol‡	0.016-256*	≤8	16	≥32
	Florfenicol§	0.03-64	≤4	N/A	N/A
Quinolones	Ciprofloxacin	0.015–64 0.002–32*	≤1	2	≥4
	Nalidixic acid	4-64 0.016–256*	≤16	32	≥64
Tetracyclines	Tetracycline	0.06-64 0.016–256*	≤4	8	≥16

*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 5.06** and page 64 **Table 5.08**, the susceptible breakpoint for florfenicol should read (MIC ≤4).

The report was corrected and made available online on February 10th, 2009 at:
http://www.cdc.gov/narms/annual/2005/NARMSAnnualReport2005.pdf

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.

RID HCNNR71H01N (Expires on 04-21 00:03 am)
Query ID lcl|Query_227629
Description CCOL wordedited
Molecule type nucleic acid
Query Length 315

Download GenBank Graphics

Campylobacter coli strain KLC5104 LpxA (lpxA) gene, partial cds
Sequence ID: gb|AY598972.1| **Length:** 573 **Number of Matches:** 1

Range 1: 71 to 384 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
564 bits(305)	4e-157	311/314(99%)	0/314(0%)	Plus/Plus

```
Query  1    GTTGTTATTGAGGCTTATGCATATGTAAGCAAAGAGGCTAAGATAGGCAATGGGGTTGTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  71   GTTGTTATTGAGGCTTATGCATATGTAAGCAAAGAGGCTAAGATAGGCAATGGGGTTGTT  130

Query  61   ATCAAACAAGGGGCGAGAATACTTTCAGATACTACCATAGGTGATCATTCTCGTGTATTT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  131  ATCAAACAAGGGGCGAGAATACTTTCAGATACTACCATAGGTGATCATTCTCGTGTATTT  190

Query  121  TCATATGCTATAGTAGGTGATATTCCTCAAGATATATCTTATAAGGACGAGCAAAAAAGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  191  TCATATGCTATAGTAGGTGATATTCCTCAAGATATATCTTATAAGGACGAGCAAAAAAGC  250

Query  181  GGAGTAATTATAGGGCAAAATTCTACTATTAGAGAATTTGCTACGATAAATTCGGGTACA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251  GGAGTAATTATAGGGCAAAATTCTACTATTAGAGAATTTGCTACGATAAATTCGGGTACA  310

Query  241  GCTAAAGGCGATGGTTTTACTCGTATAGGAGACAATGCTTTTATAATGGCATATTCTCAT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||| |||| ||||
Sbjct  311  GCTAAAGGCGATGGTTTTACTCGTATAGGAGACAATGCTTTTATAATGGCGTATTGTCAT  370

Query  301  ATCGCCCATGATTG  314
            ||||| ||||||||
Sbjct  371  ATCGCACATGATTG  384
```

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.

RID	HCNPDRC101N (Expires on 04-21 00:03 am)
Query ID	lcl|Query_18547
Description	36cjejlpxA
Molecule type	nucleic acid
Query Length	276

Campylobacter jejuni subsp. jejuni F38011, complete genome

Sequence ID: gb\|CP006851.1\| **Length:** 1691939 **Number of Matches:** 1

Range 1: 256385 to 256656 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
503 bits(272)	8e-139	272/272(100%)	0/272(0%)	Plus/Minus

```
Query  5       AACCATCTCCTTTAGCTGTACCTGAATTTATCGTTGCAAATTCTCTAATAGTTGCATTTT  64
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256656  AACCATCTCCTTTAGCTGTACCTGAATTTATCGTTGCAAATTCTCTAATAGTTGCATTTT  256597

Query  65      TCCCTATAACAACACCGCTTTTTTGCTCTTCTTTATAAGATATGTCCTGAGGAATATCGC  124
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256596  TCCCTATAACAACACCGCTTTTTTGCTCTTCTTTATAAGATATGTCCTGAGGAATATCGC  256537

Query  125     CTACTATAGCATAAGAAAATACACGAGAATGATCACCTATAGTTGTATCTGAAAGAATTC  184
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256536  CTACTATAGCATAAGAAAATACACGAGAATGATCACCTATAGTTGTATCTGAAAGAATTC  256477

Query  185     GAGCACCTTGTTTGATGACAACATTATTACCTATTTTAGCATCTTTGCTTACATAAGCAT  244
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256476  GAGCACCTTGTTTGATGACAACATTATTACCTATTTTAGCATCTTTGCTTACATAAGCAT  256417

Query  245     AAGCTTCTACTACAACATCGTCACCAAGTTGT  276
               ||||||||||||||||||||||||||||||||
Sbjct  256416  AAGCTTCTACTACAACATCGTCACCAAGTTGT  256385
```

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.

RID HCNR87E601N (Expires on 04-21 00:03 am)
Query ID lcl|Query_27361
Description 37cjejlpxA
Molecule type nucleic acid
Query Length 276

Download GenBank Graphics

Campylobacter jejuni subsp. jejuni CG8421, complete genome
Sequence ID: gb|CP005388.1| Length: 1636029 Number of Matches: 1

Range 1: 249074 to 249345 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
497 bits(269)	4e-137	271/272(99%)	0/272(0%)	Plus/Minus

```
Query  5       AACCATCTCCTTTAGCTGTACCTGAATTTATCGTTGCAAATTCTCTAATAGTTGCATTTT  64
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249345  AACCATCTCCTTTAGCTGTACCTGAATTTATCGTTGCAAATTCTCTAATAGTTGCATTTT  249286

Query  65      TCCCTATAACAACACCGCTTTTTTGCTCTTCTTTATAAGATATGTCCTGAGGAATATCGC  124
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249285  TCCCTATAACAACACCGCTTTTTTGCTCTTCTTTATAAGATATGTCCTGAGGAATATCGC  249226

Query  125     CTACTATAGCATAAGAAAATACACGAGAATGATCACCTATAGTTGTATCTGAAAGAATTC  184
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249225  CTACTATAGCATAAGAAAATACACGAGAATGATCACCTATAGTTGTATCTGAAAGAATTC  249166

Query  185     GAGCACCTTGTTTGATGACAACATTATTACCTATTTTAGCATCTTTGCTTACATAAGCAT  244
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249165  GAGCACCTTGTTTGATGACAACATTATTACCTATTTTAGCATCTTTGCTTACATAAGCAT  249106

Query  245     AAGCTTCTATTACAACATCGTCACCAAGTTGT  276
               |||||||| ||||||||||||||||||||||
Sbjct  249105  AAGCTTCTACTACAACATCGTCACCAAGTTGT  249074
```

APPENDIX 5: DETAILS OF PHENOTYPIC DRUG RESISTANCE TEST RESULTS.

LIVESTOCK SPECIES	***CAMPYLOBACTER* SPECIES**	**ANTIMICROBIALS**	Resist-ant	Interme di-ate	Suscep ti-ble	RESISTANCE PATTERNS SUMMARY		
CHICKEN	***Campylobacter jejuni***	Gentamicin	16	1	6	**Resistant to all 6 drugs**		**9 isolates**
		Chloramphenicol	13	7	3	**Resistant to 5 antimicrobials**	gentamicin, nalidixic acid, ciprofloxacin, tetracycline and erythromycin	**7 isolates**
		Nalidixic acid	19	0	4		Chloramphenicol, nalidixic acid, ciprofloxacin, tetracycline and erythromycin	**2 isolates**
		Ciprofloxacin	18	1	4	**Resistant to 4 antimicrobials**	Chloramphenicol, nalidixic acid, Tetracycline and erythromycin.	**2 isolates**
		Tetracycline	23	0	0	**Resistant to 3 antimicrobials**	Chloramphenicol, tetracycline and erythromycin	**1 isolate**

	Erythromycin	23	0	0	**Resistant to 2 antimicrobials**	Tetracycline and erythromycin	**2 isolates**
Campylobacter coli	Gentamicin	0	0	1	This was only **1 isolate** which was **resistant to 3 antimicrobials**: nalidixic acid, tetracycline and erythromycin.		
	Chloramphenicol	0	0	1			
	Nalidixic acid	1	0	0			
	Ciprofloxacin	0	0	1			
	Tetracycline	1	0	0			
	Erythromycin	1	0	0			
Other *Campylobacter* species	Gentamicin	87	0	0	**Resistant to all 6 antimicrobials**		**59 isolates**
	Chloramphenicol	69	0	0	**resistant to 5 antimicrobials**	Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin	**28 isolates**
	Nalidixic acid	100	0	1		Chloramphenicol, Nalidixic acid, Ciprofloxacin, Tetracycline	**7 isolates**

								and Erythromycin	
			Ciprofloxacin	99	0	1	**Resistant to 4 antimicrobials**	Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin	**3isolates**
			Tetracycline	108	0	1	**Resistant to 3 antimicrobials**	Ciprofloxacin, Tetracycline and Erythromycin	**1 isolate**
								Nalidixic acid, Tetracycline and Erythromycin	**2 isolates**
								Chloramphenicol, Tetracycline and Erythromycin	**3 isolates**
			Erythromycin	109	0	0	**Resistant to 2 antimicrobials**	Tetracycline and Erythromycin	**5 isolates**
LIVESTOCK SPECIES	*CAMPYLOBACTER* SPECIES	ANTIMICROBIALS		**Resistant**	**Intermediate**	**Susceptible**			
	Campylobacter jejuni	I.	Gentamicin	1	0	0	**One isolate** grew upon reviving and was **resistant to 5 antimicrobials:** Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin		
		II.	Chloramphenicol	0	0	1			

		III.	Nalidixic acid	1	0	0			
		IV.	Ciprofloxacin	1	0	0			
		V.	Tetracycline	1	0	0			
		VI.	Erythromycin	1	0	0			
GOATS									
	Other *Campylobacter* species	I.	Gentamicin	11	0	0		**Resistant to all 6 antimicrobials**	**6 isolates**
		II.	Chloramphenicol	7	0	0			
		III.	Nalidixic acid	11	0	0		**Resistant to 5 antimicrobials**	Gentamicin. Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin	**4 isolates**
		IV.	Ciprofloxacin	10	0	1			
		V.	Tetracycline	12	0	0		**Resistant to 2 antimicrobials**	Tetracycline and Erythromycin	**1 isolate**
		VI.	Erythromycin	12	0	0			

LIVESTOCK SPECIES	*CAMPYLOBACTER* SPECIES	ANTIMICROBIALS		**Resistant**	**Intermediate**	**Susceptible**		**RESISTANCE PATTERNS**		
PIGS	*Campylobacter* jejuni	VII.	Gentamicin	0	0	1		The single *Campylobacter* jejuni isolate from a pig sample was **susceptible to all antimicrobials**		
		VIII.	Chloramphenicol	0	0	1				
		IX.	Nalidixic acid	0	1	0				
		X.	Ciprofloxacin	0	1	0				
		XI.	Tetracycline	0	0	1				
		XII.	Erythromycin	0	0	1				
	Other *Campylobacter* species		Gentamicin	10				**Resistant to all 6 antimicrobials**		**6 isolates**
			Chloramphenicol	8						
			Nalidixic acid	9				**Resistant to 5 antimicrobials**	Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracyclin	**4 isolates**

									e and Erythromycin	
			Ciprofloxacin	8					Gentamicin, Chloramphenicol, Nalidixic acid, Tetracycline and Erythromycin	**1 isolate**
			Tetracycline	10				**Resistant to 4 antimicrobials**	Gentamicin, Chloramphenicol, Tetracycline and Erythromycin	**1 isolate**
			Erythromycin	10						
LIVESTOCK SPECIES	*CAMPYLOBACTER* SPECIES	ANTIMICROBIALS		**Resistant**	**Intermediate**	**Susceptible**				
	Other *Campylobacter* species		Gentamicin	1				**Resistant to 5 antimicrobials**	Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and	**1 isolate**
			Chloramphenicol	1						

								Erythromycin	
RABBITS		Nalidixic acid	2				**Resistant to 3 antimicrobials**	Chloramphenicol, Nalidixic acid and Erythromycin	**1 isolate**
		Ciprofloxacin	1						
		Tetracycline	2				**Resistant to 2 antimicrobials**	Tetracycline and Erythromycin	**1 isolate**
		Erythromycin	3						
LIVESTOCK SPECIES	*CAMPYLOBACTER* SPECIES	ANTIMICROBIALS	**Resistant**	**Intermediate**	**Susceptible**		**RESISTANCE PATTERNS**		
CATTLE	Other *Campylobacter* species	Gentamicin	1	0	0		This was only **1 isolate resistant to 5 antimicrobials**: Gentamicin, Nalidixic acid, Ciprofloxacin. Tetracycline and Erythromycin.		
		Chloramphenicol	0		0				
		Nalidixic acid	1	0	0				
		Ciprofloxacin	1	0	0				
		Tetracycline	1	0	0				
		Erythromycin	1	0	0				

LIVESTOCK SPECIES	*CAMPYLOBACTER* SPECIES	ANTIMICROBIALS	**Resistant**	**Intermediate**	**Susceptible**		**RESISTANCE PATTERN**		
DUCKS	Other *Campylobacter* species	Gentamicin	3	0	0		**Resistant to all 6 antimicrobials**		**2 isolates**
		Chloramphenicol	2						
		Nalidixic acid	3	0	0				
		Ciprofloxacin	3	0	0		**Resistant to 5 antimicrobials**	Gentamicin, Nalidixic acid, Ciprofloxacin, Tetracycline and Erythromycin	**1 isolate**
		Tetracycline	3	0	0				
		Erythromycin	3	0	0				